Assessment of DNA-Damage Repair in Breast Cancer

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July 2014

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

Abstract

Background: Current evidence indicates that DNA damage response (DDR) is a highly complex process that involves various pathways working in an orchestrated and interwoven manner in response to different types of damage to DNA. Although specific defects of DDR remain to be deciphered in cancer as a general, there is certainly an undeniable relationship between a particular dysfunction of DDR and the phenotype of tumour [1, 2]. It has been demonstrated that familial forms of breast and ovarian cancer are characterised by defects in one of the main mechanisms of DDR homologous recombination (HR) as a result of germline loss-of-function mutations in one of HR modifying genes, such as *BRCA1* and *BRCA2* [1, 3, 4]. Defects of genes involved in other DDR pathways are also associated with specific types of cancers; for instance hereditary non polyposis colorectal cancer (HNPCC) is strongly associated with specific mutations in the DNA mismatch repair pathway. Several previous studies have demonstrated that impaired DDR play a fundamental role in the pathogenesis and behaviour of breast cancer (BC). However, characterisation of this complex process, the expression and co-expression of the key proteins involved in the various DDR pathways and their prognostic significance in BC remain to be defined. In BC, it is reported that genes involved in DNA double strand breaks (DSB) repair are the most important. Two main pathways are involved in the repair of DNA-DSB; HR and Non Homologous End Joining (NHEJ) [3]. The common characteristics of global DDR are multiple genes induction directly associated with sensing and repair of DNA, arrest of cell cycle, and cell division inhibition. As a result DDR process does not only include genes activation involved in damage sensing as well as repair but additionally genes involved in control of cell-cycle [5]. Despite the fact that DDR may possibly involve activation of several pathways (such as SUMOylation (SUMO)) [6, 7] and many genes are engaged in different overlapping mechanisms, each pathway is characterised by activation and expression of a unique set of genes. This could allow discovering the active or aberrant pathway in a given tumour [1, 4, 5].

This study explores the hypothesis that investigation of alterations in the different pathways of DNA-DSB, may contribute to the characteristics of BC. Therefore, the aim was to perform a comprehensive profiling of key proteins involved in the different DNA-DSB repair pathways in the different molecular classes of BC. This approach aims to address the inherent problems arising from the complexity of DDR mechanism in BC with the potential of discovering a key pathway that is active or inactive in specific forms of BC that can be helpful to identify DNA repair status in individual BC patients.

Method: The study cohort comprises three BC groups: A) Large series of unselected primary sporadic operable invasive tumours (n=1904) in addition to B) 386 cases of oestrogen receptor (ER) negative tumours and C) a well-characterised series of BC from patients with known *BRCA*1 germline mutations (n=24). The proteins investigated in this study are known to participate in different DNA-DSB repair pathways including, DNA damage sensors (ATM and ATR), HR repair (BRCA1, BARD1, Rad51, γH2AX and

SMC6L1), DNA damage checkpoint signalling protein (CHK1 and CHK2), NHEJ repair (KU70/KU80, and DNA-PK), and SUMO (PIAS1, PIAS4, and UBC9). Because subcellular localisation of DDR proteins may affect their function, two markers that have role in nuclear transport in the cell were examined (NPM and KPNA2). The expression of these proteins was assessed using the well-established immunohistochemical technique utilising tissue microarray technology. The expression of proteins was further evaluated in various cell lines; BRCA1 deficient HeLaSilenciX® cells, and control BRCA1 proficient HeLaSilenciX®, MDA-MB-436 (BRCA1 deficient), and MCF-7 (BRCA1 proficient and ER^{+}) using Reverse Phase Protein Microarray (RPPA).

Results: Both cytoplasmic and nuclear expression was observed for expression of Rad51, SMC6L1, BRCA1, BARD1; (HR markers), PIAS1, UBC9 (SUMO markers), γH2AX (DNA-DSB marker) and CHK1 (checkpoint signalling protein). In contrast, both NHEJ markers and most of the DNA damage sensors (ATM and ATR), CHK2 and PIAS4 were mainly expressed in the nucleus. Generally, tumours that showed positive cytoplasmic/negative nuclear expression such as CHK1, PIAS1, Rad51, and BRCA1, and positive nuclear NHEJ markers showed an association with a poor outcome and adverse prognostic characteristics including high histologic grade, high mitotic frequency, high nuclear pleomorphism and larger tumour size in addition to ER negativity, and triple negative breast cancer (TNBC). Conversely, $nuclear^{+}$/$cytoplasmic^{-}$ expression showed an association the better outcome. Interestingly, ATM protein expression showed no association with the expression of the two NHEJ markers, whereas ATR showed an association with cytoplasmic expression of BRCA1 and BARD1 and was positively associated with NHEJ markers. In non-TNBC, tumours showing $BRCA1^{-}$/KU70/$KU80^{-}$ phenotype had worse breast cancer specific survival (BCSS) than positive expression ($P<0.0001$), whereas in the TN cohort, complex of KU70/$KU80^{-}$&$DNA\text{-}PK^{+}$ had the worst BCSS ($P=0.001$), and both are independent prognostic markers for BC.

KPNA2, but not NPM was highly associated with poor BCSS ($P<0.0001$). At least one of nucleocytoplasmic transport markers (NPM or KPNA2) was significantly associated with the subcellular localisation of the most of the markers that showed cytoplasmic expression including SMC6L1, γH2AX, BRCA1, BARD1, UBC9, PIAS1 ,Rad51 and CHK1. RPPA was used to investigate the protein expression in different cell lines, although the correlation between RPPA and IHC was not significant, the results of RPPA were consistent with that demonstrated by IHC further supporting the finding of the current study.

Conclusion: This study highlight the complexity of DDR related proteins and the overlap between different pathways involved in DDR. The finding of this study may help in the classification of BC and therefore, targeting active pathways in the development of drugs would enhance better patients' outcomes. Major prognostic and predictive variables can be very important in choosing suitable treatment plans, identifying the risk of recurrence and classifying patients for clinical trials. Our results show that the HR- repair marker Rad51, complex of HR and NHEJ repair markers (BRCA1&KU70/KU80) in non-TNBC, and a

complex of NHEJ markers (KU70/KU80&DNA-PK) are all independent prognostic markers for BC. In addition to expression, subcellular localisation of DDR proteins appeared to be a major factor in their role. Particularly, HR repair markers (but not NHEJ) showed worse features of cytoplasmic location of expression, whereas nuclear expression was associated with more favourable features. Finally, the results of this study provide further evidence to support combined use of IHC with the parallel analytic capability of protein microarray RPPA to investigate protein alterations in human tumours.

Acknowledgment

First of all most special thanks and grateful to God, who made all things possible.

I would like to acknowledge and extend my heart gratitude to my supervisors, Professor Ian Ellis, Dr. Andy Green, and Dr. Emad Rakha for their vital encouragement, motivation, enthusiasm, immense knowledge, support supervision, crucial contribution and their valuable advices throughout this project. Their truly scientist intuition have made them as constant supporters with limitless ideas and passions in science, I am grateful in every possible way and hope to keep up our collaboration in the future.

I am grateful to Dr Paddy Tighe and Dr Ola Negm in their fruitful collaboration in proteomic study. I am also indebted to Dr Madhusudan Srinivasan and his group for providing cell lines and help me in cell culture work.

I am also indebted to Dr Jenna Reps and Mohammed Albanghali for their critically important data analysis.

Also this research project would not have been completed without the assistance and technical support of Chris Nolan who gave me so much important advices and to all stuff in Oncology Department.

A special thanks to my family. Words cannot express how grateful I am to my parents (Mr.Tariq and Mrs. Sabah Alsharidah) for all of the sacrifices that they've made on my behalf. Their prayer for me was what sustained me thus far. Moreover, my sincere thanks go to my sisters, brothers and my friends who shared their love and experiences with me. Very special thanks to my friends Munirah Almushawah and Aliyah Aljebreen for their supports.

This study was under the financial support of the Ministry of Higher Education Kingdom of Saudi Arabia; to whom I am indebted.

Declaration

I declare that this thesis is the result of my own work and has not in this, or any other form, been presented to this, or any other university in support of an application for any degree other than for which I am now a candidate.

Alaa Alshareeda

Approval

This work has been approved by Nottingham Research Ethics Committee2

Reference number: REC C20313

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List of Abbreviations

Abbreviation	Definition
53BP1	Tumour protein p53-binding protein 1
AB	Strept ABComplex
ALK	Anaplastic lymphoma kinase
AML	Acute Myeloid Leukaemia
AP-site	Apurinic/Apyrimidinic site
APE1	AP-Endonuclease 1
AR	Androgen Receptor
ATM	Ataxia Telangiectaxia Mutation
ATR	ATM and Rad3-related
BARD1	BRCA1-associated RING Domain Protein1
BC	Breast Cancer
Bcl2	B-cell lymphoma 2
BCS	Breast Cancer Survival
BCSS	Breast Cancer Specific Survival
BER	Base Excision Repair
BFB	Breakage-Fusion-Bridge
BLBC	Basal Like Breast Cancer
BMI	Body Mass Index
BRCA1	Breast Cancer Associated Gene1
BRCA2	Breast Cancer Associated Gene2
BSA	Bovine Serum Albumin
CCND1	Cyclin D1
CDK	Cyclin-Dependent Kinases
CDKIs	CDK Inhibitors
cDNA	complementary DNA
CGH	Comparative Genomic Hybridization
CHK1	Checkpoint Kinase-1
CHK2	Checkpoint Kinase-2
CKs	Cytokeratins
CNA	Copy Number Alterations
CRM1	Chromosome Region Maintenance 1
CSA	Cockayne Syndrome type A
CSB	Cockayne Syndrome type B
CSC	Cancer Stem Cell
CtIP	CtBP-Interacting Protein
DAB	Diaminobenzidine Tetrahydrochloride
DDB	DNA-Damage Binding
DDR	DNA Damage Response
DFI	Disease Free Interval
DNA-PK	DNA- dependent Protein Kinase
DR	Direct Repeat

Abbreviation	Definition
DSBR	Double Strand Break Repair
E1	E1-activating enzyme
E2	oestradiol
E2	E2-conjugating enzyme
E3	SUMO-E3 ligases
E-cadherin	Epithelial cadherin
ECL	Enhanced Chemiluminescence.
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMA	Epithelial Membrane Antigens
EMS1	Excess Microsporocytes1
EPG	Excellent Prognostic Group
ER	Estrogen Receptor
ERCC1	Excision Repair Cross Complementing
FA	Fanconi Anaemia
FANCD2	Fanconi Anemia group D2 protein
FANCF	Fanconi Anemia, Complementation group F
FBS	Fetal Bovine Serum
FBW7γ	F-Box protein
FEN-1	Flap Endonuclease-1
FFPE	Formalin Fixed, Paraffin Embedded tissue sections
FGF	Fibroblast Growth Factor
FISH	Fluorescence In Situ Hybridization
FLARE	Fragment Length Analysis using Repair Enzymes
FOXC1	Forkhead box C1
G0	Gap 0
G1	Gap 1
G2	Gap 2
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GGR	Global Genomic Repair
GPG	Good Prognostic Group
GSTP1	Glutathione S-Transferase P1
HER-2	Human Epidermal Growth Factor Receptor
HNPCC	Hereditary Non Polyposis Colorectal Cancer
HR	Homologous Recombination
HRMA	High Resolution Melting Analysis
HRT	Hormone Replacement Therapy
H-score	Histochemical-score
IBC	Inflammatory Breast Cancer
ID4	Inhibitor of DNA-binding/differentiation proteins
IGF	Insulin Growth Factor
IHC	Immunohistochemistry
IR	Ionizing Radiation
IRIF	Ionising Radiation-Induced Foci
KAP1	KRAB-Associated Protein 1

Abbreviation	Definition
KPNA2	Karyopherin α-2
KRAB	Krüppel-Associated Box
LEF1	Lymphoid Enhancer-binding Factor 1
Lig I	Ligase I
Lig IIIα	ligase IIIα
LN	Lymph Node
MDC1	Mediator of DNA Damage Checkpoint protein1
MDN1	Midasin
MDM2	Mouse Double Minute 2 homolog
MEV	Multi Experiment Viewer
MFI	Metastasis-Free Interval
M phase	Mitosis phase
MLF1	Myeloid Leukemia Factor 1
MMR	Mismatch Repair
MRN	MRE11–RAD50–Nbs1
MTA1	Metastasis- Associated Protein
MPG I	Moderate Prognostic Group I
MPG II	Moderate Prognostic Group II
MUC1	Mucin 1
MYC	Myelocytomatosis
Nbs1	Nijmegen breakage syndrome1
NER	Nucleotide Excision Repair
NES	Nuclear Export Sequence
NGFR	Nerve Growth Factor Receptors
NHEJ	Non-Homologous End-Joining Pathway
NIH	National Institutes of Health
NIR	Near Infrared
NIBC	Non Inflammatory Breast Cancer
NICE	National Institute for health and Care Excellence
NLS	Nuclear Localisation Signal
NPCs	Nuclear Pore Complexes
NPI	Nottingham Prognostic Index
NPM	Nucleophosmin
NRG	Neuregulin
NTS	Non-Transcribed Strand
NST	No Special Type
PARP	Poly ADP Ribose Polymerase
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PgR	Progesterone Receptor
PIAS	Protein inhibitor of activated signal transducer
PIAS1-4	Protein Inhibitor of Activated STAT1-STAT4
Pol β	Polymerase β
PPG	Poor Prognostic Group

Abbreviation	Definition
PTEN	Phosphatase and Tensin Homolog
Rac1	Ras-related C3 botulinum toxin substrate 1
RING	Really Interesting New Gene
RNAi	RNA interference
RNAPII	RNA Polymerase II
RS	Recurrence Score
ROS	Reactive Oxygen Species
RPA	Replication Protein A
RPPA	Reverse Phase Protein Microarray
RTK	Receptor Tyrosine kinases
SAE1	SUMO-conjugation System Consisting of an E1 Activating Enzyme
SAP	Scaffold Attachment factor-A/B/acinus/PIAS
SCID	Severe Combined ImmunoDeficiency
SDS	Sodium Dodecyl Sulphate
SES	Socio-Economic Status
siRNA	Short interfering RNA
SMA	Smooth Muscle Actin
SMC	Structural Maintenance of Chromosome
S phase	Synthesis phase
SSB	Single Strand Break
ssDNA	single stranded DNA
STAT	Signal Transducer and Activator of Transcription
SUMO	Small Ubiquitin-Related Modifier
SVM	Support Vector Machines
TAILORx	Trial Assigning IndividuaLized Options for Treatment
TBS	Tris Buffered Saline
TCR	Transcription Coupled Repair
TDLU	Terminal Duct Lobular Unit
TEBs	Terminal End Buds
TFIIH	Transcription Factor-IIH
T1GFR	Type 1 Growth Factor Receptor
TMA	Tissue Microarray Array
TN	Triple Negative
TOPB1	DNA Topoisomerase 2-Binding Protein 1
TrkA	Tyrosine Kinase receptor type 1
TS	Transcribed Strand
UBC9	Ubiquitin-Conjugating Enzyme 9
UTRN	Utrophin
UV	Ultraviolet Light
V(D)J	Variable, Diverse, and Joining
VI	Vascular Invasion
VPG	Very Poor Prognostic Group
W.B	Western Bolt
XPC	Xeroderma Pigmentosum, complementation group C
XRCC1	X-Ray repair Cross-Complementing protein 1

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Chapter 1

1.1 Introduction

1.1.1 Normal Breast Development: Anatomy and Histology

The development of the mammary gland is complex: proliferation, apoptosis, differentiation and migration are all important in the development of the remarkably organised, branched ductal network of epithelial cells. Despite the existence of the mammary gland in embryos, the majority of the branching morphogenesis required for the development of the ductal tree appears at puberty, along with the release of ovarian hormones. At this time, the distal ends of the mammary ducts will develop into bulbous structures composed of several layers of epithelial cells, known as terminal end buds (TEBs)[8]. These types of TEBs proliferate and ramify and will attempt to invade the adipose tissue, in order to permit the development of a complex branching structure: this action occurs over a period of 10-12 weeks. Following this developmental phase, the TEBs then regress [9]. However, the complex signalling pathways regulate the proliferation of luminal cells.

There are many hormones that have a bearing on stromal cells, in terms of activating the process of branching, such as oestrogen, progesterone and prolactin [10]. During puberty, both oestrogen and epidermal growth factor (EGF) equally regulate ductal elongation and branching. Various other growth factors (for example, fibroblast growth factor (FGF) [11], insulin growth factor (IGF)[12], neuregulin (NRG), amphiregulin and receptors ERBB2/3/4 [13-15]) are also implicated in the development and branching of embryonic mammary gland morphogenesis. Hormones such as progesterone, prolactin and placental lactogens promote alveolar proliferation and differentiation during pregnancy [16]. An awareness of the macroscopic anatomy of the breast and its changes offers several clinical applications, in terms of breastfeeding/lactation support and the discovery, diagnosis and management of the removal of benign and malignant lesions. With regards to the latter, the epithelial cells lining the duct walls are the origin of the majority of breast malignancies [17], underlining the importance of an extensive understanding of the anatomy of the breast.

A couple of different hypotheses concerning cell of origin of BC have already been suggested [18-21]. The first theory states that BC stems from a common epithelial stem cell and subsequent genetic alterations determine the phenotype, while a second hypothesis postulates that BC could possibly originate from distinct cancer stem cells and progenitor cells. Consequently, the phenotype in the latter situation is partially influenced by the epithelial cell of origin differentiation. Primarily based on observational data from molecular studies, for example using gene expression arrays as well as gene transfection models, produced results in line with the theory postulating that biology of the tumour of a BC, at least to some extent, mirrors the biology of the tissue/epithelial cell of origin at the initiation time. It could possibly be that the two theories do not oppose each other and that tumours may possibly grow from different precursor cells such as occasionally coming from a stem

cell and sometimes from diversely developed progenitor cells. It is therefore hypothesised that the type of mutations acquired, the differentiation potential of the cancer cells, and the cell of origin are very likely to make a decision whether a tumour follows a Cancer Stem Cell (CSC) model [22].

In the early 1990s, clinical findings and genetic analyses of a number of cancers led to the hypothesis that six genetic mutations are essential to alter a normal somatic cell into a cancer cell [23, 24]. These six mutations showed specific features such as; self-sufficiency for growth signals, insensitivity to signals of antigrowth, apoptosis evasion, unlimited ability to replicate, sustained angiogenesis, as well as tissue invasion and metastasis. Stem cells possess the strongest potential for proliferation and a considerably longer life span compared to their progeny and as a consequence has a very higher chance to increase genetic mutations [25]. The realisation that the adult body contains small numbers of stem cells provided a substitute probability for the cancer origin. Possibly only single or two mutations, including self-sufficiency in growth or insensitivity to antigrowth signals, are required for stem cells to trigger tumorigenesis rather than six mutations, a rare event in any cell type.

In 1994, John Dick and his colleagues revealed that leukemia-initiating stem cells existing in the peripheral blood of acute myeloid leukaemia (AML) patients [26]. In 2003, a study by Clarke et al effectively demonstrated the presence of stem cells in BC [27] who used a model in which human BC cells were grown in immunocompromised mice, and observed that only a minority of cells of BC possessed the capability to develop new tumours. The authors were able to distinguish the tumorigenic (tumour initiating) from the cancer of non-tumorigenic cells depending on expression of cell surface marker. Clearly they were able to prospectively identify and isolate the tumorigenic cells as CD44+CD24−/lowLineage− in eight of nine patients. Only 100 cells with this phenotype were able to develop tumours in mice, while tens of thousands of cells with alternate phenotypes did not develop tumours. The tumorigenic subpopulation could possibly be serially passaged: each time cells inside this population developed new tumours that contain additional CD44+CD24−/lowLineage− tumorigenic cells and the phenotypically diverse combined populations of non-tumorigenic cells existing in the initial tumour. The chance to prospectively discover tumorigenic cancer cells will certainly help the pathways elucidations that control their growth as well as survival. Additionally, considering these cells drive development of tumour, strategies intended to aim for this particular population may possibly result in more efficient treatments. Thus, CD44+CD24−/lowLineage− cells coming from the majority of tumours appear to express properties of cancer stem cells. Unequivocal demonstration of the stem cell capacity of these cells will need model systems development effective at generation of tumour from an individual cell [28]. Importantly, Clarke et al show that there is certainly BC cells hierarchy in which some cells are able to proliferate substantially, while most of tumour cells that could be extracted out of this population currently have only limited proliferative potential *in vivo*.

It can be hypothesised that tumours originating from migrating uncommitted stem cells are an uncommon event otherwise individuals would develop tumours early in life in

several organs and this would very seriously threaten human viability. Nevertheless, a progenitor cell/stem cell, that is in charge of the development of breast duct (lobe specific) possessing its main influence at or after puberty, during menstrual cycle and at pregnancies/lactation, could possibly be the reason for development of the tumour in women, who already would have a chance of given birth to children, thus not necessarily reducing reproduction of humans [18].

Although the origins of breast carcinoma cells have been studied for decades, controversy remains, in regards to this. The normal parenchymal cells of the mammary gland are composed of two main functional units, ducts and lobules, which are lined by two cell layers: the inner/luminal layer and a distinct outer cell layer, which is juxtaposed to the basement membrane [29, 30]. These units are divided into two main types: luminal/glandular cells and basal/myoepithelial cells. Figure 1.1 shows the normal parenchymal cells of mammary gland. This division is based upon morphological classification (completely remodelled by using the expression profile analysis through complementary DNA (cDNA) microarray), immunophenotypes and the expression of the various markers of these cells. In general, the luminal cells of the inner layers have been linked with the expression of the low molecular weight of cytokeratins (CKs), such as CK7, CK8, CK18 and CK19 [31]. They also tend to express Oestrogen Receptors (ER) and other markers, including Progesterone Receptors (PgR), Mucin 1 (MUCI), α-6-integrin, B-cell lymphoma 2 (Bcl2) and Epithelial Membrane Antigens (EMA) [31]. It is accepted that myoepithelial cells are related to high molecular weight 'basal' cytokeratins, such as CK5; CK14; CK17; Smooth Muscle Actin (SMA) and smooth muscle myosin heavy chain; calponin; P63 [32, 33]; caldesmon; β4 integrin; CD10; P-cadherin; Nerve Growth Factor Receptors (NGFR); laminin; S-100 and caveolin 1 [31, 34, 35]. Moreover, myoepithelial cells are characteristically absent, with regards to the majority of the markers of luminal cells [36]. The term *basal-like* is used as a synonym for the basal/myoepithelial cells of the normal breast, based on the similarities between the molecular profile of these cells [37]. This term generally applies to the expression of a specific sub-population of basal CK to many cells, either in the luminal or the basal site [34].

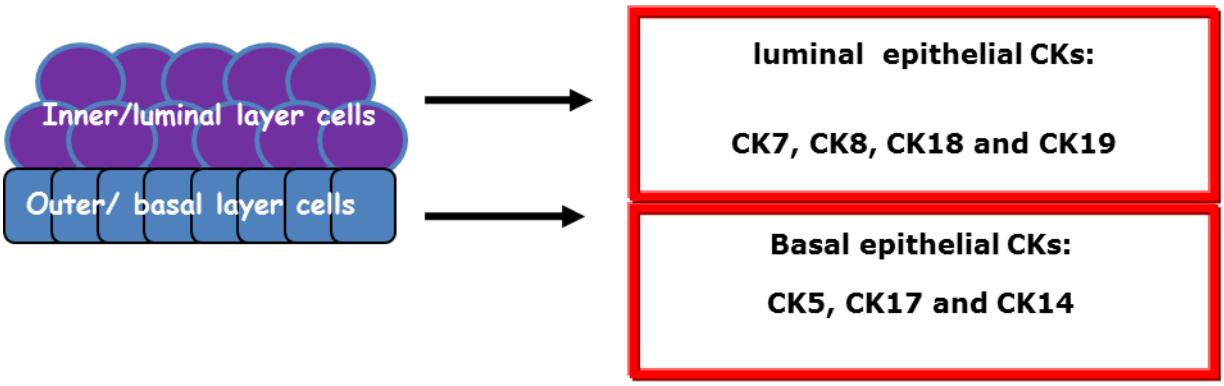

Figure 1.1 Normal parenchymal cells of mammary gland. Luminal cells of the inner layers have been linked with the expression of the low molecular weight of cytokeratins (CKs), such as CK7, CK8, CK18 and CK19, whereas the outer layers (basal) express high molecule CKs such as CK5, CK17 and CK14.

1.1.2 Breast Cancer Epidemiology

BC is a leading cause of death in women around the world. The rate varies (it is up to five times more prevalent in some countries than others), but is increasing in some areas that were previously classified as having a low occurrence of BC [38]. The incidence of this disease amongst women in Europe and North America is approximately 2.7% of the population up to the age of 55 and 7.7% in those aged 75 and over [38, 39]. However, in England, there were 41,259 new cases of clinically-diagnosed BC in 2010, a rise of 1.8% (731 cases) from 2009. In 2010, there were 126 new cases per 100,000 females, compared to 125 new cases per 100,000 females in 2009. These prevalence rates have risen by an alarming rate of 90% between 1971 and 2010. More than 9,700 women died as a result of BC in England in 2011, a rate of twenty-four deaths per 100,000 females. Between 1971 and 2011, this mortality rate dropped by 37%.

1.1.3 Pathogenesis and Aetiology

BC expresses various biological and clinical behaviours: this diversity is shown in the underlying morphologic and molecular differences, with a range of histologic features and molecular pathologic markers that are useful in predicting clinical outcomes and in selecting appropriate therapy [40].

Previously, it was understood that BCs usually originate in the mammary epithelium, through a well-defined, but non-obligatory, sequence of histological alterations; from normal epithelium through to hyperplasia, atypical hyperplasia, *in-situ* carcinoma and, finally, invasive, malignant disease [19-21]. In the standard model of multi-stage tumour growth, the development of a normal epithelial cell progresses into a premalignant atypical cell and, subsequently, clonal enlargement results in a pre-malignant lesion, *in-situ* (step 1). As time

passes, this lesion has the potential to become invasive (step 2) and to disseminate, followed by invasion of the immune system and metastases (step 3) [41]. However, at each and every step, a significant genetic/epigenetic event is thought to occur that offers the cell new features, along with causing a clonal selective advantage for that cell: this model has been verified by a number of molecular studies [41-43]. In addition, a number of studies have implemented comprehensive gene expression and genetic profiling studies in order to compare *in-situ*, invasive, and metastatic BCs but however, these studies didn't determine tumour stage–specific gene signatures [44-46]. Nevertheless, generally, these studies have targeted in depth only the tumour of epithelium cells, whereas the potential involvement of other epithelial and myoepithelial cells as well as the stroma in the progression of the tumour have not been investigated in details. Nevertheless, the rate of normal mutation is limited to the genetic differences that are usually necessary for growth of a tumour. It is thus often suggested that mutations causing genomic instability appear as the initiating event and generate pressure, in order to induce tumourigenesis [47, 48]. Generally, the majority of these hypotheses believe that genomic instability arises from mutations in the various genes associated with cellular functions, such as the repair of DNA and chromosomal segregation. Furthermore, mutations in these genes do not have a direct selective advantage or disadvantage; they only influence the rates of the mutation of other genes[41].

There is a list of many different genes that are supposedly implicated in the tumorigenesis of BC. In sporadic BC, the amplification of genes such as Myelocytomatosis (MYC), Excess Microsporocytes1 (*EMS*1), Cyclin D1 (*CCND*1) and *ERBB2* are essential in the development of such cancer. In addition, growth factors, such as EGF and IGF-1, demonstrate some role in the growth or proliferation of BC [49-54]. BC type 1 susceptibility protein (BRCA1), as a tumour suppressor, has an essential role in maintaining genomic stability. It interacts with various proteins and, in addition, the complexes formed with BRCA1-associated RING domain protein 1 (BARD1) are involved in the recognition and repair of DNA [55]. Germline mutations in *BRCA*1 confer susceptibility to breast and ovarian cancer. *BRCA*1 mutations can occur across the gene and comprise different types including insertions, deletions, frame-shifts, base substitutions or inferred regulatory mutations. In sporadic BC, *BRCA*1 is infrequently mutated and previous studies did not identify coding region mutations and non-coding regulatory region mutations are extremely rare. In contrast, reduced BRCA1 protein expression has been reported in a subset of these cancers, suggesting impaired function of BRCA1 [55-59]. However, this proposed dysfunctional status could be due to epigenetic defect or abnormalities in any pathway is directly or indirectly related to BRCA1 such as BRCA/Fanconi Anaemia (FA) pathway [60]. Evidence is accumulating that dysfunction of BRCA1, could possibly be essential in the pathogenesis of a significant proportion of sporadic, non-familial BCs. This concept arises from several lines of evidence, which includes both phenotypic analyses of tumours and mechanistic studies showing inactivation of components of these pathways. Underlying different DNA-repair defect, arising from loss of homologous recombination that has been proposed is a defining tumours property with BRCA/FA pathway inactivation. The FA proteins are implicated in similar DNA-repair pathways to BRCA1. There is an evidence shows that a significant proportion of

sporadic cancers inactivate the pathways of BRCA/FA by methylation of Fanconi Anemia, Complementation group F (FANCF) [61]. However, methylation of FANCF has been observed in different types of sporadic cancer, such as ovarian, breast and oral cancers, and non-small-cell lung and cervical carcinomas [61-63]. Other important and related genes involved in BC include *BRCA2* and *TP53*. *BRCA2* mutational inactivation is infrequent, mainly as it requires both gene copies to become mutated or even completely lost [55, 64]. Mutation of *TP53* remains the most frequent genetic change revealed in human neoplasia. Just like the *BRCA1* mutation, the *TP53* mutation is associated with more aggressive disease and poor overall survival; however, it accounts for only a small proportion of hereditary BC [65].

The subtypes of ER-α and ER-β are encoded by different mRNAs, but show a similar structural and functional domain composition [66]. ER regulates gene expression through ER-dependent and ER-independent mechanisms, ending with the stimulation of gene transcription, such as that of cell cycle control proteins. Despite the fact that the *ER-α* and *ER-β* genes show a large degree of homology, it is generally believed that their distributions and functions are significantly distinct in many tissues. The recent development of reliable antibodies to ER-β has offered an evaluation of the hypothesis that the likelihood of malignant transformation in morphologically benign breast lesions can be precisely identified by the distribution and amount of expression of ER-β, in accordance with that of ER-α [67]. The overexpression of ER-α can often be seen in the early stages of BC [68] and the relevance of ER-β in BC is much less distinct than that of ER-α. The existence of mRNA of ER-β has been revealed, both in normal and malignant mammary gland tissue [69]. However, Shaaban et al revealed that a decreased level of ER-β in accordance with ER-α is a definitive predictor of individual cases of hyperplasia of the type more likely to develop into invasive BC, thus supporting the idea that transcriptional activity of ER-α is directly controlled by ER-β [70]. However, isoforms of PgR, PgR-α and PgR-β show distinctive physiological functions, yet their separate functions in BC are not clear [71].

Despite the fact that both normal and pre-invasive breast cells show peripheral myoepithelial layers, significant differences exist between them [72]. The transition from hyperplasia to atypical hyperplasia is clinically related to a higher risk of BC and the subsequent phase is usually a marker of progression to carcinoma *in-situ* which can be defined as a proliferation of cells together with cytological features of malignancy, but without stromal invasion throughout the basement membrane [41]. Malignant tumours are recognised by aberrant cellular differentiation, together with disorganised growth and increased proliferation rates and the ability to invade [73]. Cells become invasive when they migrate from the basement membrane and invade the stroma. As a result of dissemination through the lymph vessels, invasive cells can metastasise, either to loco-regional lymph nodes or to distant organs. Most invasive breast carcinomas are ductal (75–85%) and infiltrating lobular carcinoma that comprises approximately 10% of all BCs. Although ductal and lobular terminology were derived from breast ducts and lobules to reflect the site of origin, the well-

known model in evolution of BC published by Wellings and Jensen suggested that the origin of most BCs is the Terminal Duct Lobular Unit (TDLU) [74, 75].

1.1.4 Risk Factors for Breast Cancer

Epidemiological BC studies have proven that hormonal factors play a major role in the causation of the disease, which is mainly linked to ER. However, there are many other factors that contribute to an increased risk of BC, such as early menarche, late menopause, high-levels of endogenous oestradiol and obesity in postmenopausal women [38].

1.1.4.1 Age

The age of a patient is highly related to the incidence of BC; the risk doubles every 10 years up to the menopause, when the level of increase drops significantly [76]. Indeed, in some countries, there is a flattening of the age incidence curve just after menopause [77-85]. Age-adjusted prevalence and the death rate for BC differ markedly between countries, by up to a five-fold increase. The variation between Far Eastern and Western countries is decreasing, although it remains that the death rates in Western countries are five times that of those in the Far East. In migrants from Japan to Hawaii, it was demonstrated that the rate of the incidence of BC assumed the rate of the host country over two generations. This highlights the fact that environmental factors are generally of higher importance than genetic factors. Figure 1.2 shows the incidence rate of BC by age group for women in the UK. The average woman under the age of 40 has a significantly lower chance of developing BC.

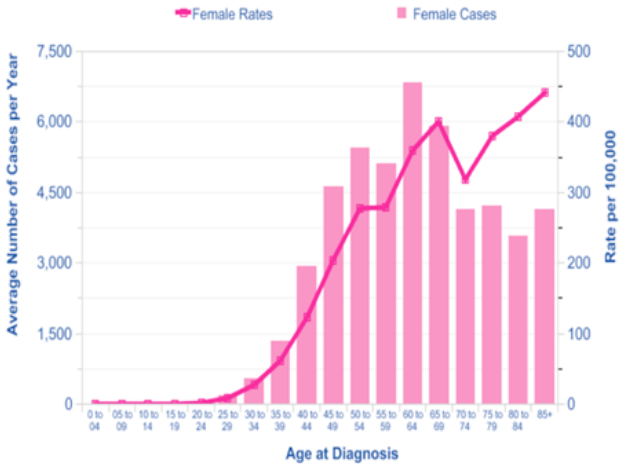

Figure 1.2 Incidence rate of breast cancer by age group for women in the UK. The average woman under the age of 40 has a significantly lower chance of developing breast cancer. Taken from Cancer Research UK.

1.1.4.2 Hormonal Factors

It has been consistently demonstrated that ER is usually induced and enhanced in mammary tumours in rodents. Women who have a bilateral oophorectomy before the age of

thirty-five have only a 40% associated risk of BC, compared to women who go through a normal menopause[86]. The increased risk of women who experience normal menopause is thus due to hormonal influence, via ER [86, 87].

Many experts have analysed the relationship between lactation and the incidence of premenopausal BC in cohort studies, with varying outcomes. Two independent studies have claimed an inverse association between lactation and the possibility of premenopausal or early-onset BC [88, 89], while two other studies showed no association [90, 91]. Such inconsistent findings may possibly be due to variation in age at diagnosis between the studies populations. However, in studies that indicate a protective association, cases were considerably younger. This is consistent with several cohort studies that observed there was no association between lactation and post-menopausal BC [90-92]. However, extended durations of lactation might confer protection for older women [93].

Recent epidemiological studies have inconsistently revealed a modestly-increased BC risk associated with hormone replacement therapy (HRT) [94] (specifically, those women using a combination of progestin with oestrogen, rather than oestrogen alone, or those who experienced long-term use of HRT [95, 96]). Limited knowledge is known, with regards to the different formulations of HRT (and progestins in particular).

1.1.4.3 Family History

It has been hypothesised that BCs in women with an inherited predisposition to such cancers tend to be phenotypically and prognostically distinct. Despite the fact that family history is a well-established aetiological risk factor for BC, its association with survival is still ambiguous. Many different studies have recorded an increased survival for females with a family history of BC [97-100], whereas other studies state little or no difference in survival rates [101-103] or, even worse, in survival [104, 105]. Susceptibility to BC is usually inherited as an autosomal dominant, with limited penetrance. Yet it is believed that only 20% to 25% of the occurrence of BC in first-degree relatives of women affected by the disease may be related to mutations in well-known genes: this includes the high-penetrance susceptibility genes *BRCA*1 and *BRCA*2, in addition to the moderate and low-penetrance genes identified up to the present time [106, 107]. In the UK, out of the whole of recognised BCs, the mutation of *BRCA*1 accounts for around 2% of the total incidence of the disease [108].

Table 1.1 shows additional common risk factors of BC.

Table 1.1 Additional Risk Factors of Breast Cancer

Risk Factors	
Socio-Economic Status (SES)	SES has been associated with most of the recognised or suspected risk factors for BC incidence and progression. SES includes access to medical care across the world, screening programmes, self-awareness and level of education[109].
Body Weight	High body mass index (BMI) has been linked to an elevated risk of BC in post-menopausal women[110]. Obese post-menopausal women present 10-30% higher risk of developing BC, whereas the risk is reduced by 20% in obese pre-menopausal women [110]. Higher serum oestrogen levels and enhanced local production of oestrogen have already been considered primary mediators of how increased body weight promotes development of BC in post-menopausal women [111]. In pre-menopausal women, the reduction in the risk would probably be as a result of an ovulatory menstrual cycle stimulated by obesity whereas, in post-menopausal women, there is trend for an oestrogen increase in the circulation as a result of hormones conversion in the fatty tissue as the main source of oestrogen in post-menopausal women [112].
Body Height	In UK, there is a highly significant trend of risk of BC with relative risk of 1.16 for every 10cm greater in height [113].
Breast Density	High breast density is a common and strong risk factor for BC [114]. Post-menopausal hormone therapy, particularly oestrogen and progestin, enhances density of the breast [115] as well as the risk of BC [116]. Regardless of whether density of breast provides a higher effect on the risk of BC for some subgroups of women identified by menopausal status and use of post-menopausal hormone therapy remains unknown [117].
Smoking and Alcohol Consumption	On the basis of epidemiological data, alcohol and tobacco has been classified as human carcinogens [118].
Diet	There is an increase of BC risk by 13% with the higher level of fat intake [119]. In a large study carried out on European women, there was a positive correlation found between intake of high fat and risk of BC and this was mainly observed among post-menopausal women [120].
Ethnicity	A number of studies have linked ethnicity with the prognosis of BC [121, 122]. It has been stated that variation in survival between different ethnic groups is a reflection of many factors such as effect of place of birth as well as residence place [123]. A study by Komenaka et al on Hispanic whites and African Americans populations showed that African American patients had poorer BC–specific survival than non-Hispanic white patients. [124]. On the other hand, Soliman et al showed that Egyptian women less than 60 years had higher mortality rate and lower rate after 60 years compared to African-American women [125].

1.1.5 Overview of the Prognostic Factors for Breast Cancer

A wide variety of clinical and pathological factors are consistently used to categorise patients with BC, in order to be able to evaluate prognosis and identify suitable therapy. Such factors are age of patient, status of axillary lymph nodes, tumour size, histological features (mainly histological grade and lymphovascular invasion), the status of hormone receptors and, finally, the status of Human Epidermal Growth Factor Receptor 2 (HER-2) [126]. Taking into account these factors in combination is of more significant clinical value than

considering each in isolation and this combined strategy creates the structure of several schemas, which are used to classify patients into risk groups. Such schemas include the St Gallen criteria [127, 128], the National Institutes of Health (NIH) consensus criteria [129] and the Nottingham Prognostic Index [130].

1.1.5.1 Clinico-pathological Factors as Prognostic Factors

1.1.5.1.1 Age at Diagnosis

Age upon diagnosis of BC has a significant effect on the rate of a patient's survival. Females under 35 years demonstrate significantly worse survival rates, in comparison to females aged 70 years and over [131, 132]. Younger patients are more likely to have poor clinical characteristics, such as larger tumour size and higher histological grade, lymphatic vessel and the involvement of lymph nodes [122, 133]. In addition, tumours in younger women are usually associated with the overexpression of mutated P53 protein, the amplification of HER-2, negativity of ER or PgR and Basal-Like BCs (BLBC)[122, 134]. Furthermore, it has been estimated that these women have a higher morbidity risk compared to other age groups [132].

1.1.5.1.2 Histological Type

The histological type of invasive BC presents valuable prognostic information [135, 136]. With regards to its relationship with BC, along with outcomes, histological typing needs to be regularly conducted for every case of invasive BC. Ductal carcinoma of no special type (NST), accounting for up to 75% of all BCs, has the worst prognosis, whereas tubular BC demonstrates the best prognosis. However, some other special types of invasive carcinoma fall in between these two prognostic extremes, including mixed ductal NST and classical lobular carcinoma, with 55% of patients experiencing a 10-year survival rate [137-139]. Inflammatory BC (IBC) has the most severe prognosis, in terms of stage III tumours, when compared to non-inflammatory BC [140]. Despite the prognostic significance of the histological type of a tumour, its part in the decision of clinical management is reasonably limited [141].

1.1.5.1.3 Tumour Size

Size of tumour is one of the most highly-effective prognostic factors and is a predictor of the behaviour of BC; the larger the tumour, the worse the patients' outcomes [142]. Indeed, the size of a tumour is an indication of how long has tumour been present. In addition, the size of a tumour can be used by radiologists as a standard tool in the screening of BC, in revealing impalpable nodules simply through mammography [143]. The rate of the occurrence of metastases to the axillary nodal in patients with tumours smaller than 1cm in

size ranges between 10-20%. Additionally, patients who are node-negative and have tumours smaller than 1cm have an approximate 90% ten-year disease-free survival rate [144]. Screening data, together with measurements of a tumour, presents population-based estimates of tumour growth, as does screen test sensitivity directly associated with the size of a tumour. There are certainly significant differences in tumour growth in BC, with faster growth rates amongst younger women [145]. The growth of a tumour can be simply determined by comparing the tumour sizes of clinically-detected and screening-detected cases; however, the applied statistical models only employ such data to some extent. Chen et al [146] applied tumour size within a classical Markov model, while Van Oortmarssen et al [147] incorporated tumour size in a simulation approach, yet each of these studies only classified size of tumour into two or three classes. Some clinical observation studies, however, completely apply measurements of tumour size, together with tumour growth, modelled as being a continuous function of tumour size [145].

1.1.5.1.4 Tumour Histological Grade

Grade of tumour is an important predictor of overall and disease-free survival [131, 148, 149] and categorisation is based on the level of similarity to normal tissue; for example, well, moderate and poorly differentiated. A modification of the Bloom-Richardson histological grading of invasive breast carcinoma, the Nottingham Grading System is one of the most powerful factors in offering important prognostic information [150]. Grading contributes to the management of treatment for patients receiving adjuvant therapy, in addition to inclusion in the Nottingham Prognostic Index (NPI) [151, 152]. The Nottingham Grading System consists of three tiers, dependent upon the reporting of three factors showing the degree of tumour differentiation: these are tubule formation level, mitotic frequency and nuclear pleomorphism. The histological grade helps to measure the biological aggressiveness of tumours and, in most cases, this does not alter as time passes [151, 152]. In studies carried out over the space of 10 years, in which the survival rates based on histological tumour grade in combination with the stage of lymph node involvement and size of tumour were assessed, patients presenting as histological grade 3 and stage 1 experienced similar survival rates as those patients with histological grade 1 and stage II disease. Regardless of the status of the lymph nodes, patients with a histological grade 1 tumour less than 1cm in size had an excellent prognosis, with an approximate 99% 5-year survival rate [152, 153].

1.1.5.1.5 Lymph Node Status (LN)

The presence of metastatic BC within the axillary lymph nodes is a very important predictor of overall and disease-free survival rates [131, 148, 149]. It has been observed that there are five variables in this, in which infiltrated lymph node staging was, by far, the identifying factor in predicting early recurrence. It was also second most prominent in gauging the probability of survival, after tumour size [148].

1.1.5.1.6 Nottingham Prognostic Index

The NPI includes three important prognostic factors (status of lymph nodes, tumour size and histological grade) and the prognostic significance has been confirmed by several studies conducted across the world [137]. The calculation of the NPI was described previously, as follows [154, 155]: lymph node (LN) stage (1–3) + Grade (1–3) + maximum diameter (cm × 0.2), offering a NPI range from 2.08 (LN negative, grade 1, 0.4 cm) to 6.8 (LN Stage 3, grade 3, size 4.9 cm). The NPI is a useful tool regarding the prognosis of patients' risk, in addition to stratification, offering improved prognostic significance more than any of its individual components. NPI predicts the survival rate of invasive breast carcinoma patients and is needed in the assigning of patients into therapeutic routines. In earlier studies, three subsets of risk groups were designated: good prognosis (≤3.4), moderate prognosis (3.41-5.4) and poor prognosis (>5.4) [155, 156]. However, Blamey et al reported six different NPI groups: an Excellent Prognostic Group (EPG), with an observed NPI range of 2.08–2.4, a Good Prognostic Group (GPG), with an observed NPI range of 2.42 to ⩽3.4, a Moderate Prognostic I Group (MPG I), with an observed NPI range of 3.42 to ⩽4.4, a Moderate Prognostic II Group (MPG II), with an observed NPI range of 4.42 to ⩽5.4, a Poor Prognostic Group (PPG), with an observed NPI range of 5.42 to ⩽6.4, and a Very Poor Prognostic Group (VPG), with an observed NPI range of 6.5–6.8 [157].

1.1.5.1.7 MammaPrint™ Assay

The MammaPrint (Agendia BV, The Netherlands) is the first fully commercialised microarray-based multigene assay constructed to individualise treatment method for BC patients. In addition it helps to assess the risk that a breast tumour will metastasise to other parts of the body. MammaPrint offers a prognostic test for most women under the age of 61 years with either ER^+ or ER^- BC with negative lymph nodes. The test of MammaPrint requires freshly prepared tissues collected into an RNA preservative solution. The 70 genes that comprise the assay of MammaPrint are focused mainly on proliferation with other genes related to invasion, metastasis, integrity of stroma and angiogenesis [158]. MammaPrint assay has advantages over other known tests such as Fluorescence In Situ Hybridization (FISH) or Polymerase Chain Reaction (PCR) mainly because, in a single analysis, MammaPrint can evaluate the expression of all of the genes that could be involved in a cancer, instead of just a few. This can make the identification of cancer subtype more accurate than single gene tests [159]. Saghatchian et al have created a MammaPrint profile from frozen tumours of patients operated from primary BC and classified the samples as genomic high risk (were mainly ductal carcinomas (93%), grade 3 (60%), ER and PgR-negative, and HER-2 positive (25%), or genomic low risk (the 5-year overall survival was 97% vs. 76% for in the genomic high risk group ($P < 0.01$); distant metastasis free survival at 5 years was 87% for genomic low risk patients and 63% for genomic high risk patients ($P < 0.01$) [160].

1.1.5.1.8 Oncotype DX Breast Cancer Assay

The Oncotype DX BC assay is a reverse transcriptase polymerase chain reaction assay on RNA extracted from sections of formalin-fixed, paraffin-embedded tissue samples. It was initially developed for women with early-stage invasive BC with ER^+ cancers and node negative. Generally in these cases, anti-hormonal therapy, such as Tamoxifen or aromatase inhibitors, is planned, and therefore, Oncotype DX can help to predict the benefit of chemotherapy and the probability of distant BC recurrence [161, 162]. Around 25,000 genes in the genome of human, Genomic Health determined 250 candidate genes possibly involved in tumour behaviour of BC. These genes were subsequently tested in over 400 patients from three independent clinical studies to be able to determine a 21 gene panel highly correlated with distant recurrence-free survival [163]. The panel includes 16 cancer genes and five reference genes used to normalise the cancer genes expression. The three clinical studies also developed the base for the Recurrence Score (RS) calculation, which helps to combine the data of gene expression from the 21-gene panel into a single result as well as addresses 3 of the 7 metastatic pathways [162]. Flanagan et al studied 42 BC cases and evaluated them by Oncotype DX. However, these cases were retrospectively reviewed to identify the age of the patient, the size of the tumour, histologic grade, ER, PgR, and HER-2 status. they found that RS is significantly correlated with tubule formation, nuclear grade, mitotic count, immunohistochemical score of ER, PgR and HER-2 status, and that the equation RS=13.424+5.420 (nuclear grade) +5.538(mitotic count) −0.045 (ER immunohistochemical score) −0.030 (PgR immunohistochemical score) +9.486 (HER-2) predicts the RS with an R^2 of 0.66, revealing that the full model accounts for 66% of the data variability. Despite the fact that the Oncotype DX RS remains potential, further validation of its independent value beyond that of histopathologic analysis is recommended before it can be applied in clinical decision making [162]. However, National Institute for health and Care Excellence (NICE) has today recommended the use of a test as an alternative that can help clinicians make a decision whether to recommend chemotherapy in people who have early BC. Oncotype DX is being assessed in lymph node negative, HER-2 negative, ER positive BCs in a prospective trial, the Trial Assigning IndividuaLized Options for Treatment (Rx) (TAILORx) [164], launched 2006 May, enrolled 10,000 people with intermediate results on the test; results are expected to be accomplished in 2014.

1.1.6 Classification of Breast Cancer

The application of high-throughput technologies, such as cDNA microarray and Tissue Micro Array technology (TMA), and analytical tools that review thousands of genes and their products in clinical breast material have created a step towards the categorisation of BC, depending on gene or protein expression patterns. Perou et al applied hierarchical clustering to a set of 65 breast tissue specimens from 42 patients (one ductal carcinoma *in-situ*, one fibroadenoma, two lobular carcinomas, 36 invasive ductal carcinomas and 2 normal breast samples) [165]. The tumours were classified into different molecular subtypes, in

accordance with similarities in gene expression, heterogeneous clinico-pathological features and responsiveness to therapy. The prognostic association of these results have been corroborated by comparable expression in other groups and the researches of Sorlie et al and others support these results [166, 167].

BC is broadly divided into two groups: ER positive (ER^+) and ER negative (ER^-), leading to subdivisions into more biologically and clinically relevant subgroups [165, 166]. ER^+ tumour subgroups are known as the luminal group, due to their expression of genes that encode the characteristics of the proteins of luminal epithelial cells. ER^- tumours are subdivided into HER-2 positive, BLBC and normal breast-like tumours [168]. BLBCs are characterised by the expression of basal epithelial cell markers (such as CK5[33], CK14 and CK17[30, 165]) and other markers, such as nestin [169], c-kit [170] and Epidermal Growth Factor Receptor (EGFR) [33]. The variation of gene expression within these subtypes is expected to characterise different tumour subtypes, influencing overall outcome and response to treatment. BCs with an HER-2 positive phenotype and basal-like categories have the poorest prognosis [34, 37, 165].

1.1.6.1 Molecular Classification of Breast Cancer

Morphologically identical BCs can show divergent clinical outcomes and responses to treatment. This can mainly be related to variations of molecular features that vary among histologically similar types of cancer. As a result, molecular classification can be as powerful as histopathology in predicting behaviour of tumours and even more powerful as a predictive for treatment response. The accumulation of genomic aberrations is an essential aspect of the development of solid-tumours and especially breast tumour. However, identification of behaviours through assessment of genomic and epigenomic characteristics of tumours may expose primary mechanisms of disease evolution and identify potential candidates for therapy intervention. Recent improvement of high-throughput molecular methods provides new chances for capturing the wide variety of genomic and biologic variability in tumours [171]. By classifying breast tumours based on their particular DNA Copy Number Alterations (CNA) patterns, three classes were revealed and known as the 1q/16q, amplifier, and complex subtypes. Tumours of the 1q/16q genomic subtype had better disease-specific survival than the amplifier and complex subtypes. In contrast, BLBC (as defined based on gene expression profile) were found mainly within the complex genomic subtype [171].

Jönsson et al have identified global DNA copy number and profiling of gene-expression breast tumours. However, they found various amplicons to co-occur, the 8p12 and 11q13.3 regions appearing the most repeated combination apart from amplicons on the same arm of the chromosome. Using unsupervised hierarchical clustering with 133 significant GISTIC regions, six genomic subtypes were revealed, termed 17q12, basal-complex, luminal-simple, luminal-complex, amplifier, and mixed subtypes. However, tumours of luminal A type were distributed in two main genomic subclasses, luminal-simple and luminal-complex, the simple group developing a greater prognosis, in contrast the complex group included as

well luminal B and most of *BRCA2*-mutated tumours. The basal-complex subtype demonstrated comprehensive genomic homogeneity and harboured the greater number of tumours with a mutation in *BRCA1* [172]. However, Cornen et al [173] have showed that mutations of *KCNB2* (Potassium voltage-gated channel subfamily B member 2) were associated with luminal B tumours while candidate tumour suppressor genes such as *Midasin* (*MDN*1*)* (6q15) and *Utrophin* (*UTRN)* (6q24), were mutated in this subtype.

Bergamaschi et al [174] have analysed ER negative tumours and demonstrated that HER-2 positive tumours showed amplification at 17q12-q21, whereas, the recurrent CNAs identified in BLBC had increases at 1q12-q41, 6p12-p25, 7q22-q36, 10p12-p15, 17q25 and 21q22, but losses at 3q12, 4p15-p32, 4q31-q35, 5q11-q31, 14q22-q23, 17q and 20p [211]. However, CNAs of some of these subtypes were harbouring *BRCA1*, *EGFR* and had a dysfunction in the pathways of *caveolin1* [174-176]. It has been shown that, within BLBC, loss of the function of *BRCA1* is more frequent than in other BC biological subtypes, might be suggestive of defective double strand DNA repair pathways [177, 178]. In regards to double strand DNA repair, [179] Gao et al have showed that *Rad*51 135G/C polymorphism may be identified as a susceptibility locus for BC. Recently, Curties et al [180] have presented an incorporated analysis of CNAs and gene expression in primary breast tumours. However, they found that inherited variants (copy number variants as well as single nucleotide polymorphisms) and additionally acquired somatic CNAs were related to expression in 40% of genes, with the landscape dominated by *cis* and *trans*-acting CNAs. In addition they have discovered genes of putative cancer, such as deletions in *PPP2R2A*, *MTAP* and *MAP2K4*. However, using paired profiles of DNA–RNA shown novel subgroups with different clinical outcomes, which reproduced in the validation cohort were identified, such as a high-risk, ER-positive 11q13/14 *cis*-acting subgroup and a good prognosis subgroup lacking of CNAs. In addition, several signalling molecules, transcription factors and genes of cell division were related in *trans* with this deletion event in the BLBC, which includes alterations in *AURKB*, *BCL2*, *BUB1*, *CDCA3*, *CDCA4*, *CDC20*, *CDC45*, *CHK1*, *FOXM1*, *HDAC2*, *IGF1R*, *KIF2C*, *KIFC1*, *MTHFD1L*, *RAD51AP1*, *TTK* and *UBE2C*. Significantly, TTK (MPS1), a dual specificity kinase that helps *AURKB* in alignment of chromosome during mitosis, and recently revealed to enhance aneuploidy in BC [181], was seen up-regulated. However, cluster analysis proposed 10 groups (based on Dunn's index); Cluster 1: many intermediate prognosis groups of predominantly ER^+ cancers were documented, such as a 17q23/20q *cis*-acting luminal B subgroup, Cluster 2: ER^+ subgroup consist of 11q13/14 *cis*-acting luminal tumours, in addition, high frequencies of amplification was found for both *CCND1* and *EMSY*. Therefore based on the findings, the 11q13/14 amplicon could possibly be influenced by a gene cassette instead of only one oncogene. Cluster 3: this group showed low genomic instability, however, it was mainly luminal A cases, and was enriched for histopathology types that usually have good prognosis, such as invasive lobular and tubular carcinomas. Cluster 4: similar to Cluster 3 by showing good prognosis, but however, it showed both ER^+ and ER^-, in addition, most of the cases were extensive lymphocytic infiltration. Cluster 5: The *ERBB2*-amplified cancers characterized by HER2-enriched (ER^-) and luminal (ER^+) cases. Cluster 6: many intermediate prognosis

groups of predominantly ER^+ cancers were documented, such as an 8p12 *cis*-acting luminal subgroup. Cluster 7: Luminal A subgroup with favourable outcome and was characterised by the lack of the 1q alteration at the same time maintaining the loss of 16p gain/16q with greater frequencies of amplification of 8q. Cluster 8: showed similar CNA profiles to cluster 7, so it was luminal A subgroup with favourable outcome and was characterised by the classical 1q gain/16q loss. Cluster 9: many intermediate prognosis groups of predominantly ER^+ cancers were documented such as an 8q *cis*-acting/20q amplified mixed subgroup. Finally Cluster 10: this was BLBC with stable and mainly high genomic instability, in general it showed relatively good long-term outcome and characteristic *cis*-acting alterations (5 loss/8q gain/10p gain/12p gain), additionally, alterations of several transcription factors and many cell division genes were observed in BLBC [180].

Although there is no international agreement or consensus regarding the definition of various molecular classes in BC, the most commonly reported molecular classes based on gene expression profiling are as follows;

1.1.6.1.1. Normal Breast-like Class

Normal breast-like subtypes have a similar gene expression profile to that of normal breast tissue, with a high level of many genes characteristic of adipose cells and other types of non-epithelial cell [165, 182]. However, there is some concern that this subtype may not exist and is due to technical artifact, where samples have mainly consisted of normal breast tissue, rather than tumour [183]. In addition, Holm et al have stated that normal-like tumours did not show unique profiles of methylation [184].

1.1.6.1.2. Luminal Class

This is the largest class of BC mainly characterised by hormone receptor expression. There are at least two main luminal subgroups, commonly known as luminal A and luminal B tumours [165, 166]. However, a luminal C subgroup has also been reported in several studies, but this is less well-defined than the luminal A and luminal B subgroups [166, 167]. Luminal A tumours are frequently characterised by the positive expression of ER and negative HER-2, whereas luminal B is related to positivity of ER^+ and overexpression of HER-2 and/or high proliferation status [166, 182]. Luminal B tumours have a significantly worse prognosis than luminal A tumours, and show lower expression of ER than luminal A [185, 186]. Luminal B tumours express luminal CK8, CK18, CK19 and genes related with the activation of ER, such as *LVI* and *cyclin D1*, although some carry mutations in *TP53*, which are usually grade 1 [165, 182]. In most cases, luminal subtypes carry a good outcome [168]. Yanagawa et al showed that luminal A cases showed lower expression of the Ki-67 antigen, compared to luminal B [187]. Luminal B BCs with Ki-67 levels of at least 14% showed a poorer prognosis for both BC recurrence and survival compared with luminal A tumours with level of Ki-67 less than 14% [188]. In addition, Feeley et al[189] have defined luminal A as being ER

positive, HER2 negative, and Ki-67 low (<14% cells positive), whereas luminal B subtype as being ER positive, HER2 negative, and Ki-67 high (≥14% cells positive). In their study, patients with luminal B tumours showed worse disease-free survival than tumours of luminal A. However, when they used status of TP53 or negativity of PgR rather than Ki-67 in order to classify ER-positive luminal tumours, a similar outcome finding to those obtained using the proliferation index was observed. Therefore, Ki-67 index, status of TP53, or negativity of PgR can be helpful to segregating ER-positive, HER2-negative tumours into prognostically significant subgroups with significantly various clinical outcomes. These biomarkers particularly in combination may possibly be applied clinically to direct patient management. However, few chromosome rearrangements have been seen in luminal A tumours, whereas, luminal B tumours showed many more rearrangements, which occurred mostly within amplicons. Additionally, BC cell lines with luminal expression patterns show frequent amplification of DNA, however, this was luminal B rather than A subtype [190].

1.1.6.1.3 HER-2-Positive Class

HER-2 is overexpressed in 10-20% of BCs and is associated with a poor outcome. *HER-2* amplification plays a direct role in the pathogenesis of BCs, which has been a focus for therapeutic agents targeted directly at this amplification. In addition, the HER-2 status is predictive, with regards to response to certain chemotherapeutic agents (for example, doxorubicin (Adriamycin) and HER2-targeted therapies (trastuzumab, pertuzumab and lapatinib [34, 191]).

Holm et al [184] have stated that HER-2-enriched molecular subtypes did not show unique profiles of methylation. Although gene expression profiling has identified a HER-2-enriched subtype, it needs to be considered that tumours of HER-2-positive are observed in all molecular subtypes of BC [192, 193], and that expression profiles of HER-2-positive tumours are very heterogeneous [194]. However, the study of Holm et al added support to the heterogeneous picture of HER-2-positive BC, and proposed that amplification of *HER-2* does not have an effective feature influence on patterns of methylation. However, BC cell lines with luminal expression patterns show frequent amplification of DNA, which typifies HER-2 subtype tumours [190]. Bignell et al have investigated amplicon associated rearrangements at the DNA sequence level. In a single *ERBB2*-amplified BC line, they determined at 17q12-q21 (*ERBB2*) the exact inverted duplication architecture predicted by a Breakage-Fusion-Bridge (BFB) process of sister chromatid. On the other hand, amplicons in other places in the genome involved direct repeats of head-to-tail, showing that different amplification mechanisms are not mutually exclusive, and can also arise inside the same cancer cell [195].

1.1.6.1.4 Basal-like Breast Cancer Class

Approximately 15% of BCs are basal-like in origin and are associated with a higher histological grade, poor overall survival and younger patient age [196, 197]. Myoepithelial cells are characteristically negative for the majority of the markers of luminal cells [36]. The term *'basal-like'* is used as a synonym for the basal/myoepithelial cells of a normal breast, based on the similarities of their molecular profiles [37], and this term generally stems from the expression of a specific sub-population of basal CK to many cells, either at the luminal or basal site [34]. In the initial gene expression profiling studies of Sorlie and Perou et al [165], both CK5 and CK17 were identified as key markers in BLBC[198]; however, CK17 protein, using immunohistochemistry, is expressed in very few tumours and in the absence of CK14 and CK5 [199]. Various studies have shown the implication of some basal-like genes in cellular proliferation, suppression of apoptosis, extracellular remodelling, cell migration, invasion and other markers of cancer [23, 37, 186].

1.1.6.1.4.1 Triple Negative Breast Cancer

Triple Negative (TN) BC and BLBC have become a key topic of research interest, due to their aggressive behaviour and lack of targeted therapy [165]. The term 'triple negative' refers to the lack of ER, PgR and HER-2 expression and is highly associated with a poor prognosis [200]. Patients with these phenotypes are unlikely to benefit from current endocrine or HER-2 targeted therapies; however, it should be considered that the characterisation of TN and BLBC remains controversial. Although the terms appear synonymous, many biological phenomena, immunophenotypes and responses to chemotherapy are different between TN and BLBC; therefore, they remain pathologically and biologically heterogeneous [201]. The refinement of the TN phenotype through the use of additional basal markers may be helpful in reducing the heterogeneity of TN tumours and expanding understanding of the clinical and biological features of these tumours. Consequently, this can justify a specific therapeutic target for each individual tumour class and thus help personalise treatment for BC patients.

Recently, several differences between TN and BLBC have been reported, through the use of $CD44^{+}/CD24^{-}$[202, 203], cell cycle regulators and immunologic markers, such as P53 and MUC2/MHC class 1 [200, 202-205]. Rakha et al supported these studies, which showed an association between a high proliferation rate and BLBC. In contrast, when compared with TN, BLBC did not significantly show a difference in tumour grade, size or vascular invasion [205]. A study of 823 BLBC patients receiving anthracyclines, defined as negative for HER-2 and ER but positive for either EGFR or CK5/6, received less benefit from the anthracyclines than those patients in the TN group, who were negative for all these markers [206]. Cheang et al also supported this result. [196]. Despite this, potential clinical trial plans are undoubtedly needed, in order to consider the advantages of various chemotherapies, with regards to BLBC.

1.1.6.1.4.2 BRCA1 and Basal-Like Breast Cancer

There is evidence that there is a link between BLBC and *BRCA1* deficiency, where the more aggressive BLBC is related to a germ-line mutation of *BRCA1* [177]. It has been confirmed that, within BLBC, loss of the function of *BRCA1* is more frequent than in other BC biological subtypes (see Table 1.2) [178]. The morphological characteristics and immunohistochemical features of BLBC are significantly similar to *BRCA1* mutation tumours, particularly in terms of the definition of the negativity of ER, PgR, the somatic mutation of *TP53* and HER-2 [58, 207].

However, Turner et al have demonstrated that the expression of basal CKs in a tumour is not likely to arise from of a dysfunction in the *BRCA1* pathway [58]. A select number of regulators involved in signal transduction have shown many promising roles in BLBC and EGFR is considered a component of BLBC [178]. The expression of EGFR in relation to *BRCA1* and the various markers of BLBC has been considered in some studies [166, 178]: Turner et al showed that the expression of EGFR was associated with positive expression of BRCA1, P-cadherin and CK5/6 with ER^-[178], whilst EGFR was significantly prognostic in patients with germ-line *BRCA1* mutations. Therefore, it is expected that BLBC segregates, together with *BRCA1* tumours, in microarray expression profile data. These two tumours are rarely harbour amplification of the *CCND1* gene [208].

The *BRCA1* pathway participates in a number of cellular processes, beside its functions in response to DNA damaging, transcriptional regulation, ER signalling and X-chromosome inactivation. Of these various functions, the inhibition of ER-α signalling is associated with the expression of exogenous *BRCA1* [209]. It has also been shown that ER-α mediated transcription is a result of the knockdown of *BRCA1* through RNA interference; therefore, it is believed that these factors may explain the development of ER-negative tumours, but the physiological features remain unclear [209]. X-chromosome inactivation is present in both *BRCA1* BC and sporadic BLBC [210].

Many transcriptional factors are preferentially expressed in BLBC, such as c-Myc, Forkhead box C1 (FOXC1) and E2F-5 [34, 211]. The stimulation of *BRCA1* expression by ER results from the influence of ER affecting the growth of breast cells. This proliferation-mediated increase may proceed through the E2F site in the proximal promoter. On the contrary, it is suggested that P53 inhibits *BRCA1* expression by preventing E2F binding to this site [212]. There are other regulators of *BRCA1*,such as GABP-α/β, which is the downstream mediator of the signalling of neuregulin in nerve cells [211].

Laminin, an extracellular matrix protein involved in cell adhesion, is also suggested to be a negative regulator of *BRCA1* expression and thus is associated with BLBC [211]. α6β4 integrin is an interacting partner for most laminins, which plays a role in modulating the signalling pathways of proliferation and survival. Lu et al highlighted the preferential expression of α6β4 integrin in BLBC, which was absent in non-basal tumours [213]. Inhibitor

of DNA-binding/differentiation protein (ID4), which is a negative regulator of *BRCA1*, is over-expressed in BLBC, leading to dysfunction in the upstream pathways that regulate the expression of *BRCA1*. This mechanism has been suggested to play a role in the suppression of *BRCA1* in sporadic BLBC [58].

Recently, a study based on the similarities between the gene expression profiles of *BRCA1* and BLBC showed a high expression of Ets1 (transcription factor that binds to DNA at the palindromic Ets-binding site) in hereditary *BRCA1* BCs. Bosman et al investigated the function of Ets1 in BLBC, in terms of the regulation of the *α/β crystallin* gene, and it was found that the *α/β crystallin* was over-expressed in BLBC, due to its Ets1 activity; silencing *Ets1* decreased the promoter activity of the *α/β crystallin*. *β* crystallin plays a role in the mechanism of cell death, suppressing apoptosis and resistance against chemotherapy [214-216]. According to the study of Bosman et al Ets1 was reported as a predictive marker, in addition to more than ten proteins, in the identification of BLBC by immunohistochemistry. It is important to study the co-expression of Ets1 and α/β crystallin, in order to evaluate their prognostic value in BLBC [216].

Previous evidence hypothesised that epigenetic alterations, such as *BRCA1* hypermethylation, may play a determinant role in tumour initiation or in the progression of malignant tumours [217]. However, in BC, the loss of gene expression through the hypermethylation of CPG islands has been determined for a number of genes, such as *P16* and Glutathione S-Transferase P1 (*GSTP1)* [202]. Many studies have thus explored these epigenetic changes as a detection marker in helping to understand the role of the inactivation of *BRCA1* in BC. Finally, the disorder of *BRCA1* may lead to a specific profile of methylation in tumours, such as in the expression of an altered P21 in *BRCA1* negative cell lines, which has been associated with the regulation of DNA methylation and its role in the management of the cell-cycle [217]. Although many studies highlighted the decrease in *BRCA1* mRNA expression and methylation, others did not identify this association [218, 219]. Matros et al studied the expression of the *BRCA1* promoter methylation in different breast tumours and, in BLBC, there was infrequent *BRCA1* methylation, while the expression of *BRCA1* and the mitotic rate were high [167].

BRCA1 also interacts with diverse groups of proteins, including BARD1. Inactivation of BARD1 induces the basal-like phenotype (ER^-, PgR, $HER\text{-}2^-$, $CK5^+$, $CK14^+$, P53 lesion and vimentin), with an occurrence, pathology and latency that are identical to those that develop in *BRCA1* and *BRCA1*/*BARD1* mutant mice [220]. Although this shows the function of *BARD1* as a tumour suppressor, it should be noted that it was a null mutation, whereas all *BARD1* lesions reported so far in humans were of a missense mutation [221, 222]. Disagreement remains, with regards to the similarity between *BRCA1* and BLBC, as to whether this is due to a specific decrease in the expression of *BRCA1* in BLBC or is just a general marker of high-grade tumours [178].

Table 1.2 Similarities between BLBC and *BRCA1*Mutation Tumours in Previous Studies

Parameters	*BRCA1* Phenotype	Non-basal	BLBC	Basal Definition	References
Grade III	High grade 3	Mixed	High grade 3	ER^-/PgR^-& $CK14^+$	[37, 223-226]
	ND	46% luminal-A	66%	Combined	[204, 227, 228]
	74%	46% (ER^+)	100%	ND	[229]
Proliferation	High	Luminal B>A	High	Combined	[37, 165, 185, 230]
HER-2	Rare	Luminal A^+	Rare	TN	[224, 231]
	$HER\text{-}2^-$	Luminal A^+	$HER\text{-}2^-$	TN	[165, 228, 232]
	$HER\text{-}2^-$	$HER\text{-}2^+$ (26%)	100% $HER\text{-}2^-$	ER^-, $HER\text{-}2^-$ /low,$CK5/6^+$ and/or $EGFR^+$	[207]
EGFR	Overexpression	ND	Overexpression	TN and $EGFR^+$	[37, 224, 227, 233-237]
	60-70%	8%	60-80%	TN and $EGFR^+$	
	(67%, 14/21)	Luminal (100%) $HER\text{-}2^+$ (58.3%)	15-35%	ER^-, $HER\text{-}2^-$ low,$CK5/6^+$ and/or $EGFR^+$	
Basal CKs	Basal CK^+ 88%	Basal CK^+ (7.3%)	Basal CK^+	TN, $CK5/6^+$ and/or $CK14^+$ and/or $CK17^+$	[165, 182, 207, 224, 238-240]
	57%-88%	ND	ND	$CK5/6^+$ and/or $CK14^+$ and/or $CK17^+$	[207, 238]
TN	90%	ND	90-80%	Any Basal CK^+	[37, 165, 238]
ER	ER^-	ER^+	ER^-	TN and basal CK^+	[165, 224, 230, 238]
	ER^-(36%, 96/268)	ND	ND	ND	[228]
	ND	ER^+ (100%)	ER^+ (0%)	Combined	[227]
PgR	PgR^-	PgR^+	PgR^-	TN	[224, 228, 231]

This table shows different studies regarding the similarities between BLBC and *BRCA1* mutation tumours. TN= triple Negative BC. ER= oestrogen receptor, PgR= progesterone receptor. Combined=TN and any positive basal-like biomarker including, but not limited to basal CK. ND= no data.

1.1.7 DNA Damage Signalling and Repair

The stability of the genome, maintained by the DNA molecule, is not certain for the entire lifetime of human beings. The contribution of DNA damage to human disease is exemplified in a set of uncommon genetic diseases that have, at their basis, various aspects of DNA metabolism [241]. DNA is regularly open to danger, as a result of exogenous and endogenous agents. Exogenous aggressors include Ultraviolet Light (UV) light, irradiation

and genotoxic agents, such as those found in cigarette smoke. Endogenous agents originate from the products of normal cellular metabolism (e.g., Reactive oxygen species (ROS)) and the spontaneous disintegration, under physiological conditions, of some chemical bonds in DNA [242]. ROS is able to stimulate base and sugar modification, base loss, strand cleavage and DNA protein cross-links [243].

The value of DNA repair is underlined by any deficiency of DNA repair, which is definitely related to hypersensitivity to DNA-damaging agents, bringing about accumulation of mutations in the genome [244] and also with genomic instability syndromes, which usually significantly improve the incidence of cancer [245]. Thus, the epidemiology of DNA repair and its influence on the susceptibility of human beings to cancer is an important area of research. A number of pathways that result in genomic instability have already been identified, such as the Base, or Nucleotide Excision Repair disruption (BER)(NER) respectively, Double Strand Break Repair (DSBR), Mismatch Repair (MMR) and DNA damage signalling pathways [245, 246]. One fundamental factor to consider is that breast (mammary) tissue has a higher chance of DNA damage occurring, due to the massive remodelling of such tissue throughout a woman's life.

1.1.7.1 Base Excision Repair

BER pathway is the primary mechanism of repairing DNA damage, especially that of an endogenous origin or an exogenous origin [247]. BER is initiated by damage specific DNA glycosylases that generate the damaged base through hydrolysis of the N-glycosylic bond linking the base of DNA to the backbone of sugar phosphate. Thus, the developing abasic site (Apurinic/Apyrimidinic-site (AP-site)) is highly processed by AP-Endonuclease 1 (APE1), which cleaves the phosphodiester bond 5'- to the AP-site, bringing in a DNA single strand break (SSB) and a 5'-sugar phosphate. Upon this, SSB is repaired by a DNA repair complex that comprises of DNA polymerase β (Pol β), X-Ray repair Cross-Complementing protein 1(XRCC1), in addition to DNA ligase IIIα (Lig IIIα) [248, 249]. Pol β has AP lyase activity, which erases the 5'-sugar phosphate, in addition to working as a polymerase for DNA, bringing a single nucleotide to the 3'-end of the sugar phosphate of arising single-nucleotide [250]. Finally, Lig III finalises and seals the ends of DNA, consequently finishing the repair of DNA [5, 251, 252]. This particular process is often known as the short and simple patch pathway of BER (Figure 1.3), through which the majority of cells achieve repair [253, 254]. When the 5'-sugar phosphate is resistant to cleavage by Pol β, a move to Pol δ/ε arises, which introduces 2-8 additional nucleotides directly into the gap of the repair; for this reason, any resulting structure of flap is simply eliminated by Flap Endonuclease-1 (FEN-1), in a Proliferating Cell Nuclear Antigen (PCNA)-dependent process. DNA ligase I subsequently seals the remaining nick in the actual backbone of DNA, which is known as long-patch BER [255, 256] (see Figure 1.3).

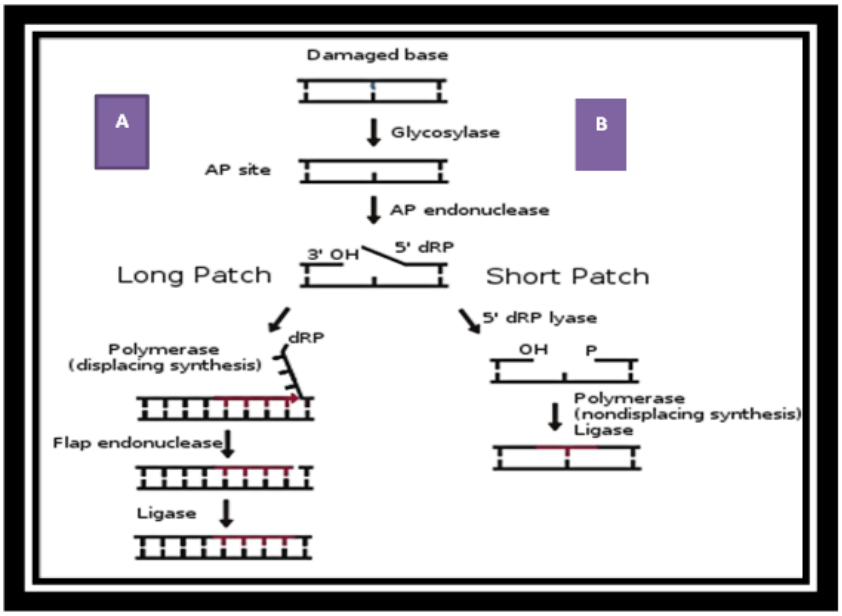

Figure 1.3 Basic Pathways of Base Excision Repair (BER). Where A; shows the long patch and B; short patch. It starts by DNA glycosylase recognising and removing the damaged base. If 5' sugar phosphate resistance to cleavage by Pol β then Pol δ/ε will take place and start via the long patch pathway.

1.1.7.2 Nucleotide Excision Repair

The NER is probably the most versatile and flexible of all DNA repair pathways and is the major repair mechanism in eliminating the plethora/bulk of structurally unrelated DNA lesions, such as UV-light-induced photolesions, cyclobutane pyrimidine dimers, cross-links of intrastrand, massive chemical adducts and bulky adducts, produced as a result of exposure to genotoxic agents and oxidative damage [257, 258]. BER is recognised as the main pathway for these lesions, but NER may be considered as a backup system [259, 260].

A powerful property of NER is that it is usually paired to transcription, commonly contributing to the favoured repair associated with the transcribed strand (TS) (over that of the non-transcribed strand (NTS) in active genes), a sub-pathway known as transcription coupled repair (TCR). The mechanical aspects of TCR continue to be ambiguous, despite the fact that it is usually considered that RNA polymerase II (RNAPII) acts as a damage sensor that alerts the pathway of NER once it encounters a blocking lesion in the TS [261]. As a result, RNAPII can easily alternate to Xeroderma pigmentosum, complementation group C (XPC) and DNA-Damage Binding (DDB) complexes, in recognition of the lesion. Furthermore, XP group C patients, who are deficient in global genomic repair (GGR), still retain TCR. GGR regulates the non-transcribed domains repair of the genome, while TCR eliminates any lesions resulting from the transcribed strand of active genes. Usually, the initial step of NER is the realisation of damaged residues, in addition to the formation of a small bubble structure with or without damaged bases, completed by XPC-hHR23B (human Rad23B homolog) and the 9 subunits of transcription factor-IIH (TFIIH), XPA and

Replication Protein A (RPA) respectively. The double incision of the damaged DNA strands 5' and 3' to the lesion is performed by two endonucleases, XPG and ERCC1 (excision repair cross complementing)-XPF. DNA Polδ and Polε with each other, utilising the sliding clamp, PCNA, the pentameric clamp loader, and the DNA ligase I (Lig I). These are accountable for the generation of an oligonucleotide that contains the lesion, synthesis and the ligation of the producing gap. With the exclusion of XPC-hHR23B, the genes engaged in GGR are also necessary for TCR. Furthermore, TCR requires other genes, such as *CSA* (Cockayne syndrome type A) and *CSB* (Cockayne syndrome type B) [5, 251, 262, 263](see Figure 1.4).

Deficiencies in the pathway of TCR can lead to a variety of many other genetic diseases, such as Cockayne Syndrome [251, 252], where the patients are certainly not susceptible to cancer, yet are afflicted by developmental defects and several neurological problems, usually serious, from an early age.

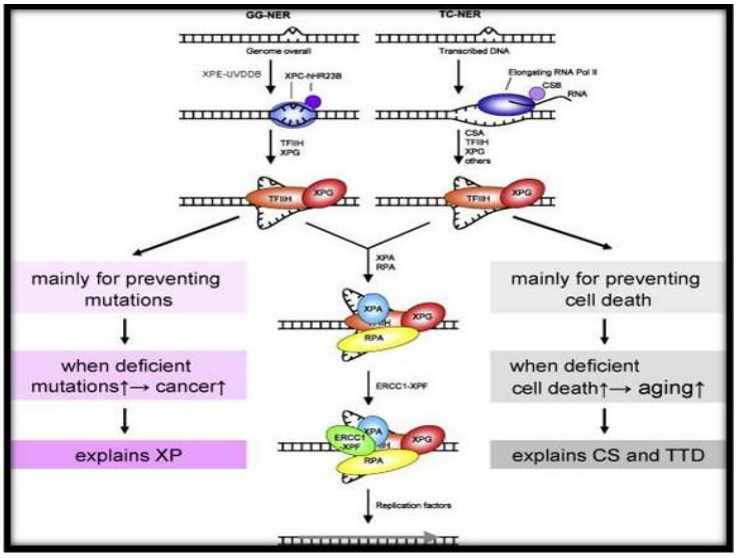

Figure 1.4 Pathways for Nucliotide Excision Repair (NER) . It removes ssDNA that contains the damage and undamaged remains as template to synthesis a short complementry. Global genomic repair (GGR) regulates the non-transcribed domains repair of the genome, while transcription coupled repair (TCR) eliminates any lesions resulting from the transcribed strand of active genes.

1.1.7.3 Double Strand Break Repair

Breaks in both DNA strands give rise to the most serious lesion that threatens genome stability. There are approximately 10 DSBs occurring per day per cell, with regards to the metaphase chromosome and breaks of chromatid in early passage primary human or mouse fibroblasts [264-266]. In mitosis phase multi-cellular eukaryote cells, DSBs are typically pathological (accidental), apart from the specific subgroup of physiologic DSBs of the vertebrate immune system and, particularly, in early lymphocytes. The main pathological causes of DSBs inside wild type cells include replication across a nick, causing an increase in breaks of chromatid all through the Synthesis phase (S phase). These types of DSBs are usually (and preferably) repaired by Homologous Recombination (HR), through the use of

the surrounding sister chromatid. Each of the remaining pathological types of DSBs are generally repaired by Non Homologous End Joining (NHEJ), due to the fact that they normally arise once there is no nearby homology donor and/or simply because they take place outside of the S phase. Factors in this include ROS from oxidative metabolism, ionising radiation (IR) and the inadvertent action of nuclear enzymes [267]. A number of genes identified as BC susceptibility genes, including *BRCA1, BRCA2, ATM, P53 and CHK2*, are very important DSBR genes [262]. Details of these pathways are presented in Chapter 4.

1.2 Hypothesis of the Study

Deficiencies in the signalling of DNA-damage response (DDR) pathways play fundamental roles in the pathogenesis of several human cancers, including hereditary BC. There is compiling evidence that DDR play an important role in the pathogenesis and behaviour of sporadic BC and those DDR mechanisms are different among various tumour classes. Targeting DDR in hereditary BC has proven successful using specific DDR protein inhibitors such as Poly ADP Ribose Polymerase (PARP) inhibitors. Therefore, assessment of impaired DDR in sporadic BC will have major implication in the management of BC taking into account that BC with defective DDR show aggressive features and poor response to current treatment modalities. Identification of subclasses with defective DDR may improve treatment of these aggressive tumours using commercially available or novel DDR-related protein inhibitors. However, the complexity of DDR in addition to the molecular heterogeneity of BC make studying individual genes related to DDR in an unselected series is less likely to identify the target subclass. As a result, the hypothesis of this study is that investigating the expression patterns of a large panel of DDR-related biomarkers in a well-characterised annotated series of sporadic BC could help in deciphering the process of DDR, identify the active pathway that can be targeted in specific subclasses, improve our understanding of its role in the different molecular classes and add prognostic and predictive information.

1.3 Aims of the Study

The aim of this study is therefore to investigate alterations in the different pathways of DDR occur in a large clinically and molecularly annotated series of BC which will help identify tumours with impaired DDR, improve our understanding of its role in the different molecular classes, narrow down the target pathway-related markers and may have potential therapeutic implications. This study will investigate different clinical and molecular types of BC, with regards to expression of proteins involved in the DDR pathway (in particular, DNA damage sensors and those proteins involved in DNA-DSB pathways including HR, NHEJ repair, and SUMO). This will include assessment of expression, subcellular localisation and interaction, using immunohistochemistry (IHC) and tissue microarray (TMA) utilising primary invasive early stage sporadic BC. In addition, a subset of hereditary BC cases will be used as a control group for BRCA1 deficiency. Reverse Phase Protein Microarray (RPPA)

will be used to perform a comprehensive profiling of DDR pathways in cell lines corresponding to the main molecular classes of BC. This will determine associations with clinico-pathological characteristics, assess the impact of DDR protein expression and determine their impact on the response to therapy (long-term follow-up and treatment information were available). This is the first large study to investigate the expression of different proteins in different DDR pathway and their subcellular localisation in BC.

Chapter 2

2.1 Materials and Methods

2.1.1 Patient Cohorts

The study cohort comprised of three groups: A) 1904 unselected cases of female primary operable invasive tumours between 1986 and 1998, B) 386 cases selected from a consecutive series of primary operable ER negative tumours between 1998 and 2007 and C) 24 well-characterised series of breast tumours from patients with known *BRCA1* gene mutations. However, High Resolution Melting Analysis (HRMA) with employing PCR was used for *BRCA1* mutation detection in group C (this was performed by Dr Ahmed Benhasouna). All cases were obtained from the well-characterised Nottingham Tenovus primary breast carcinoma series. All patients were measured in a standardised manner, in terms of clinical history and characteristics of tumour. This was based on age, menopause status, tumour size, tumour type, histological grade, nodal status, lymphovascular invasion and the NPI. In 1988, tumour characteristics considered in the management of patients were NPI and ER status. Cases with a score of NPI≤ 3.4 received no adjuvant therapy, but those patients with NPI> 3.4 received Tamoxifen if they expressed ER^+ (+/-Zoladex in pre-menopausal patients or ovarian irradiation) [268]. Classical cyclophosphamide, methotrexate and 5-flurouracil were used if the patients were ER^- and were fit enough to receive chemotherapy. In early 1990's, the same protocol was applied in addition, patients who were grade II, or III, and node positive have been given prophylactic irradiation to the axilla following surgery. However, It was clear that not all patients required radiotherapy, because most of un-irradiated patients did not suffer local recurrence [154].

Survival data were collected using a prospective method and included BC specific survival (BCSS), defined as the interval from the date of primary treatment to the time of death due to BC, in addition to any disease-free interval (DFI), identified as the interval from the date of primary treatment to the first loco-regional recurrence. Additionally, Metastasis-Free Interval (MFI) is defined as the duration from the date of primary surgery to the appearance of distant metastasis. All these parameters were measured in months.

The sporadic patients cohort (n=2290) comprised of 780 (34.3%) cases ≤50 years old and 1495 (65.7%) patients >50 years old. Characteristics of disease differed between the cohorts; at primary diagnosis, 346 (15.2%) were grade I, 670 (29.5%) were grade II and 1257 (55.3%) were grade III. The majority of the cohort 1486 (65.8%) demonstrated a tumour size greater than 1.5cm and 580 (35.1%) had definite vascular invasion. Data relating to follow-ups were collected initially at 3 monthly intervals, followed by intervals of 6 months and, subsequently, 12 months (median 116 months; range from 2 to 247 months for BCSS). Within this period, recurrence and distant metastases occurred in 809 (36.4%) and 642 (28.5%) patients respectively. A total of 564 (29.3%) cases died from BC. Data of some cases was missing, due to patients lost to follow up.

Table 2.1, Table 2.2, and Table 2.3 summarise the frequencies of clinico-pathological features, treatment plan for the patients, and patient's outcome respectively in the different cohorts, which are classified based on ER and BRCA1 protein expression in sporadic and hereditary BCs.

Nottingham Research Ethics Committee 2 approved this study.

Table 2.1 Frequency of Clinico-Pathological Features in the Different Cohorts of Breast Cancer.

Parameters		Sporadic ER^- &$BRCA1^-$	Sporadic ER^+& $BRCA1^+$	Hereditary ER^-	Hereditary ER^+
Menopause Status	**Pre-menopausal**	247(47.6)	255(36.8)	ND*	
	Post-menopausal	272(52.4)	437(63.2)		
Age (years)	**<50**	241(45.8)	217(31.4)	13(76.5)	5(83.3)
	≥50	285(54.2)	475(68.6)	4(23.5)	1(16.7)
Tumour Size (cm)	**≤1.5**	124(24.2)	279(40.3)	3(20)	2(33.3)
	>1.5	388(75.8)	414(59.7)	12(80)	4(66.7)
Stage	**1**	329 (62.8)	448(64.6)	ND*	
	2	142(27.1)	199(28.7)		
	3	53(10.1)	46(6.6)		
Grade	**1**	3 (0.6)	176(25.4)	0	0
	2	44 (8.4)	335(48.3)	1(6.3)	1(16.7)
	3	479 (91.1)	182(26.3)	15(93.8)	5(83.3)
Tubule Formation	**1**	2(0.4)	50(7.4)	0	0
	2	70(13.5)	279(41.4)	3(20)	1(16.7)
	3	445(86.1)	345(51.2)	12(80)	5(83.3)
Pleomorphism	**1**	1(0.2)	21(3.1)	0	0
	2	11(2.1)	400(59.4)	0	0
	3	504(97.7)	252(37.4)	15(100)	6(100)
Mitosis	**1**	27(5.2)	365(54.1)	0	0
	2	71(13.7)	148(22)	2(13.3)	2(33.3)
	3	419(81.0)	161(23.9)	13(86.7)	4(66.7)
Vascular Invasion	**No**	129(61.4)	397(67.3)	6(35.3)	2(33.3)
	Yes	81(38.6)	193(32.7)	11(64.7)	4(66.7)
Tumour Type	**Medullary**	24(4.6)	1(0.1)	1(5.9)	0
	Invasive Ductal/No Special	463(89)	281(41)	5(88.2)	5(83.1)
	lobular	5(1)	89(13)	0	0
	Mixed**	16(3.1)	256(38.7)	0	0
	other***	12(2.3)	49(7.2)	1(5.9)	1(16.7)

ND*= no data available. Four cohorts are classified based on the protein expression of nuclear ER and BRCA1 in hereditary and sporadic BCs. ** Lobular or tubular mixed BCs. *** Mucinous, Alveolar Lobular, Miscellaneous including Metaplastic, Adenoid Cystic, Spindle, and Tubulolobular. Hereditary= Known *BRCA1* germline mutation.

Table 2.2 Frequency of Treatment Received in the Different Cohorts of Breast Cancer.

Treatment		Sporadic ER$^-$ & BRCA1$^-$	Sporadic ER$^+$& BRCA1$^+$	Hereditary ER$^-$	Hereditary ER$^+$
Chemotherapy	**No**	170(35.4)	612(91.2)	ND*	
	Yes	310(64.6)	59(8.8)		
Endocrine Treatment	**No**	432(89.6)	400(59.6)		
	Yes	50(10.4)	271(40.4)		
Radiotherapy local	**No**	58(27.8)	314(46.9)		
	Yes	151(72.2)	356(53.1)		
Radiotherapy nodes	**No**	151(72.2)	561(83.6)		
	Yes	58(27.8)	110(16.4)		

Four cohorts are classified based on the protein expression of nuclear ER and BRCA1 in hereditary and sporadic BCs. Although some cases are ER positive tumours, they did not receive endocrine therapy, because the treatment plan for those cases with a score of NPI≤ 3.4 and therefore received no adjuvant therapy. In addition, even if the cases were scored as ER negative, if they showed any positivity (very weak expression of ER), then they received endocrine therapy. ND*= no data available. Hereditary= Known *BRCA1* germline mutation.

Table 2.3 Patients' outcomes in the Different Cohorts of Breast Cancer.

Status		Sporadic ER$^-$ & BRCA1$^-$	Sporadic ER$^+$& BRCA1$^+$	Hereditary ER$^-$	Hereditary ER$^+$
Survival from BC*	**Alive**	362(76.1)	426(75.9)	ND^{++}	
	Dead	114(23.9)	135(24.1)		
Disease Recurrence**	**No**	377(73.8)	431(63.3)		
	Yes	134(26.2)	250(36.7)		
Distance Metastasis***	**No**	391(75.3)	522(75.4)		
	Yes	128(24.7)	170(24.6)		
NPI$^+$	**Excellent**	2(0.4)	127(18.5)	0	0
	Good	25(4.8)	182(26.5)	0	1(16.7)
	Moderate 1	202(38.9)	196(28.5)	3(20)	2 (33.3)
	Moderate2	171(32.9)	113(16.4)	5(33.3)	1(16.7)
	Poor	85(16.4)	57(8.3)	7(46.7)	2(33.3)
	Very poor	34(6.6)	12(1.7)	0	0

Four cohorts are classified based on the protein expression of nuclear ER and BRCA1 in hereditary and sporadic BCs. Breast cancer specific survival*, defined as the interval from the date of primary treatment to the time of death due to BC. Only patients who died from BC were considered.
Disease free interval**, identified as the interval from the date of primary treatment to the first loco-regional recurrence. Metastasis free interval*** is defined as the duration from the date of primary surgery to the appearance of distant metastasis. NPI$^+$= Nottingham Prognostic Index: an excellent prognostic group; NPI range of 2.08–2.4, a good prognostic group; NPI range of 2.42 to ⩽3.4, a moderate prognostic I group; NPI range of 3.42 to ⩽4.4, a moderate prognostic II group; NPI range of 4.42 to ⩽5.4, a poor prognostic group; NPI range of 5.42 to ⩽6.4, and a very poor prognostic group; NPI range of 6.5–6.8. ND^{++}= no data available. Hereditary= Known *BRCA1* germline mutation.

2.1.1.1 Available Biomarkers' Data

Data on a wide range of biomarkers of known clinical and biological relevance to BC were accessible and saved on a web-based interface (Distiller; Slidepath Ltd, Dublin, Ireland). These include, ER, PgR, HER-2 [269], CK5, CK17, CK14 [198], tumour suppressor proteins (P53 and PTEN), [270-272] cell proliferative marker (Ki-67), cell cycle progression/arrest regulator markers (P21, P27) [273-275], down regulator proteins for BRCA1; ID4 and MTA1. All markers were stained on TMA except Ki-67 which was performed on full face sections.

2.1.2 Tissue Microarray Construction

TMA can be a time-consuming, labour-intensive, and economically costly technique. However, there are some advantages to using this high-throughput technique for immunohistochemistry, particularly as all tissue samples are exposed to the same conditions of the experiment, leading to more consistent and rigorous results. In addition, TMA provides efficient management of tissue archives as large number of tumour samples could be screened together allowing preservation of tissue resources for further research [276, 277].
Paraffin-embedded formalin-fixed specimens of BC were identified from the Nottingham-Tenovus primary breast carcinoma series (the three groups in section 2.1.1). Table 2.4 summarises the details of the TMA construction. TMAs for the ER negative series and previous primary series as described previously [278]. However, TMAs for the new primary series and BRCA1 tumours were constructed as follows:

2.1.2.1 Preparation of the Donor Blocks and Their Corresponding Slides

Sampling the representative site from the donor block is very critical for construction of TMAs. Full-face sections were prepared from each donor block and stained with haematoxylin and eosin and assessed using light microscopy to identify the most representative areas in the tumour, this step was achieved by a pathologist (Dr Dena Jerjees). Only invasive tumour was considered and avoiding *in-situ* lesions and necrotic or haemorrhagic tumour zones. However, priority was given to blocks that contain enough tumour tissue that had not been frequently sectioned for previous studies. These tumour areas were marked on top of the slide and then transferred to the corresponding donor paraffin block for future sampling using the tissue arrayer needle.

2.1.2.2 Designing the Array

Tumours were arrayed where each block contained up to 150 cores (10x15 layouts). The cores orientation on the array slide is very crucial mainly because uncertainty regarding their orientation can certainly threaten the experiment's evaluation. Therefore, in each array block three normal kidney tissue cores were used at the beginning of the first row, and three normal liver tissue cores at the end of the last row to identify the start and the end of the array as well as to guarantee correct orientation.

2.1.2.3 Construction of the Array

An automated GrandMaster TMA arrayer (3DHISTECH Ltd, UK) was used with a 0.6mm core needle. It is a simultaneous loading, imaging, drilling and punching microarrayer. In addition, it automatically measures the block height measurement to ensure

the embedded cores are in alignment with the recipient block surface, and label image capture for reference. TMA construction was performed by: Alaa Alshareeda, Dr Rezvan Feysal, Dr Dena Akram, and Mr Glynn Donovan. Copies of the TMA were made with 1 core per patient included on each donor block. A total of eight copies were made with 4 from peripheral and 4 from central zones of the tumour. For this study, those TMAs with cores sampled from the periphery were used.

2.1.2.4 Sectioning of the TMA Blocks

Prior to sectioning for the first time after construction, the TMA blocks were incubated at 37°C for 15min; to allow both the surrounding paraffin and cores to hybridise. For immunohistochemistry, 4 µm tissue sections were cut from each TMA block using a microtome and placed onto Xtra slides (Surgipath Ltd) by Mr Christopher Nolan.

Table 2.4 The Details of TMA Construction Used in This Study

TMA	Method	Markers	Acknowledgments
TMA of ER-negative tumours (386 cases).	TMAs were prepared by a manual TMA arrayer as described previously [278-280]. Briefly, cores of 0.6 mm thickness were obtained from the most representative areas of the tumours then re-embedded in microarray blocks. Each case was represented once on each TMA; TMAs of 150 cases per block were made.	All markers used in this study	Ahmed Benhasouna.
Previous TMA of unselected primary series (1,944 cases)		Rad51, BRCA1, PIAS4, PIAS1, CHK1, and ATM.	Ahmed Benhasouna, Mohammed Aleskandarany, and Claire Paish.
New TMA of Unselected Primary series (1,904 cases)	As described in Sections 2.1.2.1 to 2.1.2.4	ATR,CHK2, γH2AX, BARD1, SMC6L1, DNA-PK, KU70/KU80, UBC9, KPNA2, and NPM	Alaa Alshareeda, Rezvan Feysal, Dena Akram, and Glynn Donovan.
TMA of breast tumours from patients with known *BRCA1* gene mutations (24 cases).		All markers used in this study	Woolston Caroline.
For unselected primary series, 2 different sets were used, because the previous one was exhausted and lost the tissue so, a new set on the same cases was made. The new TMA was fewer than the previous because some of donor blocks were exhausted. Only the matched cases were considered in this study and excluded missing one. Although manual and automated methods were used to construct the TMA, this has no effect on the step of selecting the tumour area, since in both methods this step was done manually by pathologists. In this study, TMA from periphery area of the tumour was used mainly in the new, the previous unselected primary series and also used for ER-negative tumours, whereas, central TMA was used for known *BRCA1* gene mutation TMA.			

2.1.3 Immunohistochemistry

Immunohistochemistry has become an established tool for both research and diagnostic purposes. It represents the process of detecting antigens (in the present study; proteins) in a tissue's cells by exploiting the principle of antibodies binding specifically to antigens in biological tissues. IHC offers fast results (within 1-2 days), fairly inexpensive, allows co-localisation of an antigen and the lesion it has produced, leading to increase diagnostic accuracy [281]. In this study, 16 antibodies were investigated; ATM, ATR, CHK1, CHK2, γH2AX, Rad51, BRCA1, BARD1, SMC6L1, KU70/KU80, DNA-PK, UBC9, PIAS1, PIAS4, KPNA2, and NPM (Table 2.5).

2.1.3.1 Immunohistochemical Antibody Labelling Using the Novolink Detection Method

The slides were placed on a 60^{o}C hotplate for 10 minutes, in order to ensure that the TMA cores adhered firmly to the slides. They were then allowed to cool and were de-waxed, in two xylene baths (Genta Medica, York, UK), for 5 minutes each, followed by rehydration in alcohol baths for 3x 2 minutes each. The slides were then washed under running water for 5 minutes (this step was performed by IHC autostainer machine, ©Leica Biosystems).

Antigen retrieval was performed by microwaving the slides at 800W for 20 minutes in citrate buffer (1M sodium citrate at pH6.0) and then cooling the slides under running tap water. Slides then were loaded into Shandon Sequenza coverplates (Thermo Scientific), making sure that there were no air bubbles present (this was done in tap water bath under water surface), and subsequently placed into Sequenza trays. Slides were rinsed with TBS (Tris Buffered Saline pH7.6). Endogenous peroxidise activity was blocked by applying Hydrogen Peroxidise (NovoLink™ Detection System (Leica, RE7150-K)) for 5 minutes, followed by rinsing with TBS for 2x 5 minutes. Protein block (NovoLink™ Detection System (Leica, RE7150-K)) was then applied for 5 minutes. After the slides were washed with TBS for 2x 5 minutes, the primary antibody for each target (optimally diluted in Leica antibody diluent) was applied and incubated for 60 minutes at room temperature or overnight at 4 ^{o}C depend on the antibody used. The slides were rinsed with TBS for 2x 5 minutes and were then incubated with post primary block NovoLink™ Detection System (Leica, RE7150-K) for 30 minutes, before being rinsed again with TBS for 2x 5 minutes. Novolink polymer was then added for 30 minutes. The washing step, using TBS, was repeated and followed by freshly-prepared Diaminobenzidine tetrahydrochloride (DAB) working solution (1:20 DAB chromogen in DAB substrate) NovoLink™ Detection System (Leica, RE7150-K) being added to the slides for 5 minutes; the slides were then once again washed with TBS for 2x 5 minutes. Slides were counter-stained with haematoxylin (NovoLink™ Detection System (Leica, RE7150-K) for 6 minutes, therefore, the slides were removed from the coverplates.
Slides were then rinsed under running water for 5 minutes. Finally, the slides were dehydrated by immersing them in IMS alcohol for 3x 2 minutes. They were then cleared in xylene (Genta Medica, York, UK) for 2x 5 minutes (again these steps were performed by

autostainer machine, ©Leica Biosystems), followed by mounting, with a glass cover slip, in DPX (BDH, Poole, UK).

2.1.3.2 Optimisation of Antibodies Used for IHC

All the antibodies were optimised on TMA before the staining on the whole series, by changing different variables in the protocol of the staining until the optimum result was obtained; such as no/less background staining, and high degree of expression heterogeneity, starting with the dilution recommended by the supplier's datasheet in addition to three or more dilutions above and below the recommended dilution. In addition antigen retrieval solutions were used, all the antibodies used in this study were subsequently pre-treated in 0.1M citrate buffer at pH 6.0.

In addition to Western blotting, negative controls (with omission of the primary antibody) were used in each experiment to ensure the specificity of the antibodies. In addition, specific positive controls, as advised by the antibody manufacturer or choosing a specific tissue from the human protein atlas (http://www.proteinatlas.org/) to observe the pattern and intensity of the protein expressions on TMA were included in the IHC experiments. However, all negative controls used in this study were applied on BC tissue. Positive controls used for each antibody is presented in the specific results Chapters.

All the markers used in this study have been previously stained on TMA, reference for each antibody is represented in the specific chapter. Moreover, some control TMA slides containing a variety of BC cases with some containing cores from different areas of the same cases in addition to normal parenchymal elements were used during optimisation to assess the degree of expression heterogeneity.

2.1.3.3 Immunohistochemical Scoring

Stained TMA slides were scanned using high-resolution digital images (Nanozoomer; Hamamatsu Photonics, Welwyn Garden City, UK) at x20 magnification and were scored visually on high-resolution monitors, using a web-based interface (Distiller, Slidepath Ltd., Dublin, Ireland). However, the following markers were scored using light microscopy: ATM, ATR, CHK1, CHK2, Rad51, PIAS4, KU70/KU80 and BRCA1.

Only staining of invasive cancer cells within the tissue cores was considered taking care not to score any *in-situ* components. The pattern of expression was visually recorded and considered any cellular localisation (e.g. nucleus or cytoplasm). For evaluation of IHC, a modified Histochemical-score (H-score) was used [282]. For H-score, both the intensity of staining and the percentage of stained cells were considered within each tissue core. Staining intensity was scored as 0, 1, 2 or 3 for negative, weak, moderate and strong, respectively. The

percentage of positive cells for each intensity was subjectively estimated. Multiplication of the two indices (intensity and percentage positive cells) provided final scores that ranged from 0 to 300. All cases were scored without prior knowledge of the patients' pathological or outcome data.

The author re-scored each marker with at least 30% of a randomly chosen subset of cases (details are summarised in each chapter). A statistical agreement test was performed (Kappa value) for each marker, where there was good agreement (≥0.5), and an average was taken. If there were discrepancies, the highest scoring was taken. However, if only one core was informative (because of either loss or absence of tumour tissues), then the score applied was that of the remaining core.

2.1.4 Cell Line and Culture Media

Cell lines and reagent were taken from the group of Dr Madhusudan Srinivasan as collaborative project. BRCA1 deficient HeLaSilenciX® cells and control BRCA1 proficient HeLaSilenciX® cells (Cervical carcinoma cell line) were obtained from Tebu-Bio (www.tebu-bio.com). Cells of SilenciX were grown in DMEM medium (with 580mg/L of L-Glutamine, 4500 mg/L D- Glucose with 110mg/L Sodium Pyruvate) supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin and 125 µg/ml Hygromycin B (Life Technologies). BC cell line MDA-MB-436 (BRCA1 deficient) was grown in DMEM (Sigma, UK), while MCF-7 (BRCA1 proficient and ER^+) and MDA-MB-231 (ER^- and $EGFR^+$) were grown in RPMI1640 (Sigma, UK). All of the media applied to culture cell lines were supplemented with 10% FBS (PAA, UK) and 1% Penicillin/Streptomycin. MCF-7 and MDA-MB-231 were purchased from ATCC and MDA-MB-436 cell line was purchased from CLS.

2.1.4.1 Preparation of Cell Lysates

Thawing and freezing procedures were carried out by Nada Albarakati. Cells were grown to 80% confluency and were washed with sterile Phosphate Buffer Saline (PBS). The cell monolayer was disrupted by a 5-minute incubation with 2mL 10% trypsin/EDTA in PBS, pre-warmed to 37ºC. Flasks were gently shaken to help disrupt the cell monolayer. Cells were suspended in fresh media, at a concentration of $1x10^6$ cells per ml. They were then pelletted through centrifugation at 500*g* for 5 minutes. 1 mL of cell suspension was pipetted and washed once with PBS. Therefore, lysis of the cells was performed by adding RIPA buffer (25mM Tris, 150mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate and 0.1% Sodium Dodecyl Sulfate (SDS)) to a phosphatase and protease inhibitor cocktail (Pierce, Thermo Fisher Scientific, UK), therefore, the lysate was incubated on ice for 10 minutes, with frequent shaking. Lysates were centrifuged for 20 minutes at 4°C at 13000*g*, in order to remove cell debris. Finally, the supernatant was stored at -20 °C, until use.

2.1.4.2 Cell Passage Procedure

The first part of this procedure was identical to that described in section 2.1.4.1. Following the centrifugation step, the supernatant was discarded and the cell pellet re-suspended in 10 mL fresh growth medium (pre-warmed to 37ºC). A small amount (10 µL) of the resulting suspension was pipetted into each chamber of a Neubauer haemocytometer, which has 25 central squares, and allowed to settle for 1 min before counting. All cells within the 5 x 5 division grids of both chambers were counted. The mean count was calculated and the numbers of cells were calculated using the following equation: Number of cells/mL = Mean cell count x dilution factor (10) x 10^4. The suspension was diluted with fresh growth medium and poured into cell culture plates or cell culture flasks. The passages number used in this study were as follows: for HeLa BRCA1; between passage 21 and 30, HeLa BRCA1 control; between passage 15 and 20, MCF-7; between passage 25 and 32, MDA-MB-231; passage 15 and 24, finally for MDA-MB-436; between passage 12 and 20.

2.1.4.3 Cell Lysate Protein Quantification (Bradford Assay)

The concentration of cell lysate protein was calculated using the BioRad Bradford assay, using bovine serum albumin (BSA) as a standard. 10µL of a protein was mixed with 250mL of diluted and filtered assay solution in a 96-well plate leading to a differential colour change occurs in response to the protein concentration. Consequently, OD595nm was established using microplate reader; FLUOstar OPTIMA from BMG LABTECH Ltd., Aylesbury, UK, to determine the absorbance value. Therefore, a standard curve was created using BSA diluted in lysis buffer by plotting the values of absorbance against concentrations of corresponding protein. The absorbance of unknown protein lysates was plotted on the standard curve to evaluate the concentration of the proteins in each cell lysate, and, this step was performed in triplicate. The determination of the concentration of the protein, in individual cell lysates, was performed to help load equal amount of protein.

2.1.4.4 Specificity of Antibodies by Western Blot (W.B)

The specificity of the following antibodies was achieved through Western blot (Table 2.5) with the exception of PIAS4, ATR, BRCA1, and BARD1. These markers were stained before starting cell culture's work; details are given in each chapter. Western blotting validated the specificity of some antibodies, however, this was deemed to be validated by a single band at the correct protein size. The pre-stained marker 'full range rainbow marker' (Invitrogen Life Technologies) was used as a molecular weight standard.

A mixture of different cell lysates to detect the specificity of an antibody has previously been applied in different studies and showed its reliability [283-285]. In this study, KU70/KU80, and Rad51 were tested by W.B on MCF-7 cell lines, whereas, the remaining

antibodies (ATM, CHK1, CHK2, γH2AX, SMC6L1, PIAS1, UBC9, KPNA2, and NPM) were detected in a mixture of different lysates (MCF-7, MDA-MB-231 and HeLa BRCA1 and its control). All the antibodies tested in this study shared the same positive controls which were MCF-7, HeLa BRCA1 cell lines. http://www.proteinatlas.org/ provides profile data for positive controls of all the markers used in this study.

2.1.4.5 Denaturing Polyacrylamide Gel Electrophoresis

The separation of proteins was achieved by SDS-PAGE, in accordance with the Laemmli method. Proteins were solubilised in SDS loading buffer. One part of the 2X SDS buffer (1x50mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue and 1% (v/v) b-mercaptoethanol) was added to the same volume of the sample and was heated for 5 minutes at 100°C. Equal amounts of protein (20μg) were loaded into each well of a precast 10-15% Tris Glycine Gel (Bio-Rad). Gels were run at 150V for 90 minutes in Tris/Glycine/SDS running buffer (Bio-Rad), using 'Criterion' (Bio-Rad) gel equipment. The pre-stained marker 'full range rainbow marker' (Invitrogen Life Technologies) was used as a molecular weight standard.

After electrophoresis, proteins were transferred to nitrocellulose membranes (Hybond-C extra, Amersham Pharmacia Biotech) by wet transfer, using a Bio-Rad trans-Blot cell. Electro-blotting was performed at 25V for 90 minutes in transfer buffer (20% (v/v) methanol, 50mM Tris-HCl and 380mM glycine). Membranes were blocked in 10mL of blocking buffer (PBS contained 0.1% Tween-20 and 5% non-fat dry milk) for 1 hour at room temperature. The blot was washed 3 times (for duration of 5 minutes per wash) in TBS-Tween-20 and was incubated with primary antibodies at 4°C overnight, or at room temperature for a period of 1 hour. After washing three times with TBS-Tween-20 detection of the transferred proteins were obtained by Enhanced Chemiluminescence (ECL). The secondary antibody (Anti-rabbit or anti mouse monoclonal horseradish peroxidase conjugate, Dako, Carpinteria, CA, USA), diluted 1:2000 in blocking buffer, was added for 1 hour at room temperature. Following an additional wash, the membrane was incubated with ECL detection reagent (GE Healthcare, UK) for 1 minute and then removed from the solution and wrapped in cling film. The membrane was put into a film case and brought to the dark room. A sheet of Kodak film (Kodak Bio Max Film) was overlaid on the blot for different exposure times: 2 minutes, except KU70/KU80 which was for 20 seconds. The film was soaked in developer (Sigma Aldrich, UK) for 2 minutes, fixed in fixing solution (Sigma Aldrich, UK) for 2 minutes, then washed in water for 2 minutes and allowed to dry. Details of the dilution of primary antibodies are given in Table 2.6

Table 2.5 List of Antibody Tested by Western Blot on Different Cell Lines.

Antibody	Cell lines used	Specific positive cell lines*
Rad51	Single cell line MCF-7	MCF-7 or HeLa BRCA1 cell lines
KU70/KU80	Single cell line MCF-7	MCF-7 or HeLa BRCA1 cell lines
SMC6L1	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
DNA-PK	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
ATM	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
CHK1	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
CHK2	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
γH2AX	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
PIAS1	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
UBC9	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
KPNA2	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
NPM	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines

Passages, Bradford assay and gel electrophoresis were done by the author. Passages used in W.B. were as follows; HeLa BRCA1; passages 29&30, HeLa BRCA1 control; passage 15&16, MCF-7; passages 25&26, MDA-MB-231; passage 15&16. * Available at http://www.proteinatlas.org/.

2.1.5 Reverse Phase Protein Microarray (RPPA)

Cell lines preparation and protein extraction and interpretation of the results were carried out by the author. However, the RPPA and analysis was carried out by Dr Ola Nejm (Immunology, School of Life Sciences, University Hospital, Nottingham, UK) as a collaborative project.

Three different passages were used in RPPA per experiment, and each run was duplicated. The passages numbers used were as follows: for HeLa BRCA1; between passage 21and 30, HeLa BRCA1 control; between passage 15and 20, MCF-7; between passage 25and 32, finally for MDA-MB-436; between passage 12 and 20. Cell line lysates were solubilised in a 4x SDS sample buffer, with a ratio of 1:3 respectively, and were boiled for 5 minutes at 95°C. Samples were loaded onto a 384-well plate (Genetix, UK), where each sample was serially diluted 5 times in 1x SDS buffer. Samples were robotically spotted, in duplicate, onto nitrocellulose-coated glass slides (GraceBiolab, USA), using a microarrayer (MicroGridII). Slides were incubated overnight in blocking solution (0.2% I-block (Tropix, Bedford, MA, USA) and 0.1% Tween-20 in PBS) at 4°C, with shaking. After washing three times for 5 minutes each (TBS-Tween-20), the slides were incubated overnight with the primary antibodies (details of the dilution of primary antibodies are given in Table 2.6) and optimally diluted in antibody diluents, in order to reduce background (DAKO). In addition, mouse anti-Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) antibody (BioLegend, UK), diluted 1:250 in the same diluent was used as house-keeping protein, in terms of control for protein loading. Slides were incubated overnight at 4°C, with shaking. Following washing, as described before, the slides were incubated with diluted infrared (1:5000 in washing buffer) secondary antibodies ((800CW anti-rabbit and 700CW anti-mouse antibodies) Dako, Carpinteria, CA, USA) for 30 minutes at room temperature in the dark, with shaking. The slides were washed as before and were dried through centrifugation at 500*g* for 5 minutes, before being scanned with a Licor Odyssey scanner at 21μm resolution at 800nm (green) and 700nm (red). The resultant TIFF images were processed with Axon Genepix Pro-6 Microarray Image Analysis software (Molecular Services Inc.), in order to obtain fluorescence data for each feature and to generate gpr files. Protein signals were finally

determined, with background subtraction and normalisation to the internal housekeeping targets, using RPPanalyzer, a module within the statistical language on the CRAN (http://cran.r-project.org/). Finally, a heat map was created using Multi Experiment Viewer (MEV) software.

All the antibodies were tested by RPPA, except PIAS4, this marker was previously stained by the author before starting the collaboration with Dr Ola Nejm, the aim was to validate RPPA on the cell lines and compare it with IHC's findings, and this was achieved on the other 15 antibodies (ATM, ATR, CHK1, CHK2, γH2AX, Rad51, BRCA1, BARD1, SMC6L1, KU70/KU80, DNA-PK, UBC9, PIAS1, KPNA2, and NPM).

2.1.6 Statistical Analyses

All statistical analyses were carried out using IBM SPSS statistic 21.0 software. All statistical analysis was carried out by Alaa Alshareeda except the RPPA results which were performed by Dr Ola Nejm. Mohammad Albanghali and Dr Jenna Reps provided advice regarding selecting the most suitable statistics tests. For all statistical tests, a two-sided *P-value* of <0.01 was considered statistically significant.

2.1.6.1 The Determination of the Optimal Cut-offs

Cut-off values for the positivity of biomarkers were individually assessed and determined based on previous publications [273]. Table 2.6 shows the details of primary antibodies; such as source, cut off and pre-treatment, used in this study. The cut-off of the biomarkers were dichotomised and obtained using different approaches: a) using the mean or median of the H-score of the staining according to distribution pattern whether normally or not normally distributed, or b) using x-tile software (version 3.6.1, 2003-2005, Yale University, USA http://x-tile.software.informer.com) (Table 2.6). x-tile plot shows the presence of significant tumour classes and illustrates the robustness of the relationship between a biomarker and outcome by construction of a two dimensional projection of every possible subpopulation. x-tile software randomly splits the total cohort of the patient into two separate identical training and validation sets by creating separate data of "censored" and "uncensored" observations, ranked by follow up time of the patients. However, those patients who died from BC were considered as an event (uncensored), whereas patients died from other causes or lost to follow-up during this study were censored during the analysis at that event time. Therefore, the optimal cut-points were identified by locating the brightest pixel on the diagram of x-tile plot of the training set. Statistical significance was investigated by validating the obtained cut-point to the validation set.

In the present study, x-tile was used if the value of mean or median was very high (e.g H-score >200). In the present study, it was used for Rad51 CHK1, γH2AX, ATM, ATR

[286], PIAS1, PIAS4 [285], KU70/KU80 [287], BARD1, DNA-PK, and NPM. However, for the reaming markers (CHK2, BRCA1, SMC6L1, UBC9 and KPNA2), regardless of cellular localisation, the cut-offs were based on the frequency distribution.

The following statistical methods were used:

2.1.6.2 Univariate Analysis with Clinico-Pathological al Parameters and Tumour Markers

All statistical analyses were performed using IBM SPSS 21.0. Analysis of continuous variables was performed using the appropriate statistical test (e.g. Pearson's correlation and ANOVA test [administered at 1% level of significance]). One way ANOVA test was used to find out which of different BC classes (by IHC or cell lines) were significantly different from each other (post hoc test; Tukey).

The differences between all categorical markers, with regards to clinico-pathological features, or with other tumour markers were analysed using the Pearson Chi-Squared test (x^2). Consequently, x^2 was also used in order to examine the inter-relations between markers themselves.

2.1.6.3 Univariate Analysis with Patients' Outcome

Patients still alive or those that died for any reason other than BC were censored. The Kaplan-Meier method (IBM SPSS 21.0) was used to generate a univariate survival curve and the differences in survival among the biomarkers were evaluated using the log-rank test.

2.1.6.4 Multivariate Analysis with Patients' Outcome

If a marker in univariate analysis was statistically significant with patient's outcome, then Cox regression (IBM SPSS 21.0) was applied for multivariate analyses to test for confounders and prognostic or predictive independency of the investigated biomarker from standard prognostic/predictive factors including tumour grade, tumour stage, and tumour size.

Table 2.6 Sources, Dilution, Cut-offs Point and Pre-treatment Conditions Used of the Primary Antibodies Used in this Study.

Antibody	Clone	Source	Dilution IHC	Dilution W.B RPPA	Distribution*	Cut-offs	IHC kit
DNA Damage Sensors and Signal Transducers							
ATM	Ab 32420	Abcam	1:100 overnight	1:1,000 1:500	Nuclear	≥75% x-tile	Novolink
ATR	1E9	Novus Biologicals	1:20 overnight	NT 1:500	Nuclear	≥18 H-score, x-tile[286]	Novolink
CHK1(Phospho S345)	Ab58567	Abcam	1:150 1h	1:1,000 1:6,000	Nuclear/cytoplasmic	≥20 H-score nuclear, ≥80 H-score cytoplasmic , x-tile.	Novolink
CHK2	Ab 47433	Abcam	1:100 1h	1:500 1:6,000	Nuclear	≥105 H-score, median.	Novolink
γH2AX (phospho S139)	Ab22551	Abcam	1:600 1h	1:2,000 1:1000	Nuclear/cytoplasmic	≥40 H-score nuclear, ≥120 H-score cytoplasmic, x-tile.	Novolink
Homologous Recombination Repair							
BARD1	NBP1-19636	Novus Biologicals	1:50 1h	NT 1:200	Nuclear/cytoplasmic	≥130 H-score cytoplasmic, >0 H-score nuclear, x-tile.	Novolink
BRCA1	Ab-1 (MS110)	Calbiochem	1:150 1h	NT 1:200	Nuclear/cytoplasmic	≥93 H-score nuclear, ≥40 H-score cytoplasmic, means	ABC[288]
Rad51	Ab88572	Abcam	1:70 1h	1:1,000 1:100	Nuclear/cytoplasmic	≥8 H-score nuclear ≥80 H-score cytoplasmic, x-tile.	Novolink
SMC6L1	AB57759	Abcam	1:100 1h	1:1,000 1:250	Nuclear/cytoplasmic	>240 H-score nuclear, mean, ≥230 H-score cytoplasmic, median.	Novolink
Non Homologous End Joining Repair							
KU70/KU80	Ab3108	Abcam	1:2500 1h	1:1,000 1:500	Nuclear	≥90 H-score, x-tile.	Novolink
DNA-PK	3H6	Cell signaling	1:28 1h	1:2,000 1:150	Nuclear	≥150 H-score, x-tile.	Novolink
Basal Cytokeratins							
Ck5	5Ab-1xM26	TBC	1:50 1h	NT NT	Cytoplasmic	≥10% [198, 273].	Novolink
CK17	VPC-413	Vector laboratories	1:100 1h	NT NT	Cytoplasmic	≥1% [198, 273].	Novolink
CK14	LL002	Novocastra	1:100 1h	NT NT	Cytoplasmic	≥10% [198, 273].	Novolink
Epidermal Growth Factor Receptor Family Members							
HER-2	Polyclonal	Dako-Cytomation	1:100 1h	NT NT	Membranous	≥10% [273].	ABC[268].
Hormone Receptors							
ER	1D5	Dako-Cytomation	1:200 1h	NT NT	Nuclear	≥1% [273].	ABC[268].
PgR	PgR	Dako-Cytomation	1:150 1h	NT NT	Nuclear	≥1% [273].	ABC[268].
BRCA1 Down Regulators Proteins							
MTA1	Ab84136	Abcam	1:200 1h	NT NT	Nuclear/cytoplasmic	Nuclear ≥50 H-score& Cytoplasm ≥120 H-score, x-tile.	Novolink
ID4	Ab77345	Abcam	1:100 1h	NT NT	Nuclear/cytoplasmic	Nuclear ≥12 H-score, mean &Cytoplasm ≥100 H-score, median.	Novolink
Proliferative Marker							
Ki-67**	MIB1 M7240	Dako-Cytomation	1:100 1h	NT NT	Nuclear	>34% mean.	ABC[268].

NT= not tested. IHC= immunohistochemistry. W.B= western blot. RPPA= Reverse Phase Protein Microarray. *Cellular localisation. **Ki-67 Performed on full face formalin fixed, paraffin embedded tissue sections (FFPE, 4μm thick), whereas all other antibodies were stained on TMA.MTA1, ID4, Ki-67, ER, PgR, HER-2, basal CKs were accessible and saved on a web-based interface (Distiller; Slidepath Ltd, Dublin, Ireland).

Table 2.6 Sources, Dilution, Cut-offs Point and Pre-treatment Conditions Used of The Antibodies Used in This Study Continued.

Antibody	Clone	Source	Dilution IHC	Dilution W.B RPPA	Distribution*	Cut-offs	IHC kit
Cell Cycle Progression/arrest Regulator Markers							
P21	EA10	Abcam	1:25 1h	NT NT	Nuclear	≥1% [273].	ABC[268].
P27	SX53G8	Dako-Cytomation	1:10 1h	NT NT	Nuclear	≥70 H-score [273].	ABC[268].
SUMO Markers							
PIAS4	NBP1-31215	Novus Biologicals	1:250 1h	NT NT	Nuclear	≥160 H-score, x-tile	Novolink
PIAS1	Ab32219	Abcam	1:425 1h	1:1,000 1:1,000	Nuclear/cytoplasmic	Nuclear ≥35H-score Cytoplasm ≥95H-score, x-tile	Novolink
UBC9	Ep2938Y	Novus Biologicals	1:225 1h	1:500 1:250	Nuclear/cytoplasmic	Nuclear ≥160H-score, median Cytoplasm ≥200H-score, mean.	Novolink
Tumour Suppressor Markers							
P53	DO7	Novocastra	1:50 1h	NT NT	Nuclear	≥5% [273].	ABC[268].
PTEN	MMAC1 Ab-4 (Clone 17.A)	Thermo	1:501h	NT 1:500	Nuclear	PTEN; ≥1 H-score, mean.	Novolink
Nucleocytoplasmic Transport Markers							
Nucleophosmin	Ab55708	Abcam	1:400 1h	1:5,000 1:500	Nuclear	≥180 H-score, x-tile.	Novolink
KPNA2	Ab84440	Abcam	1:400 1h	1:1,000 1:500	Nuclear	≥30 H-score, median.	Novolink

All the antibodies were pre-treated in citrate antigen retrieval pH=6.0 in microwave for 20 minutes and stained on TMA. NT= not tested. IHC= immunohistochemistry. W.B= western blot. RPPA= Reverse Phase Protein Microarray. *Cellular localisation. P53, PTEN, P21 and P27 were accessible and saved on a web-based interface (Distiller; Slidepath Ltd, Dublin, Ireland).

Chapter 3

3.1 Introduction

3.1.1 DNA Damage Response in Relation to Cell Cycle Control Checkpoints

The majority of human cells do not usually actively progress through a cell cycle; they generally remain in an 'out-of-cycle' state. A minority of cells do tend to be actively proliferating (cycling) and these are largely found in the stem-transit amplifying compartments of self-renewing tissues; for example, the epithelia and bone marrow [289]. The majority of functional cells are irreversibly withdrawn from the cell-division cycle directly into terminally differentiated states, such as the surface epithelial cells of skin/mucosa, or have reversibly withdrawn into a quiescent (G0) state, such as hepatocytes [290, 291].

The cell cycle has four distinct phases and the most essential phases are the synthesis (S) phase, when replication of the DNA commences, and the M (mitosis) phase, when the cell splits into two daughter cells. Two gap phases, referred to as G1 and G2, separate the S and M phases. G1 is the very first phase within the interphase, beginning from the end of the M phase and right up until DNA synthesis begins; this is a time when the cell becomes sensitive to positive and negative discriminative stimulus from networks of growth signalling. G2 refers to the gap immediately after the S phase and ensures that the cell is ready to enter the M phase and divide. G0 represents a state where cells possess reversibly reclusive rights from the cell division cycle in response to cells of high density, or maybe deprivation of mitogen [292]. However, cells may irreversibly recede from the cell cycle into terminally differentiated or senescent out-of-cycle states. Progression and transition from one phase to another through the cell cycle is monitored by sensor mechanisms, referred to as checkpoints, which usually manage the appropriate order of events [293]. If the sensor systems recognise aberrant or even incomplete cell cycle events, such as DNA damage, checkpoint pathways carry the signal directly to effectors, leading to the induced arrest of the cell cycle until it is repaired [294, 295]. Effector proteins comprise of Cyclin-Dependent Kinases inhibitors (CDKIs), which can often reversibly stop the progression of the cell cycle. Cell cycle engine deregulation underlies uncontrolled cell proliferation, which will subsequently characterise the phenotype of cancer [296, 297].

Following the detection of DNA lesions, DDR organises the repair of DNA, the control of the cell-cycle checkpoint and specific programmes, such as apoptosis and senescence, in order to increase genomic integrity and thus suppress tumorigenesis [298]. The decision as to which system of repair to use is dependent upon type of lesion and phase of the cell cycle.

As outlined in Chapter 1, the DNA-DSB lesion is the most lethal: it may be cytotoxic or cytostatic and produces oncogenic translocations [299, 300]. In the cell, this lesion is

usually repaired by two major pathways, the NHEJ and HR pathways. HR is cell-cycle dependent: it is controlled by Cyclin-Dependent Kinases (CDK) activity and, sometimes, simply by the expression of cell cycle-dependent or the stability of the required factors [301]. HR readily repairs DNA-DSB in cells in the S and G2 phases, whereas repair by NHEJ occurs during the G1 phase [302]. As cells develop directly into G2–M, the chromosomes are condensed into a highly-ordered structure of chromatin, which would make a homology search by sister chromatid difficult [302].

Checkpoints consist of cellular monitoring, in addition to signalling pathways that organise the repair of DNA, the metabolism of chromosomes and transitions of the cell-cycle [303, 304]. Checkpoint proteins tend to be recruited to DNA lesions through repair complexes that create the intermediate structures of DNA, which act as signals to trigger the response of checkpoints. For instance, in mammals, the MRE11–RAD50–Nbs1 (MRN) complex processes DSBs to create long single-stranded (ss) DNA regions, which stimulates the response of checkpoints [305, 306]. Following activation, the transducers of checkpoints transmit and enhance the signal of checkpoints to downstream targets; for example, the DNA-repair apparatus and the machinery of the cell-cycle [303]. The signal transmission or activation of these targets can often be completed by distinct phosphorylation events: this influence the level of transcription or activity of repair genes, in addition to regulating the cell-cycle transitions by affecting the stability of the activity of other proteins involved in the maintenance of checkpoints or progression of the cell-cycle. A major factor in the machinery of checkpoints are the phosphoinositide 3-kinase related kinases; Ataxia Telangiectaxia mutation (ATM), ATM-Rad3-related (ATR) and DNA- dependent Protein Kinase (DNA-PK). ATM and DNA-PK largely act in response to DSBs, whereas ssDNA activates ATR [304]. The activation and recruitment of these kinases to DNA lesions are the result of direct interactions with Nijmegen Breakage Syndrom1 (NBS1) (for ATM), ATRIP (for ATR) and KU80 (for DNA-PK) [307, 308]. The resection of DSBs, which can be much less effective in G1 and are restricted by CDK activity [305, 306, 309], additionally results in the activation of ATR13. Despite the fact that ATM and ATR respond to distinct lesions, recent evidence indicates that ATR is also stimulated by IR-induced DSBs, in a cell-cycle regulated manner [306]. The activation of ATR by DSBs requires ATM as well as MRN–CtIP (CtBP-interacting protein); thus, it only arises throughout the S and G2 phases and is eliminated by the inhibition of CDK [306, 310]. These findings are in-line with previous results demonstrating, in terms of IR-induced foci formation, that the HR protein (Rad51), in both yeast and human cells, is restricted to the S and G2 phases and is dependent on checkpoint activity [311-313]. The relevant CDK targets in this pathway, in addition to CtIP [310], remain to be determined.

As soon as ATM and ATR are recruited to damaged sites, they target many different substrates, such as checkpoint kinase-1 (CHK1) and CHK2 [314]. It is demonstrated that these signalling modules play a role in the organisation of the response of checkpoints to the repair of DNA [304, 314]. Generally, in terms of response to the DNA-DSB, ATM is recruited to damaged sites by the MRN sensor complex. Thus, CHK2 is activated at the

damage site after phosphorylation by ATM, which, with other ATM substrates, subsequently activate a cascade of proteins including histone H2AX, mediator of DNA damage checkpoint 1 (MDC1), 53BP1 and BRCA1 [315, 316]. In terms of short sequences of single-strand breaks created by DSBs, the Replication Protein A (RPA)-coated DNA recruits ATR and its interacting protein, ATRIP, with assistance at the site of the lesion from Rad17, Rad9, Rad1 and complexes of Hus1 [317, 318]. Subsequent ATR phosphorylation stimulates the mediator DNA Topoisomerase 2-Binding Protein 1 (TopBP1), leading to interaction and the phosphorylation of additional mediator proteins, such as H2AX, claspin and BRCA1, which eventually induces the activation of CHK1 [319].

CHK1 phosphorylation of Rad51 is required for HR in mammalian cells; in addition, CHK1 affects the replacement of RPA on ssDNA, together with Rad51 and Rad52, in a process that triggers the formation of Rad51 presynaptic filaments and HR-mediated (DSB repair) initiation [320]. The ATM-dependent phosphorylation of the Nbs1 subunit forms a complex of MRN and Fanconi Anemia group D2 protein (FANCD2) [321] from the crosslink-repair pathway of Fanconi Anaemia (FA), which is believed to enhance the repair of HR during the S phase [322]. A common response to DSBs, chromatin relaxation is promoted by ATM phosphorylation of KRAB-associated protein 1 (KAP1) [323]. The ATM-dependent phosphorylation of histone H2AX is an early event that is essential in the efficient repair of DSBs and potentially plays a role in repair recruitment, cohesion and checkpoint factors [324].

H2AX is a key component in DNA repair, which is readily phosphorylated on serine four residues from the carboxyl terminus (serine c-4), forming γH2AX at nascent sites of DSB. Within 30 minutes of the formation of a DSB, vast quantities of γH2AX form in the chromatin located around the break site, developing a focus on where proteins participated in the repair of DNA and on the accumulation of chromatin remodelling [325]. The HR functions of H2AX are mediated by γH2AX interaction, along with the chromatin-associated adaptor protein MDC1. H2AX is most likely susceptible to further post-translational modifications related to the response of DNA damage, along with additional functions of chromatin [326]. γH2AX is essential for sister-chromatid homologous recombination, most likely in assisting the connections between sister chromatids [327]. A summary of the most common markers in response to DNA damage is shown in Figure 3.1.

3.2 Hypothesis

Cellular response to any genotoxic stress is a highly complex process, and it usually begins with one of two systems; the “sensing” or “detection” of the damage of DNA, then a number of events that include signal transduction and transcription factors activation. The activated transcription factors stimulate expressions of several genes which usually have roles in different cellular functions such as DNA repair, cell cycle arrest, and apoptosis. The response to DNA damage enhances genomic integrity and the ability for replication and thus

suppresses tumuorigenesis. Correct genetic information transmission from one cell to its progeny is based mostly on processes within the cell to check any defects within its genome as well as to repair these deficiencies in order not to pass them to following generations. These processes are mainly controlled through an array of DNA damage response proteins such as DNA damage sensors, signal transducers and effectors. Sensors, such as ATM and ATR, have the ability to identify damage areas and trigger signal transducers, which either activate or inactivate effectors. Effector proteins trigger checkpoints of cell cycle such as; CHK1 and CHK2, and then the cell may efficiently repair the damage or carry on towards apoptosis if these types of damages are irreparable. However, these molecules are not only essential for surveillance of occasional non-lethal DNA damage, yet are likewise necessary for the cell survival as well as the organism [328]. It is thus hypothesised that alterations of the DNA damage sensors may contribute to the development and progression of at least a proportion of sporadic breast carcinoma particularly those associated with features similar to breast carcinoma arising in patients with *BRCA*1 germline mutations [329, 330]. Despite the fact that the previous studies have described the sequence of events associated with DNA damage sensing, signalling transduction and repair in cells, the whole process appears to be complex and its characterisation in BC remains to be explored.

3.3 Aim

This chapter aims to underline the importance of the DNA damage sensors (ATM and ATR) signal transducers (CHK1 and CHK2) and DNA damage repair factor protein such as γH2AX in BC by using IHC; TMA in the context of specific, well-defined patient subgroups and RPPA in different cell lines. In addition, the association between cell-cycle markers, pathological features, expression of tumour biomarkers and clinical outcome will be determined.

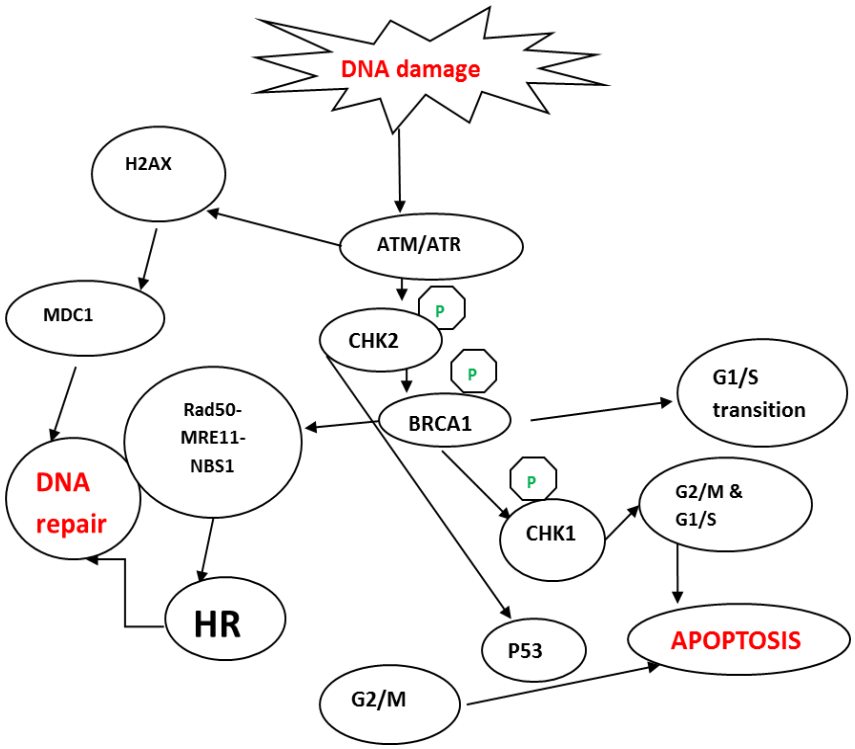

Figure 3.1 The roles of CHK1 and CHK 2 in cell cycle and DNA repair. In response to DNA damage, ATM (usually targets DNA- DSB), and/or ATR (usually targets ssDNA) trigger the activation of checkpoint CHK2 and CHK1 respectively that leads to cell cycle arrest or delay. Checkpoints pathways are characterised by cascade of protein phosphorylation events (indicated with P) that alter the activity, and even localisation of modified proteins. This leads to one of two reactions either apoptosis or DNA repair.

3.4 Materials and Methods

A previously described in Chapter 2

3.4.1 Patient Samples

All data are as previously described in Chapter 2 Section 2.1.1. Three cohorts were used: A) 1904 unselected cases of female primary operable invasive tumours between 1986 and 1998, B) 386 cases selected from a consecutive series of primary operable ER negative tumours between 1998 and 2007 and C) 24 well-characterised series of breast tumours from patients with known *BRCA1* germline mutations. However, HRMA with employing PCR was used for *BRCA1* mutation detection in group C (this was performed by Dr Ahmed Benhasouna). All cases were obtained from the well-characterised Nottingham Tenovus primary breast carcinoma series.

3.4.2 Available Biomarkers' Data

Data on a wide range of biomarkers of known clinical and biological relevance to BC were accessible and saved on a web-based interface (Distiller; Slidepath Ltd, Dublin, Ireland). These include, ER, PgR, HER-2, CK5, CK17, CK14, tumour suppressor proteins (P53 and PTEN), and cell proliferative marker (Ki-67) [270-272, 331].

3.4.3 Immunohistochemistry

As previously described in Chapter 2 Section 2.1.3. Five markers of DNA damage sensors and signal transducers, and a repair factor protein (ATM, ATR, CHK1, CHK2 and γH2AX) were investigated in this chapter.

3.4.3.1 Immunohistochemical Antibody Labelling Using the Novolink Detection Method

As previously described in Chapter2 Section 2.1.3.1. In this chapter, CHK1 and γH2AX were stained by the author as data on CHK2, ATR, and ATM expression were already available (Acknowledgement: Mr Paul Moseley). All these markers have already been previously successfully stained on TMA [332-337].

3.4.3.2 Optimisation of Antibodies Used for IHC

As previously described in Chapter 2 Section 2.1.3.2. In addition to Western blotting, specificity of staining was confirmed by application of negative (with omission of the primary antibody) and positive controls. Positive controls were used according to the manufacturer's datasheet and/or from the human protein atlas available at http://www.proteinatlas.org/. This helped not only to test the specificity of staining but also to assess the pattern and intensity of protein expressions in the appropriate tissue. Details of the negative and positive controls used are summarised in Table 3.1. Moreover, some control TMA slides containing a variety of BC cases with some containing cores from different areas of the same cases in addition to normal parenchymal elements were used during optimisation to assess the degree of expression heterogeneity.

Table 3.1 Immunohistochemistry Positive and Negative Controls of Antibodies Used in this Chapter.

Antibody	Positive control	Negative control	Reference
ATR	Ovarian cancer	BC tissue	Novus Biologicals/ human protein atlas available at http://www.proteinatlas.org/
ATM	BC tissue	BC tissue	Abcam/ human protein atlas available at http://www.proteinatlas.org/
CHK1	BC tissue	BC tissue	[332]
CHK2	BC tissue	BC tissue	Abcam/ human protein atlas available at http://www.proteinatlas.org/
γH2AX	BC tissue	BC tissue	Abcam/ human protein atlas available at http://www.proteinatlas.org/
Stating of positive and negative controls was performed together in the same run. Negative staining was performed without adding the antibody which showed no staining. All were performed on TMA. All these markers have already been previously successfully stained on TMA [332-337].			

3.4.3.3 Immunohistochemistry Scoring

As previously described in Chapter 2 Section 2.1.3.3. For evaluation of IHC of the TMA, a modified histochemical score (H-score) was used [282]. For H-score, both the intensity of staining and the percentage of stained cells were considered within each tissue core. Staining intensity was scored as 0, 1, 2 or 3 for negative, weak, moderate and strong, respectively. The proportion (percentage) of positive cells for each intensity was subjectively estimated. Multiplication of the two indices (intensity and percentage positive cells) provided final scores that range from 0 to 300.

The author re-scored each marker with at least 30% of a randomly chosen subset of cases. A statistical agreement test was performed (Kappa value) for each marker, where there was good agreement ($\geq$0.5), and an average was taken. If there were discrepancies, the highest scoring was taken. Kappa values are summarised in Table 3.2. ATM, ATR, CHK2, and CHK1 were scored using light microscopy, whereas γH2AX were scored visually using high-resolution digital images using a web-based interface (Distiller, Slidepath Ltd., Dublin, Ireland).

Scoring of ATM, ATR and CHK2 was carried out by Dr Tarek Abdel-Fatah, which 30% were rescored by the author. However, CHK1, and γH2AX were scored and re-scored by the author (100%, 30% of the cases were rescored respectively).

Table 3.2 The Statistical Agreement between Different Scoring of Antibodies Used in this Chapter.

Markers	Percentage of re-scoring	Kappa value
γH2AX.n	30%	0.83
γH2AX.c	30%	0.86
CHK1.n	100%	0.69
CHK1.c	100%	0.6
CHK2	30%	0.72
ATR	30%	0.70
ATM	30%	0.63
Kappa test was performed on IBM SPSS 21.0 software. An average was taken after re-scoring.		

3.4.4 Specificity of the Antibodies by Western Blot

As previously described in Chapter 2 Section 2.1.4.4. Western blot was used on all markers except ATR. ATR marker was previously stained by a collaborative group who used IHC negative and positive controls for this marker (Acknowledgement: Mr Paul Moseley). A mixture of different cell lysates to detect only the specificity of an antibody has been applied in different studies and showed its reliability [283, 284]. In the present study, ATM, CHK1, CHK2, and γH2AX were detected in a mixture of different lysates (MCF-7, MDA-MB-231, HeLa BRCA1 and its control). All the antibodies tested in this chapter share the same positive controls which were MCF-7 and HeLa BRCA1 cell lines. The pre-stained marker 'full range rainbow marker' (Invitrogen Life Technologies) was used as a molecular weight standard. http://www.proteinatlas.org/ provides profile data for positive controls of all the markers used in this study. Table 3.3 summarises the details of W.B for each marker.

Table 3.3 List of Antibodies Tested by Western Blot on a Mixture of Different Cell Lines.

Antibody	Cell lines	Specific positive cell lines*
ATM	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
CHK1	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
CHK2	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
γH2AX	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
Cell lines and reagent were obtained from the group of Dr Madhusudan Srinivasan. Thawing and freezing procedures were done by Nada Albarakati. Passages, Bradford assay and gel electrophoresis were done by the author. Passages used in W.B were as follows; HeLa BRCA1; passages 29&30, HeLa BRCA1 control; passages 15&16, MCF-7; passages 25&26, MDA-MB-231; passages 15&16. *Data available at http://www.proteinatlas.org/		

3.4.5 Reverse Phase Protein Microarray

RPPA was carried out as previously described in Chapter 2 Section 2.1.5. Cell line preparation and protein extraction and interpretation of the results were carried out by the author. However, RPPA run and analysis was carried out by Dr Ola Nejm (Immunology, School of Life Sciences, University Hospital, Nottingham, UK) as a collaborative project.

3.5 Statistical Analysis

All statistical analyses were done by the author using IBM SPSS 21.0 software. For all statistical tests, a two-sided *P-value* of <0.01 was considered statistically significant.

3.5.1 The Determination of the Optimal Cut-offs

As described previously in Chapter 2 Section 2.1.6.1. Biomarker expression were dichotomised using different approaches: a) using the mean or median of the H-score of the staining according to distribution pattern whether normally or not normally distributed, or b) using x-tile software (version 3.6.1, 2003-2005, Yale University, USA). If the cut-off by

mean or median was very high (e.g H-score >200) then x-tile was considered such as in, CHK1, γH2AX, ATM [286] and ATR. The median for CHK2 was used as cut off. Table 3.4 shows the details of the antibodies used in this chapter. Details of H-score histograms are presented in Appendix 1.

Table 3.4 Sources, Dilution, Cut-offs Point and Pre-Treatment Conditions of the Antibodies Used in this Chapter.

Antibody	Clone	Source	Dilution IHC	Dilution W.B RPPA	[+]Distribution	Cut-offs	IHC kit
ATM	Ab 32420	Abcam	1:100 overnight	1:1,000 1:500	Nuclear	≥75% x-tile.	Novolink
ATR	1E9	Novus Biologicals	1:20 overnight	NT 1:500	Nuclear	≥18 H-score, x-tile. [286]	Novolink
CHK1(Phospho S345)	Ab58567	Abcam	1:150 1h	1:1,000 1:6,000	Nuclear/cytoplasmic	Nuclear ≥20 H-score, Cytoplasm ≥80 H-score, x-tile.	Novolink
CHK2	Ab 47433	Abcam	1:100 1h	1:500 1:6,000	Nuclear	≥105 H-score, median.	Novolink
γH2AX (phospho S139)	Ab22551	Abcam	1:600 1h	1:2,000 1:1,000	Nuclear/cytoplasmic	Nuclear ≥40 H-score Cytoplasm ≥120 H-score, x-tile.	Novolink

IHC= immunohistochemistry. W.B= western blotting. NT= not tested. All the antibodies were pre-treated in citrate antigen retrieval pH=6.0 in microwave for 20 minutes and stained on TMA. CHK1, CHK2, and γH2AX were incubated at room temperature for 1 hour, whereas ATR and ATR were incubated at 4^{o}C overnight. [+] Cellular localisation.

3.5.2 Univariate Analysis with Clinico-Pathological al Parameters and Tumour Markers

The differences between all markers, with regards to clinico-pathological features, or with other tumour markers were analysed using the Pearson Chi-Squared test (x^2). Consequently, x^2 was also used in order to examine the inter-relations between markers themselves. In addition analysis of continuous variables was performed using the appropriate statistical test Pearson's correlation and ANOVA. One way ANOVA was used to find out which of different BC classes (by IHC or RPPA) were significantly different from each other (post hoc test; Tukey).

3.5.3 Univariate Analysis with Patients' Outcome

Patients who were alive or those who died for any reason other than BC were not included. The Kaplan-Meier method was used to generate a univariate survival curve and the differences in survival among the biomarkers were evaluated using the log-rank test.

3.5.4 Multivariate Analysis with Patients' Outcome

If a marker in univariate analysis was statistically significant with patient's outcome, then Cox regression was applied for multivariate analyses to test for confounders and prognostic or predictive independency of the investigated biomarker from standard prognostic/predictive factors such as tumour grade, tumour stage, and tumour size.

3.6 Results

3.6.1 Expression of DNA Damage Sensors and Signal Transducers Proteins in Invasive Breast Cancer

Western blotting validated the specificity of some antibodies in the DNA damage sensors pathway, this was deemed to be validated by a single band at the correct protein size (see Figure 3.2). In invasive tumours, CHK2, ATM and ATR showed nuclear (n) staining, whereas γH2AX and CHK1 showed both nuclear and cytoplasmic (c) staining. Although CHK1 and γH2AX are mainly localised in the nucleus, cytoplasm expression has been mentioned previously [338-340]. Figures 3.3 and 3.4 show the immunostaining expression of DNA damage sensors and signal transducers proteins. Table 3.5 outlines the frequencies of DNA damage sensors and signal transducers proteins in sporadic and known *BRCA1* germline mutations BCs (hereditary), while Figure 3.5 represents the distribution (mean) of these proteins in the different classes of BC by IHC. The 4 classes included were classified based on BRCA1 and ER proteins expression. Class 1; sporadic BRCA1 negative and ER negative, class 2; sporadic BRCA1 positive and ER positive, class 3; known *BRCA1* germline mutation BC that showing ER negativity and finally class 4; known *BRCA1* germline mutation BC and showing ER positivity.

ATM, CHK2, CHK1.n, and γH2AX.n staining showed stronger expression in sporadic ER^+ cancers than sporadic ER^- or known *BRCA1* germline mutations showing ER^-. In contrast, ATR protein was higher in known *BRCA1* germline mutations regardless of ER status than sporadic BRCA1/ER tumours. In contrast, CHK1.c showed lower expression in sporadic ER/BRCA1 tumours (regardless of ER status) than sporadic BC. γH2AX.c expression was almost similar among the classes (Figure 3.5). There was a strong difference between sporadic $BRCA1^-/ER^-$ and sporadic $BRCA1^+/ER^+$ among CHK2, CHK1.n, ATM, and γH2AX.n (all $P<0.0001$, Figure 3.5). In addition a strong statistical difference was seen between sporadic $BRCA1^-/ER^-$ and hereditary BC showing ER^- among CHK1.c and ATR ($P<0.0001$, and $P=0.006$ respectively, Figure 3.5). However, only CHK1.c showed a statistical difference between sporadic ER^-/ $BRCA1^-$ and hereditary BC showing ER^+ ($P<0.0001$, Figure 3.5). Sporadic ER^+/ $BRCA1^+$ vs. Hereditary ER^- was significantly different among CHK1.n ($P<0.0001$, Figure 3.5), CHK1.c ($P<0.0001$), ATM ($P<0.0001$), ATR ($P=0.001$), CHK2 ($P=0.009$), and γH2AX.n ($P=0.002$) (all Figure 3.5). Only CHK1.c showed

a significant association between sporadic ER^+ $BRCA1^+$ vs. hereditary BC showing ER^+ (P=0.0003).

The large error bars of Figure 3.5 is expected, the mean of H-score does not explain repeated observation, it shows the distribution of different cases share ER and BRCA1 status but with other factors such as grade, stage and size of the tumour which may have some effects on the expression of the markers.

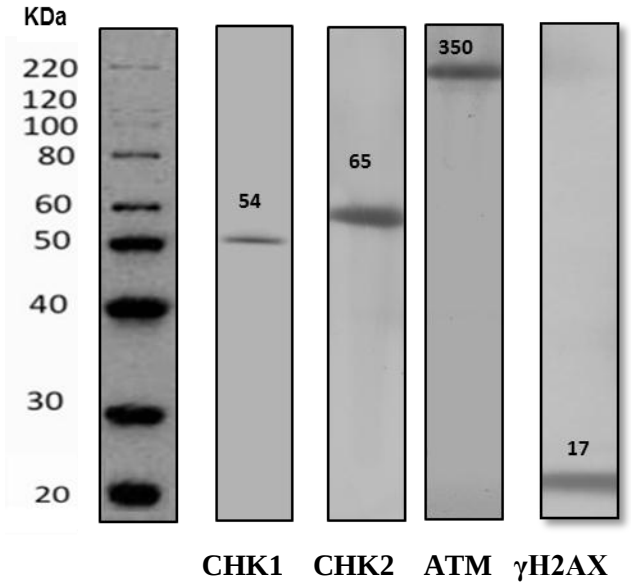

Figure 3.2 Detection of DNA damage sensors and signal transducers proteins level by Western blot in a mixture of cell lines, MDA-MB-231, MCF-7, HeLa BRCA1 and its control. The predicted size of each protein is labelled on the band. Passages used in W.B were as follows; HeLa BRCA1; passages29&30, HeLa BRCA1 control; passages 15&16, MCF-7; passages 25&26, and MDA-MB-231; passages 15&16.

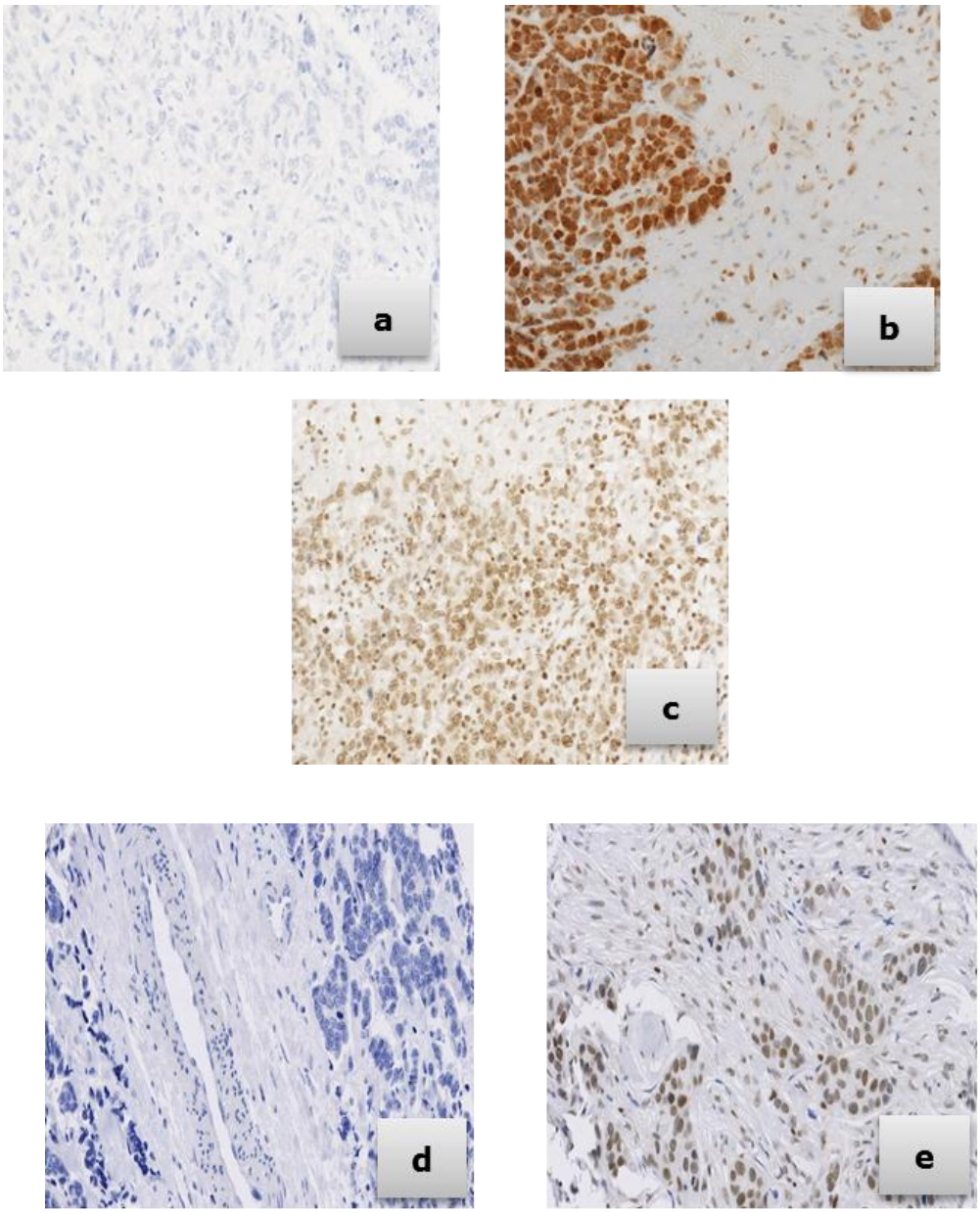

Figure 3.3 Expressions of ATR and ATM on TMA. a; a negative control for ATR in invasive ductal carcinoma of no special type stage 1 and grade 3 breast cancer. b; a positive control for ATR (nuclear expression) in ovarian cancer. c; nuclear expression of ATR in known *BRCA1* germline mutation, (stage data is missing) grade 3 ductal invasive carcinoma of no special type breast cancer. d; a negative control for ATM in invasive ductal carcinoma of no special type; stage 1 and grade 2. e; nuclear expression of ATM in lobular mixed breast cancer; stage 2 and grade 2 which is also a positive control for ATM. Magnification x20.

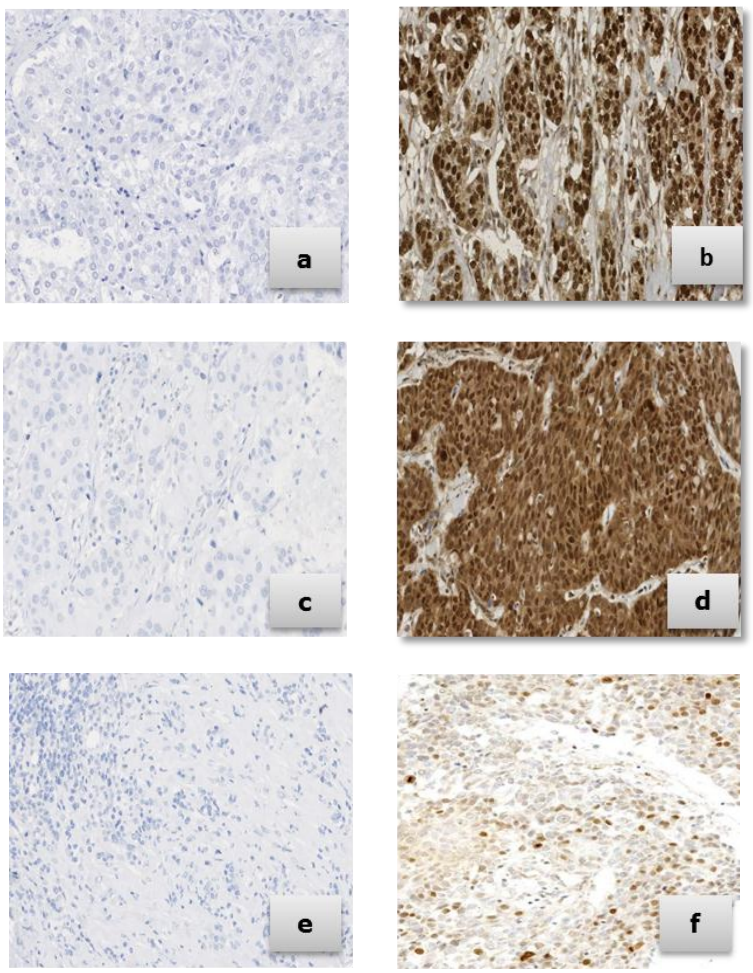

Figure 3.4 Expression of γH2AX, CHK1, and CHK2 proteins by IHC. Where a; a negative control for γH2AX in invasive ductal breast carcinoma of no special type; grade2 and stage 1. b; γH2AX nuclear and cytoplasmic expression in invasive ductal carcinoma of no special type; grade 1 and stage 1. c; a negative control for CHK1 in invasive ductal breast carcinoma of no special type; grade 2 and stage 1. d; CHK1 nuclear and cytoplasmic expression in invasive ductal carcinoma of no special type; grade 2 and stage 1. e; a negative control for CHK2 in invasive ductal breast carcinoma of no special type; grade3 and stage 1.f; nuclear expression of CHK2 in known *BRCA1* germline mutation, invasive ductal breast carcinoma of no special type; grade 3 (data for stage is missing). γH2AX, CHK1, and CHK2 used breast cancer as a positive control. Magnification x20.

Table 3.5 Frequencies of DNA Damage Sensors and Signal Transducers Proteins Expression in Breast Cancer.

Markers	Sporadic BC		Known *BRCA1* germline mutation BC	
	(%)	Frequency	(%)	Frequency
CHK1				
Nuclear	26.9	355/1322	5.3	1/19
Cytoplasmic	89.6	1183/1322	63.2	17/19
CHK2	50.9	543/1066	0	0/24
ATM	45.7	636/1392	8.3	2/24
ATR	44	566/1285	73.9	17/23
γH2AX				
Nuclear	87.6	1137/1299	89.5	17/19
Cytoplasmic	88.9	1155/1299	84.2	16/19
Sporadic BC includes both unselected and ER-negative BC cases. The number of cases may be reduced due to loss of cases during preparation of tissue for staining (TMA sectioning or IHC procedure).				

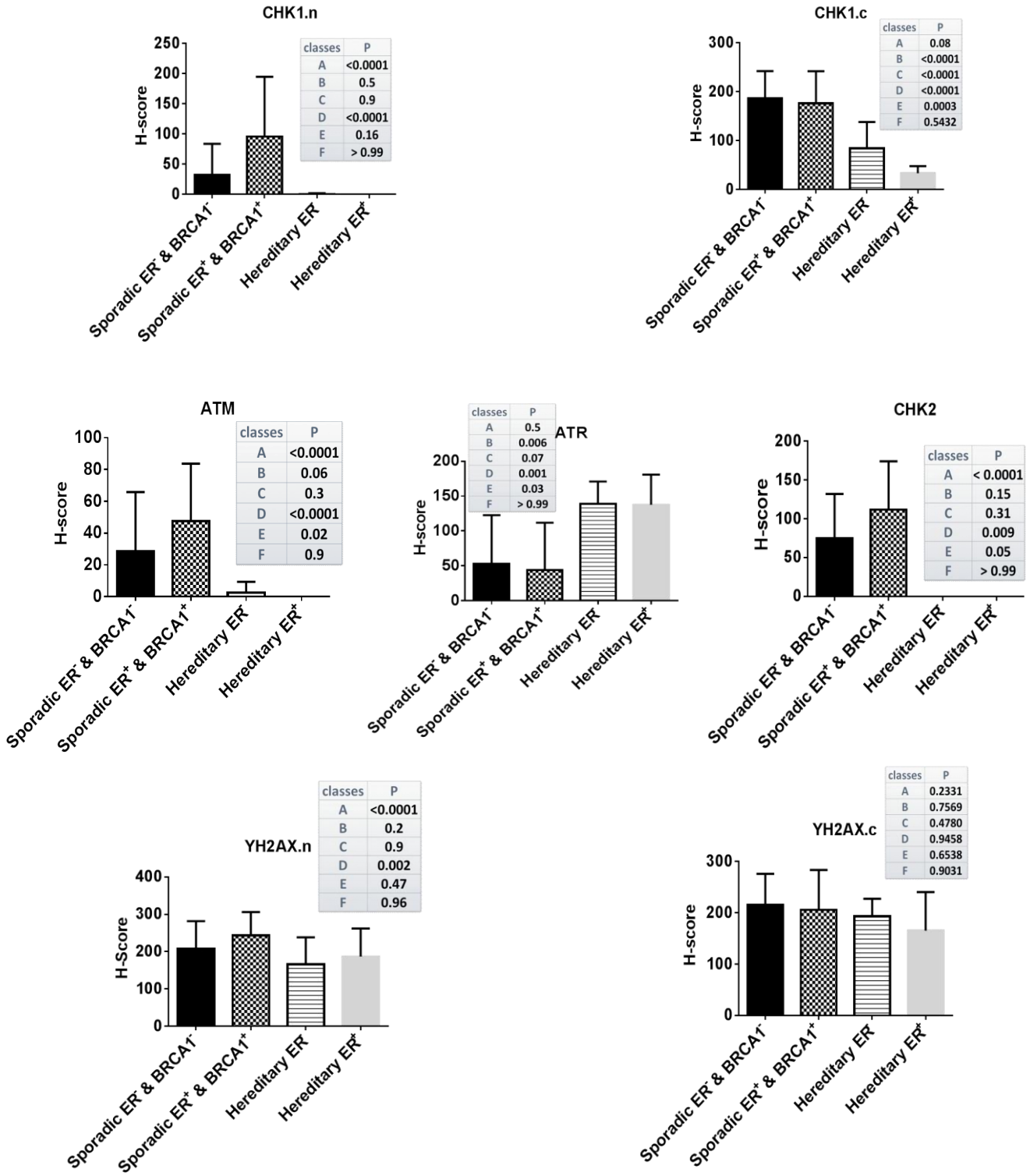

Figure 3.5 DNA damage sensor and signal transducers protein levels detected by IHC on TMA. Each bar represents different class based on hereditary or sporadic BRCA1 and ER status. Where n. is nuclear expression and c. is cytoplasmic expression. Error bars represent Mean (SD) and was created on H-score (ranges 0-300). A= sporadic cases [ER^- & $BRCA1^-$] vs. sporadic cases [ER^+ & $BRCA1^+$], B= sporadic cases [ER^- & $BRCA1^-$] vs. Hereditary cases [ER^-], C= sporadic cases [ER^- & $BRCA1^-$]vs. Hereditary cases [ER^+], D= sporadic cases [ER^+ & $BRCA1^+$]vs. Hereditary cases [ER^-], E= sporadic cases [ER^+ & $BRCA1^+$] vs. Hereditary cases [ER^+], and F= Hereditary cases [ER^-] vs. Hereditary cases [ER^+]. One way ANOVA test was used for each marker within the classes.

3.6.2 Correlation of DNA Damage Sensors and Signal Transducers Proteins

The associations between co-expression of cellular localisation of DNA damage sensors and signal transducers proteins are summarised in Table 3.6 (Pearson X^2). $\gamma H2AXn^+.c^+$ showed a high negative association with CHK1.n and positive associations with CHK1.c and CHK2 (all P<0.0001). $CHK1n^-.c^+$ had an association with both ATM and ATR; P<0.0001, and P=0.001 respectively. ATR showed a negative association with γH2AX.n (P=0.007). CHK2 showed a direct association with ATM (P=0.002), and ATR (P=0.004). Significant and non-significant correlations of DNA-damage sensors and signal transducers proteins are detailed in Appendix1.

Table 3.6 The Correlation between Co-expression of Cellular Localisation of DNA Damage Sensors and Signal Transducers Proteins.

γH2AX							
Parameters		**c^-n^- N (%)**	**c^+n^+ N (%)**	**c^+n^- N (%)**	**c^-n^+ N (%)**	***X²***	**P**
CHK1.n	**Negative**	11(84.6)	496(76.5)	83(84.7)	47(58)	19	<0.0001
	Positive	2(15.4)	152(23.5)	15(15.3)	34(42)		
CHK1.c	**Negative**	6(46.2)	47(7.3)	10(10.2)	16(19.8)	34	<0.0001
	Positive	7(53.8)	600(92.7)	88(89.8)	65(80.2)		
CHK2	**Negative**	7(63.6)	274(45.4)	63(84)	35(50)	41	<0.0001
	Positive	4(36.4)	330(54.6)	12(16)	35(50)		
CHK1							
Parameters		**c^-n^- N (%)**	**c^+n^+ N (%)**	**c^+n^- N (%)**	**c^-n^+ N (%)**	***X²***	**P**
ATM	**Negative**	26(66.7)	80 (40.4)	368 (59.8)	19 (47.5)	26	<0.0001
	Positive	13(33.3)	118 (59.6)	247 (40.2)	21 (52.5)		
ATR	**Negative**	26 (45.6)	109(67.3)	264(50.6)	19(51.4)	16	0.001
	Positive	31(54.4)	53(32.7)	258(49.4)	18(48.6)		
ATR							
Parameters		**Negative N (%)**		**Positive N (%)**		***X²***	**P**
γH2AX.n	**Negative**	66(13.4)		37(8)		7	0.007
	Positive	427(86.6)		425(92)			
CHK2							
Parameters		**Negative N (%)**		**Positive N (%)**		***X²***	**P**
ATM	**Negative**	203(59.9)		147(47.6)		10	0.002
	Positive	136(40.1)		162(52.4)			
ATR	**Negative**	257(57.1)		219(47.6)		8	0.004
	Positive	193(42.9)		241(52.4)			

N= number of cases. c. = cytoplasmic, n. = nuclear expression. The cut off points of positivity were as follows: ≥ 20 H-score for CHK1.n and ≥80H-score for CHK1.c, ≥105 H-score for CHK2, ≥75% for ATM, ≥18 H-score or ATR. This table shows the co-expression of cellular localisation of γH2AX and CHK1.

3.6.3 Correlation of DNA Damage Sensors and Signal Transducers Proteins with Other Tumour Markers

The correlations between categorical (Pearson X^2) DNA damage sensors and signal transducers proteins and other tumour biomarkers (regarding co-expression of cellular localisation of CHK1 and γH2AX) are summarised in Tables 3.7a-b. In addition, Table 3.8 showed similar correlation but using continuous data (Pearson's correlation). Some correlations between categorical and continuous data were different therefore, only these categorical correlations were re-analysed, but regardless co-expression of cellular localisation, in order to compare each cellular localisation separately (Table 3.9).

In regards to hormone receptors, there was a highly significant association between ER^- with; ATM^-, $CHK1n^-.c^+$ and $\gamma H2AXn^+.c^+$; all P<0.0001 and CHK2; P=0.007, in addition PgR^- with (ATM, CHK1 and γH2AX; all P<0.0001, Tables 3.7a-b) and finally TN tumours with; ATM, CHK1 and γH2AX; all P<0.0001, Tables 3.7a-b. Only CHK1 showed a significant negative association with HER-2 (P=0.005, Tables 3.7b).

There was a highly significant association between the DNA damage sensors and signal transducers proteins (except CHK2) and at least one of the basal cytokeratins (CK5 or CK17) or with BLBC, as defined by the negative expression of ER, PgR and HER-2 in addition to the positive expression of the basal CKs (CK5, CK17, and CK14). CHK1 and γH2AX with BLBC; both P<0.0001 and ATM; P=0.002, (Tables 3.7a-b). ATR with CK17; P=0.001, CHK1 with CK5; P=0.001 and γH2AX with CK5; P<0.0001, Tables 3.7a-b). There was a high significant association between $\gamma H2AXn^+.c^+$ (P=0.001), $CHK1n^-.c^+$ (P=0.001), and $CHK2^-$ (P<0.0001) with $PTEN^-$ (Tables 3.7a-b), but not with ATM or ATR. Significant and non-significant correlations of DNA-damage sensors and signal transducers proteins are detailed in Appendix1.

Table 3.8 (Pearson's correlation) shows the correlation on continuous data of DNA damage sensors and signal transducers proteins with other tumour markers. Regression analysis of continuous data (Table 3.8) confirmed the categorical data (Tables 3.7 and 3.9). However, when γH2AX.c was analysed as a continuous variable, some correlations showed different results. γH2AX.c had a significant correlation with CHK1.n, PgR and ER in categorical data, P=0.001, P=0.001 and P<0.0001 respectively, Table 3.9), while the correlation lost significance in continuous data (P=0.2, P=0.5 and P=0.1 respectively, Table 3.8). Similarly was seen between CHK1.c and PgR (categorical; P<0.0001, and continuous; P=0.26, Tables 3.9 and 3.8 respectively), whereas, continuous analysis increased the significance of the correlation between CHK1.n and CK5 (categorical; P=0.2, and continuous; P=0.008, Tables 3.9 and 3.8).

Table 3.7a The Correlation between Co-expression of Cellular Localisation of γH2AX with other Tumour Markers.

γH2AX							
Parameters		**c^-n^- N (%)**	**c^+n^+ N (%)**	**c^+n^- N (%)**	**c^-n^+ N (%)**	***X²***	**P**
ER	**Negative**	9(52.9)	426(43)	91(67.4)	30(24.8)	49	<0.0001
	Positive	8(47.1)	564(57)	44(32.6)	91(75.2)		
PgR	**Negative**	14(73.7)	509(54.1)	101(75.4)	41(35.7)	43	<0.0001
	Positive	5(26.3)	432(45.9)	33(24.6)	74(64.3)		
TN	**Negative**	10(55.6)	690(72)	61(45.9)	97(84.3)	52	<0.0001
	Positive	8(44.4)	268(28)	72(54.1)	18(15.7)		
CK5	**Negative**	9(75)	560(76)	62(55.4)	74(83.1)	26	<0.0001
	Positive	3(25)	117(24)	50(44.6)	15(16.9)		
BLBC	**Negative**	9(64.3)	674(78)	63(53.4)	95(86.4)	42.5	<0.0001
	Positive	5(35.7)	190(22)	55(46.6)	15(13.6)		
Ki-67	**Negative**	6(40)	287(32.8)	21(17.4)	56(52.8)	32.5	<0.0001
	Positive	9(60)	588(67.2)	100(82.6)	50(47.2)		
PTEN	**Negative**	10 (100)	423 (79.7)	64 (94.1)	43 (69.4)	15.6	0.001
	Positive	0	108 (20.3)	4 (5.9)	19 (30.6)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. The cut off points are presented in the next table. This table shows the co-expression of cellular localisation of γH2AX.

Table 3.7b The Correlation between DNA Damage Sensors and Signal Transducers Proteins with other Tumour Markers.

CHK1							
Markers		**c^-n^- N (%)**	**c^+n^+ N (%)**	**c^+n^- N (%)**	**c^-n^+ N (%)**	***X²***	**P**
ER	**Negative**	16(21.9)	100(35.6	397(46)	11(18.6)	35	<0.0001
	Positive	57(78.1)	181(64.4	466(54)	48(81.4)		
PgR	**Negative**	28(73.3)	119(44.9	467(56.3)	19(33.3)	26	<0.0001
	Positive	47(62.7)	146(55.1	362(43.7)	38(66.7)		
TN	**Negative**	62(86.1)	213(78.6	581(69.2)	49(84.5)	20	<0.0001
	Positive	10(13.9)	58(21.4)	258(30.8)	9(15.5)		
HER-2	**Negative**	66(93)	241(86.1	703(82)	55(94.8)	13	0.005
	Positive	5(7)	39(13.9)	154(18)	3(5.2)		
CK5	**Negative**	48(92.3)	159(75.4	543(72.7)	39(92.9)	17.5	0.001
	Positive	4(7.7)	52(24.6)	204(27.3)	3(7.1)		
BLBC	**Negative**	56(90.3)	207(81.2	582(74.8)	51(96.2)	22	<0.0001
	Positive	6(9.7)	48(18.8)	196(25.2)	2(3.8)		
P53	**Negative**	58(81.7)	175(63.6	516(61)	46(79.3)	19	<0.0001
	Positive	13(18.3)	100(36.4	330(39)	12(20.7)		
Ki-67	**Negative**	32(51.6)	95(39.4)	240(31.9)	29(55.8)	22	<0.0001
	Positive	30(48.4)	146(60.6	513(68.1)	23(44.2)		
PTEN	**Negative**	36 (83.7)	99 (79.8)	359 (82.7)	11 (47.8)	17.5	0.001
	Positive	7 (16.3)	25 (20.2)	75 (17.3)	12 (52.2)		

ATM					
Markers		**Negative N (%)**	**Positive N (%)**	***X²***	**P**
ER	**Negative**	362 (49.3)	221 (34.9)	29	<0.0001
	Positive	372 (50.7)	413 (65.1)		
PgR	**Negative**	415 (58.7)	265 (44.1)	27.7	<0.0001
	Positive	292 (41.3)	336 (55.9)		
TN	**Negative**	475 (66.5)	485 (78.9)	25	<0.0001
	Positive	239 (33.5)	130 (21.1)		
BLBC	**Negative**	478 (74.9)	456 (82.2)	9	0.002
	Positive	160 (25.1)	99 (17.8)		
Ki-67	**Negative**	221 (34.7)	233 (43.8)	10	0.001
	Positive	416 (65.3)	299(56.2)		

ATR					
Markers		**Negative N (%)**	**Positive N (%)**	***X²***	**P**
CK17	**Negative**	425(89.9)	314(81.8)	12	0.001
	Positive	48(10.1)	70(18.2)		
Ki-67	**Negative**	278(51.1)	151(31.8)	39	<0.0001
	Positive	266(48.9)	324(68.2)		

CHK2					
Markers		**Negative N (%)**	**Positive N (%)**	***X²***	**P**
ER	**Negative**	169(33.6)	135(25.9)	7	0.007
	Positive	334(66.4)	386(74.1)		
PTEN	**Negative**	328 (89.1)	226 (72.4)	31	<0.0001
	Positive	40 (10.9)	86 (27.6)		

N= number of cases. c = cytoplasmic, n = nuclear expression. The cut off points of positivity were the same as previously published [273], and were as follows: ≥1% for ER and PR; 3+ of HercepTest for HER2; ≥10% for CK5, >34% for Ki-67, ≥ 20 H-score for CHK1.n and ≥80H-score for CHK1.c, and ≥5% for P53. TN= Triple negative (ER, PgR and HER-2). Basal Like BC (BLBC) as defined by Triple negative +positive expression of CK5 and CK14 and CK17. PTEN; ≥1 H-score. This table shows the co-expression of cellular localisation of CHK1.

Table 3.8 Pearson's correlations between DNA Damage Sensors and Signal Transducers Proteins with other Tumour Markers.

Markers		CHK1.c	CHK1.n	CHK2	ATR	ATM	γH2AX.c	γH2AX.n
CHK1.n	**R**	-0.100	*	0.171	-0.053	0.125	-0.042	0.155
	P	<0.0001		<0.0001	0.138	<0.0001	0.222	<0.0001
	N	1321		665	779	892	839	839
CHK2	**R**	0.053	0.171	*	0.112	0.107	0.016	0.354
	P	0.171	<0.0001		0.001	0.006	0.665	<0.0001
	N	665	665		912	648	760	760
ATR	**R**	-0.016	-0.053	0.112	*	-0.021	0.084	0.116
	P	0.654	0.138	0.001		0.549	0.009	<0.0001
	N	779	779	912		789	955	955
ATM	**R**	0.013	0.125	0.107	-0.021	*	0.039	0.180
	P	0.700	<0.0001	0.006	0.549		0.257	<0.0001
	N	892	892	648	789		866	866
γH2AX.c	**R**	0.195	-0.042	0.016	0.084	0.039	*	0.250
	P	<0.0001	0.222	0.665	0.009	0.257		<0.0001
	N	839	839	760	955	866		1298
γH2AX.n	**R**	0.114	0.155	0.354	0.116	0.180	0.250	*
	P	0.001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	
	N	839	839	760	955	866	1298	
PgR	**R**	-0.035	0.088	0.139	-0.016	0.125	-0.021	0.173
	P	0.260	0.005	<0.0001	0.571	<0.0001	0.515	<0.0001
	N	1015	1015	1003	1197	1059	959	959
ER	**R**	-0.087	0.133	0.168	0.040	0.156	-0.049	0.239
	P	0.005	<0.0001	<0.0001	0.164	<0.0001	0.127	<0.0001
	N	1025	1025	1019	1218	1079	967	967
Ki-67	**R**	0.119	-0.128	-0.098	0.191	-0.077	0.105	-0.249
	P	<0.0001	<0.0001	0.004	<0.0001	0.009	<0.0001	<0.0001
	N	1108	1108	860	1019	1169	1117	1117
P53	**R**	0.095	-0.054	-0.016	0.019	-0.053	-0.023	-0.117
	P	0.002	0.083	0.623	0.508	0.079	0.466	<0.0001
	N	1014	1014	1005	1203	1087	965	965
CK5	**R**	0.114	-0.090	-0.045	-0.011	-0.048	0.054	-0.131
	P	0.001	0.008	0.200	0.744	0.169	0.134	<0.0001
	N	858	858	805	939	809	768	768
CK17	**R**	0.077	-0.060	-0.011	0.112	0.008	0.031	0.003
	P	0.013	0.056	0.768	0.001	0.806	0.336	0.926
	N	1026	1026	748	857	979	952	952
PTEN	**R**	-0.105	0.201	0.249	-0.016	0.003	-0.048	0.240
	P	0.008	<0.0001	<0.0001	0.641	0.944	0.214	<0.0001
	N	624	624	680	816	588	671	671

The table represents continuous data for all the markers. N= number of cases. R= Pearson's correlation, P=Probability value. c. = cytoplasmic, n. = nuclear expression. * Analysis between the marker itself.

Table 3.9 Correlation between DNA Damage Sensors and Signal Transducers Proteins with other Tumour Markers Regardless Co-expression of Cellular Localisation.

γH2AX.c				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
CHK1.n	**Negative**	58 (61.7)	580 (77.6)	0.001 11.5
	Positive	36 (38.3)	167 (22.4)	
CHK1.c	**Negative**	22 (23.4)	57 (7.6)	<0.0001 24
	Positive	72 (76.6)	689 (92.4)	
PgR				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
CHK1.c	**Negative**	47 (7.4)	85 (14.3)	<0.0001 15
	Positive	586 (92.6)	508 (85.7)	
CHK1.n	**Negative**	495 (78.1)	409 (69)	<0.0001 13
	Positive	139 (21.9)	184 (31)	
γH2AX.c	**Negative**	55 (8.3)	79 (14.5)	0.001 12
	Positive	611 (91.7)	465 (85.5)	
γH2AX.n	**Negative**	115 (17.3)	38(7)	<0.0001 28.7
	Positive	550(82.7)	506 (93)	
P53				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
CHK1.n	**Negative**	574 (72.1)	343 (75.4)	0.2 1.5
	Positive	222 (27.9)	112 (24.6)	
CHK1.c	**Negative**	104 (13.1)	25 (5.5)	<0.0001 18
	Positive	691 (86.9)	430 (94.5)	
ER				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
γH2AX.n	**Negative**	100(18)	52 (7.4)	<0.0001 33
	Positive	456 (82)	655 (92.6)	
γH2AX.c	**Negative**	39 (7)	99 (14)	<0.0001 15.7
	Positive	518 (93)	608 (86)	
CK5				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
γH2AX.c	**Negative**	83 (11.8)	18 (7.3)	0.05 4
	Positive	622 (88.2)	228 (92.7)	
γH2AX.n	**Negative**	71 (10.1)	53 (21.6)	<0.0001 21
	Positive	634 (89.9)	192 (78.4)	
CHK1.n	**Negative**	591 (74.9)	208 (78.8)	0.2 1.6
	Positive	198 (25.1)	56 (21.2)	
CHK1.c	**Negative**	87 (11)	7 (2.7)	<0.0001 17
	Positive	702 (89)	256 (97.3)	
PTEN				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
γH2AX.n	**Negative**	74 (13.7)	4 (3.1)	0.001 11.6
	Positive	466 (86.3)	127 (96.9)	
γH2AX.c	**Negative**	53 (9.8)	19 (14.5)	0.1 2
	Positive	487 (90.2)	112 (85.5)	

N= number of cases. c. = cytoplasmic, n. = nuclear expression. This tabled does not consider co-expression of subcellular localisation. Because there was a difference in the correlations between Table 3.7 (co-expression of cellular localisation; categorical data) and Table 3.8 (continuous data), therefore, these correlations were re-analysed but regardless co-expression of nuclear and cytoplasmic expression, in order to compare each cellular localisation separately.

3.6.4 Correlation of DNA Damage Sensors and Signal Transducers Proteins with Clinico-Pathological Features

Table 3.10 summarises the association between the DNA damage sensors and signal transducers proteins (CHK1, CHK2, ATM, ATR and γH2AX) and the various clinico-pathological features (Pearson X^2). The majority of tumours with poor prognostic features, such as high tumour grade, tubule formation, large tumour size, high mitotic frequency and higher nuclear pleomorphism, were associated with low levels of ATM (all P<0.0001, except tumour size P=0.002), ATR (all P<0.0001, except tubular formation; P=0.002 and tumour size; P=0.005) and CHK1.n^-.c^+ (P<0.0001, P=0.01, P=0.008, P<0.0001, and P<0.0001 respectively). In contrast, CHK2 was not associated with any of the clinico-pathological features.

The double positive expression of γH2AX, irrespective of cellular localisation, showed a significant positive association with all these poor clinico-pathological features (all P<0.0001 except tumour size P=0.002). All DNA damage sensors and signal transducers proteins except CHK2 were highly associated with the moderate I NPI (all P<0.0001). Only ATM^- (P=0.002) and ATR^- (P<0.0001) showed a significant association with stage 1 tumours. γH2AX, CHK1, ATM, and ATR, were highly associated with tumour type (invasive ductal/ no special type P<0.0001 all). Details for significant and non-signification correlations are detailed in Appendix 1.

The high value of X^2 can be referred to, a) a bias in the patients' population, b) after statistical advise and checking the data, it does not seem to have any assumption issues, the main problem is generally when one of the values of expectation is five or less, but however, in the results presented here a large chi squared value is resulted when the expectations are not less than five. However, the data just seem to show that it is very likely that the association is not due to chance.

Table 3.10 Relationship between DNA Damage Sensors and Signal Transducers Proteins with Clinico-Pathological Features.

Parameters		γH2AX					
		$c^- n^-$ N (%)	$c^+ n^+$ N (%)	$c^+ n^-$ N (%)	$c^- n^+$ N (%)	X^2	P
Size	**≤ 1.5cm**	4(22.2)	292(29.1)	25(18.2)	48(39.3)	14	0.002
	>1.5cm	14(77.8)	713(70.9)	112(81.8)	74(60.7)		
Grade	**1**	1(5)	118(11.7)	4(2.9)	13(10.6)	58	<0.0001
	2	4(20)	267(26.4)	14(10)	54(43.9)		
	3	15(75)	626(61.9)	122(87.1)	56(45.5)		
Tubules	**1**	0	39(3.9)	0	6(4.9)	27.5	<0.0001
	2	4(21.1)	305(30.5)	19(13.9)	29(23.8)		
	3	15(78.9)	657(65.5)	118(86.1)	87(71.3)		
Pleomorphism	**1**	0	15(1.5)	1(0.7)	2(1.6)	48	<0.0001
	2	7(36.8)	270(27)	13(9.5)	57(46.7)		
	3	12(63.2)	715(71.5)	123(89.8)	63(51.6)		
Mitosis	**1**	1(5.3)	264(26.4)	7(5.1)	49(40.2)	64.5	<0.0001
	2	5(26.3)	180(18)	16(11.7)	25(20.5)		
	3	13(68.4)	557(55.6)	114(83.2)	48(39.3)		
NPI	**Excellent**	1(6.7)	72(7.2)	2(1.4)	9(7.3)	47.5	<0.0001
	Good	1(6.7)	152(15.2)	4(2.9)	30(24.4)		
	Moderate I	8(53.3)	319(31.8)	50(35.7)	44(35.8)		
	Moderate II	3(20)	276(27.5)	47(33.6)	26(21.1)		
	Poor	1(6.7)	131(13.1)	31(22.1)	11(8.9)		
	Very Poor	1(6.7)	52(5.2)	6(4.3)	3(2.4)		
Tumour Type	**Invasive Ductal/NST**	15(78.9)	699(69.8)	106(76.8)	69(58)	92	<0.0001
	lobular	1(5.3)	24(2.4)	4(2.9)	20(16.8)		
	Atypical Medullary	0	22(2.2)	12(8.7)	2(1.7)		
	***Mixed**	3(15.8)	221(22.1)	14(10.1)	25(21)		
	****other**	0	35(3.5)	2(1.4)	3(2.5)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. * Lobular or tubular mixed BCs. ** Mucinous, Alveolar Lobular, Miscellaneous including Metaplastic, Adenoid Cystic, Spindle, and Tubulolobular. NST= no special type. NPI= Nottingham Prognostic Index. Excellent NPI (2.08–2.4), good NPI (2.42 to ⩽3.4), a moderate prognostic I NPI (3.42 to ⩽4.4), moderate prognostic II NPI (4.42 to ⩽5.4), poor NPI (5.42 to ⩽6.4), and a very poor NPI (6.5–6.8). Co-expression of cellular localisation of γH2AX was considered in this table.

Table 3.10 Relationship between DNA Damage Sensors and Signal Transducers Proteins with Clinico-Pathological Features Continued.

CHK1							
Parameters		**c^-n^- N (%)**	**c^+n^+ N (%)**	**c^+n^- N (%)**	**c^-n^+ N (%)**	**X^2**	**P**
Size	**≤ 1.5cm**	31(40.3)	111(37.9)	257(29.3)	24(40.7)	12	0.008
	>1.5cm	46(59.7)	182(62.1)	620(70.7)	35(59.3)		
Grade	**1**	14(18.2)	60(20.4)	84(9.5)	12(20.3)	59	<0.0001
	2	33(42.9)	83(28.2)	237(26.8)	29(49.2)		
	3	30(39)	151(51.4)	563(63.7)	18(30.5)		
Tubules	**1**	3(4.1)	19(6.8)	27(3.1)	3(5.1)	16	0.01
	2	28(38.4)	97(34.5)	241(28)	18(30.5)		
	3	42(57.5)	165(58.7)	593(68.9)	38(64.4)		
Pleomorphism	**1**	2(2.7)	9(3.2)	5(0.6)	3(5.1)	77	<0.0001
	2	40(54.8)	112(40)	214(24.9)	30(50.8)		
	3	31(42.5)	159(56.8)	641(74.5)	26(44.1)		
Mitosis	**1**	28(38.4)	90(32)	214(24.9)	34(57.6)	39	<0.0001
	2	14(19.2)	58(20.6)	156(18.1)	6(10.2)		
	3	31(42.5)	133(47.3)	491(57)	19(32.2)		
NPI	**Excellent**	8(10.5)	42(14.4)	55(6.3)	9(15.3)	57	<0.0001
	Good	18(23.7)	57(19.6)	119(13.6)	15(25.4)		
	Moderate I	27(35.5)	95(32.6)	276(31.4)	18(30.5)		
	Moderate II	10(13.2)	59(20.3)	262(29.8)	10(16.9)		
	Poor	11(14.5)	24(8.2)	122(13.9)	7(11.9)		
	Very Poor	2(2.6)	14(4.8)	44(5)	0		
Tumour Type	**Invasive Ductal/NST**	39(52)	165(57.1)	630(72)	17(29.3)	85	<0.0001
	lobular	7(9.3)	15(5.2)	40(4.6)	12(20.7)		
	Atypical Medullary	2(2.7)	7(2.4)	21(2.4)	2(3.4)		
	***Mixed**	22(29.3)	84(29.1)	165(18.9)	25(43.1)		
	****other**	5(6.7)	18(6.2)	19(2.2)	2(3.4)		

Parameters		**ATM**				**ATR**			
		Negative N (%)	**Positive N (%)**	***X²***	**P**	**Negative N (%)**	**Positive N (%)**	***X²***	**P**
Size	**≤ 1.5cm**	222(29.9)	241 (38)	10	0.002	245(34.2)	151(26.9)	8	0.005
	>1.5cm	520(70.1)	393 (62)			471(65.8)	410(73.1)		
Stage	**1**	444(59.7)	441(68.7)	12.5	0.002	479(66.9)	314(56)	17	<0.0001
	2	224(30.1)	155(24.1)			185(25.8)	185(33)		
	3	76 (10.2)	46 (7.2)			52(7.3)	62(11.1)		
Grade	**1**	61 (8.2)	135 (21)	55.3	<0.0001	152(21.2)	56(10)	40	<0.0001
	2	195(26.2)	186(28.9)			235(32.8)	163(29.1)		
	3	489(65.6)	322(50.1)			329(45.9)	342(61)		
Tubules	**1**	32 (4.4)	37 (5.9)	30	<0.0001	56(8.2)	19(3.4)	13	0.002
	2	170(23.2)	225(35.7)			216(31.7)	185(33)		
	3	532(72.5)	369(58.5)			410(60.1)	357(63.6)		
Pleomorphism	**1**	11 (1.5)	20(3.2)	31	<0.0001	24(3.5)	4(0.7)	29	<0.0001
	2	187(25.5)	239(38)			281(41.5)	174(31)		
	3	535 (73)	370(58.8)			372(54.9)	383(68.3)		
Mitosis	**1**	168(22.9)	240 (38)	40.5	<0.0001	269(39.4)	144(25.7)	28	<0.0001
	2	129(17.6)	110(17.4)			119(17.4)	103(18.4)		
	3	437(59.5)	281(44.5)			294(43.1)	314(56)		
NPI	**Excellent**	49(6.6)	95(15)	56	<0.0001	105(14.7)	35(6.4)	57	<0.0001
	Good	101(13.7)	114(18)			139(19.5)	88(16)		
	Moderate I	241(32.7)	213(33.6)			229(32.1)	143(26)		
	Moderate II	200(27.1)	155(24.4)			161(22.6)	159(28.9)		
	Poor	112(15.2)	39(6.2)			63(8.8)	96(17.5)		
	Very Poor	34(4.6)	18(2.8)			16(2.2)	29(5.3)		
Vascular Invasion	**Negative**	297(59.6)	330(70.2)	11.8	0.001	429(67.5)	269(55.6)	16	<0.0001
	Positive	201(40.4)	140(29.8)			207(32.5)	215(44.4)		
Tumour Type	**Invasive Ductal/NST**	525(71)	383(60.3)	30	<0.0001	397(56.6)	367(66.2)	21	<0.0001
	lobular	44(6)	33(5.2)			41(5.8)	27(4.9)		
	Atypical Medullary	25(3.4)	15(2.4)			25(3.6)	10(1.8)		
	***Mixed**	114(15.4)	170(26.8)			190(27.1)	136(24.5)		
	****other**	31(4.2)	34(5.4)			48(6.8)	14(2.5)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. * Lobular or tubular mixed BCs. ** Mucinous, Alveolar Lobular, Miscellaneous including Metaplastic, Adenoid Cystic, Spindle, and Tubulolobular. NST= no special type. NPI= Nottingham Prognostic Index. Excellent NPI (2.08–2.4), good NPI (2.42 to ⩽3.4), a moderate prognostic I NPI (3.42 to ⩽4.4), moderate prognostic II NPI (4.42 to ⩽5.4), poor NPI (5.42 to ⩽6.4), and a very poor NPI (6.5–6.8). Co-expression of cellular localisation of CHK1 was considered here.

3.6.5 Relationship between DNA Damage Sensors and Signal Transducers Proteins and Patients' Outcome by Univariate Analysis

Figures 3.6 to 3.12 show the association between the expression of DNA damage sensors and signal transducers proteins and patients' outcomes.

Positive nuclear (n) expression of CHK1 showed a longer BCSS (P=0.017, Figure 3.6a) than negative expression, although it was not of statistical significant, whereas the positive cytoplasmic (c) expression of CHK1 showed poorer BCSS than negative one (P=0.028, Figure 3.6b. finally, the co-expression of nuclear$^-$ and cytoplasmic$^+$ CHK1 had the worst outcome, while nuclear$^+$/cytoplasmic$^-$ CHK1 had the best BCSS (P=0.025, Figure 3.6c). Interestingly, cases showing CHK1.n$^-$ treated with chemotherapy (unselected cases) (P=0.007, Figure 3.7a) or endocrine therapy (among ER$^+$ patients) (P=0.008, Figure 3.7c) demonstrated worse BCSS than those who did not receive these treatments. In addition, cases of CHK1.c$^-$ treated with endocrine therapy (amongst them ER$^+$ patients) (P=0.04, Figure 3.7d) showed a trend for increased BCSS over the other groups, yet patients treated with chemotherapy and demonstrating a low cytoplasmic expression of CHK1 had worse survival rates than those patients who did not receive this treatment, although it was not of statistical significant (P=0.018, Figure 3.7b).

CHK2 demonstrated no effect on patient survival (P=0.4, Figure 3.8a); however, similar to nuclear CHK1, tumours having CHK2$^-$ and received chemotherapy (unselected cases) or treated with endocrine therapy in patients with tumours expressing ER$^+$ demonstrated poor BCSS, when compared to untreated cases (P=0.08; Figure 3.8b, P=0.002, Figure 3.8c respectively).

Positive ATM conferred an improved BCSS (P<0.0001, Figure 3.9a) or longer time to induce distant metastasis (P<0.0001, Figure 3.9b) over ATM negativity. Interestingly, low ATM expression levels in patients that received chemotherapy had worse BCSS than those who did not receive it (unselected cases; (P<0.0001, Figure 3.9c). Furthermore, patients receiving endocrine treatment (amongst ER$^+$ patients) had worse BCSS than patients who did not receive the treatment (P<0.0001, Figure 3.9d).

Unlike ATM, patients with positive ATR tumours demonstrated a trend for poorer BCSS (P=0.01, Figure 3.10a) than ATR negative tumours. In contrast, high expression levels of ATR demonstrated worse BCSS in those patients who received chemotherapy (unselected cases) (P=0.01, Figure 3.10b) or endocrine treatment (amongst ER$^+$ patients) (P=0.001, Figure 3.10c) than those who did not.

Although the co-expression of γH2AXn$^-$.c$^-$ demonstrated the shortest BCSS, it was not of statistical significance (P=0.097, Figure 3.11a). Nuclear or cytoplasmic expression of γH2AX showed no significant association with patient's outcome (Figure 3.11b-3.11d).

Interestingly, patients who were treated with chemotherapy and expressed nuclear (P=0.007, Figure 3.12a) or cytoplasmic γH2AX (P<0.0001, Figure 3.12b) demonstrated worse BCSS than those who did not receive the treatment. A similar effect was seen with endocrine treated ER-positive patients Figure 3.12c for nuclear (P<0.0001) and 3.12d for cytoplasmic γH2AX (P=0.029).

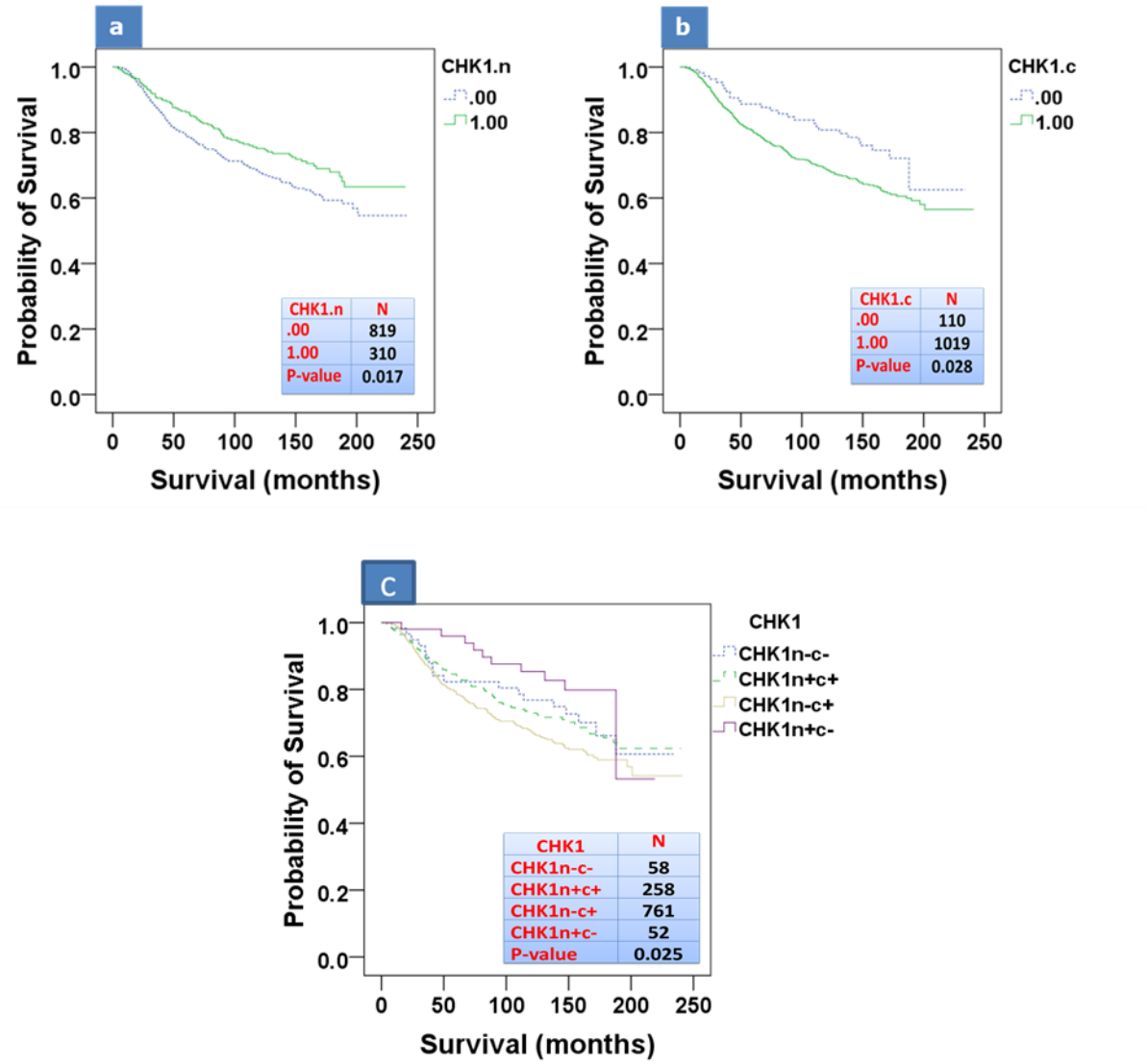

Figure 3.6 The associations between CHK1 with BCSS. 0= negative and 1= positive expression of CHK1. Where n= nuclear expression and c= cytoplasmic expression of CHK1 and N; number of cases. Only patients who died from breast cancer were considered. a; shows association between nuclear expression of CHK1 and BCSS, whereas b; shows cytoplasmic expression. c; co-expression of nuclear and cytoplasmic expression of CHK1 and its association with BCSS. P<0.01 was considered significant.

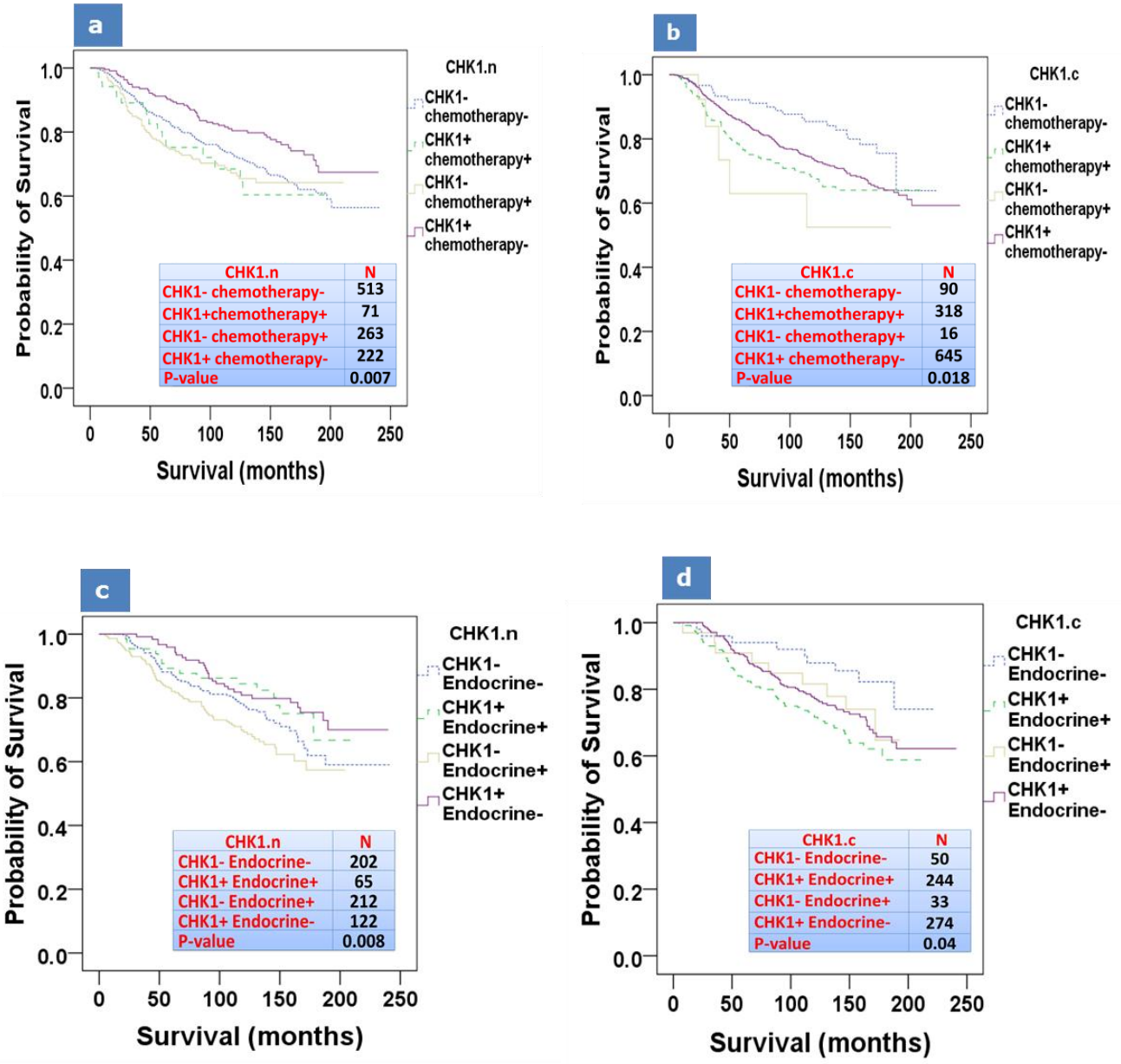

Figure 3.7 The associations between CHK1 with BCSS and the effect of treatment on patient's outcome. N; number of cases. n= nuclear expression and c= cytoplasmic expression. Only patients who died from breast cancer were considered. a; association between nuclear expression of CHK1 and BCSS based on chemotherapy in unselected cases and b; cytoplasmic expression of CHK1 and BCSS based on chemotherapy in unselected cases. c; nuclear expression of CHK1 and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. d; cytoplasmic expression of CHK1 and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. P<0.01 was considered significant. Some ER-positive tumours did not receive endocrine therapy, because the treatment plans for those cases was as patients with a score of NPI≤ 3.4 received no adjuvant therapy.

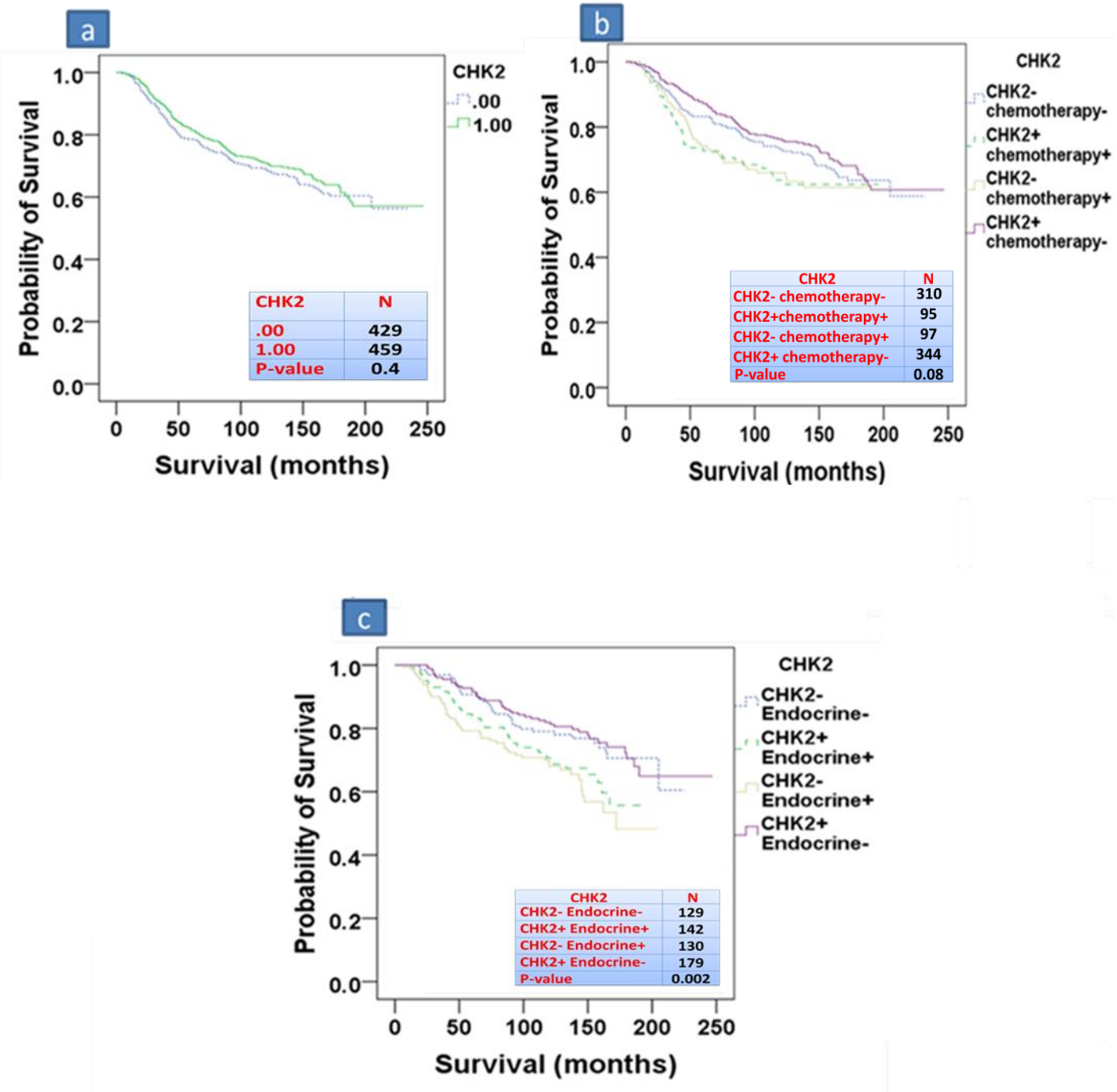

Figure 3.8 The associations between CHK2 with BCSS and the effect of treatment on patient's outcome. N; number of cases. Only patients who died from breast cancer were considered. a; shows association between CHK2 and BCSS , where 0= negative and 1= positive expression. b; expression of CHK2 and BCSS based on chemotherapy in unselected cases. c; expression of CHK2 and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. P<0.01 was considered significant. Some ER-positive tumours did not receive endocrine therapy, because the treatment plans for those cases was as patients with a score of NPI≤ 3.4 received no adjuvant therapy.

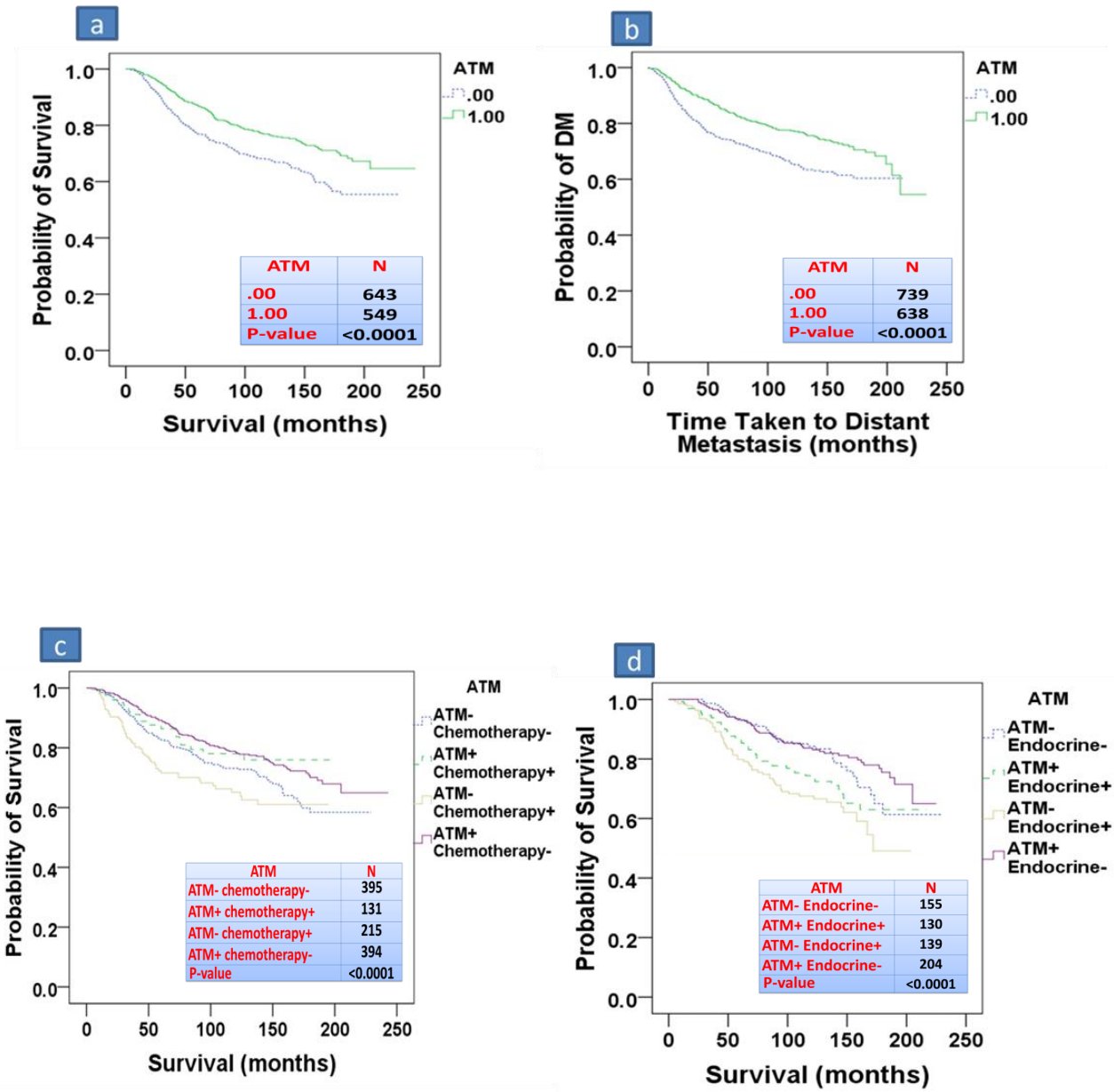

Figure 3.9 The associations between ATM with BCSS or distant metastasis and the effect of treatment on patient's outcome. N; number of cases. Only patients who died from breast cancer were considered. a; shows association between ATM and BCSS, and b; with distant metastasis, where 0= negative and 1= positive expression. c; expression of ATM and BCSS based on chemotherapy in unselected cases. d; expression of ATM and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. P<0.01 was considered significant. Some ER-positive tumours did not receive endocrine therapy, because the treatment plans for those cases with a score of NPI≤ 3.4 received no adjuvant therapy.

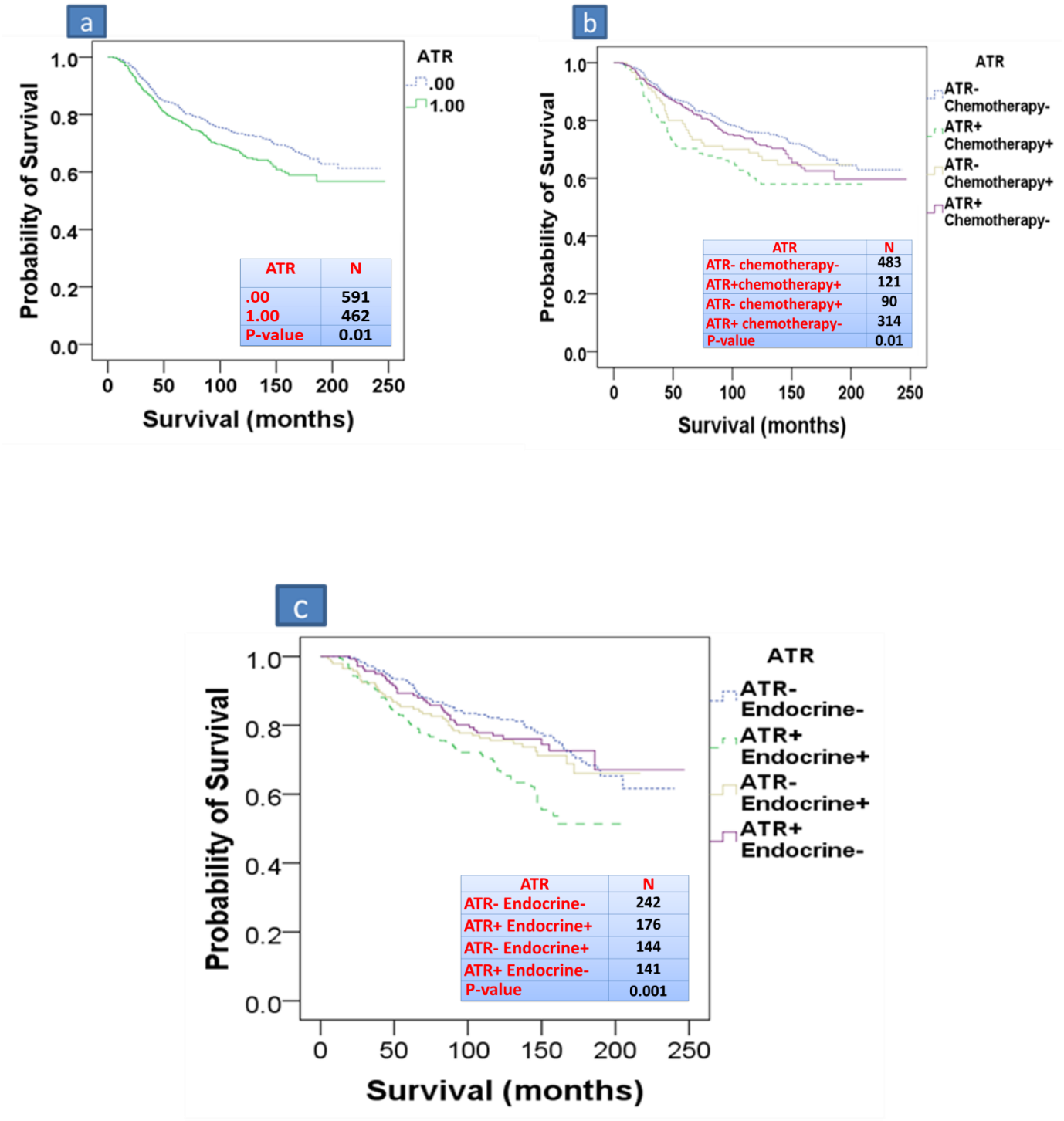

Figure 3.10 The associations between ATR with BCSS and the effect of treatment on patient's outcome. N; number of cases. Only patients who died from breast cancer were considered. a; shows association between ATR and BCSS, where 0= negative and 1= positive expression. b; expression of ATR and BCSS based on chemotherapy in unselected cases. c; expression of ATR and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. P<0.01 was considered significant. Some ER-positive tumours did not receive endocrine therapy, because the treatment plans for those cases with a score of NPI≤ 3.4 received no adjuvant therapy.

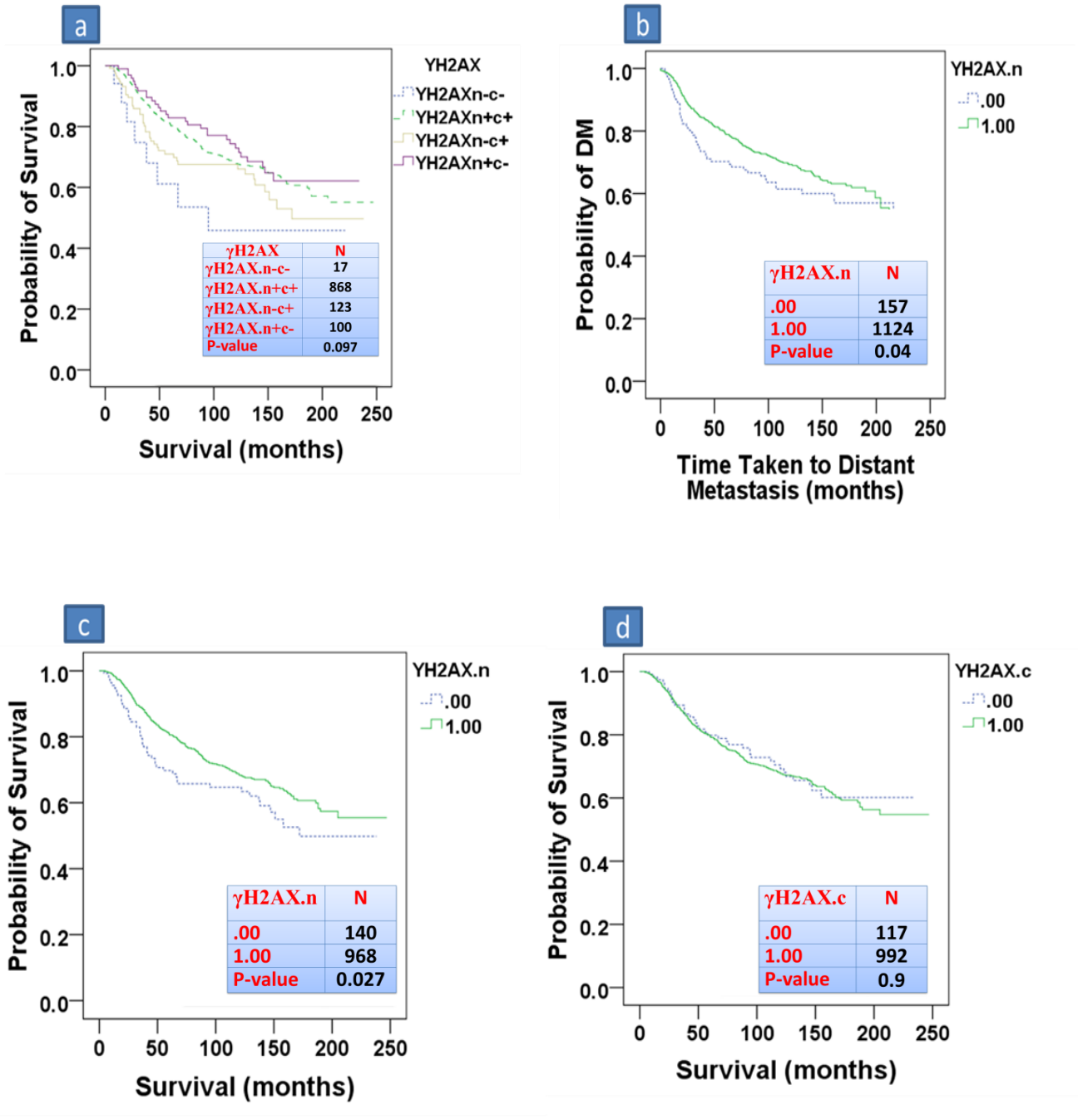

Figure 3.11 The associations between γH2AX with BCSS or distant metastasis. 0= negative and 1= positive expression of γH2AX. Where n= nuclear expression and c= cytoplasmic expression of γH2AX and N; number of cases. Only patients who died from breast cancer were considered. a; co-expression of nuclear and cytoplasmic expression of γH2AX and its association with BCSS. b; shows association between nuclear expression of γH2AX and distant metastasis and c; with BCSS. d; shows cytoplasmic expression of γH2AX with BCSS. $P<0.01$ was considered significant.

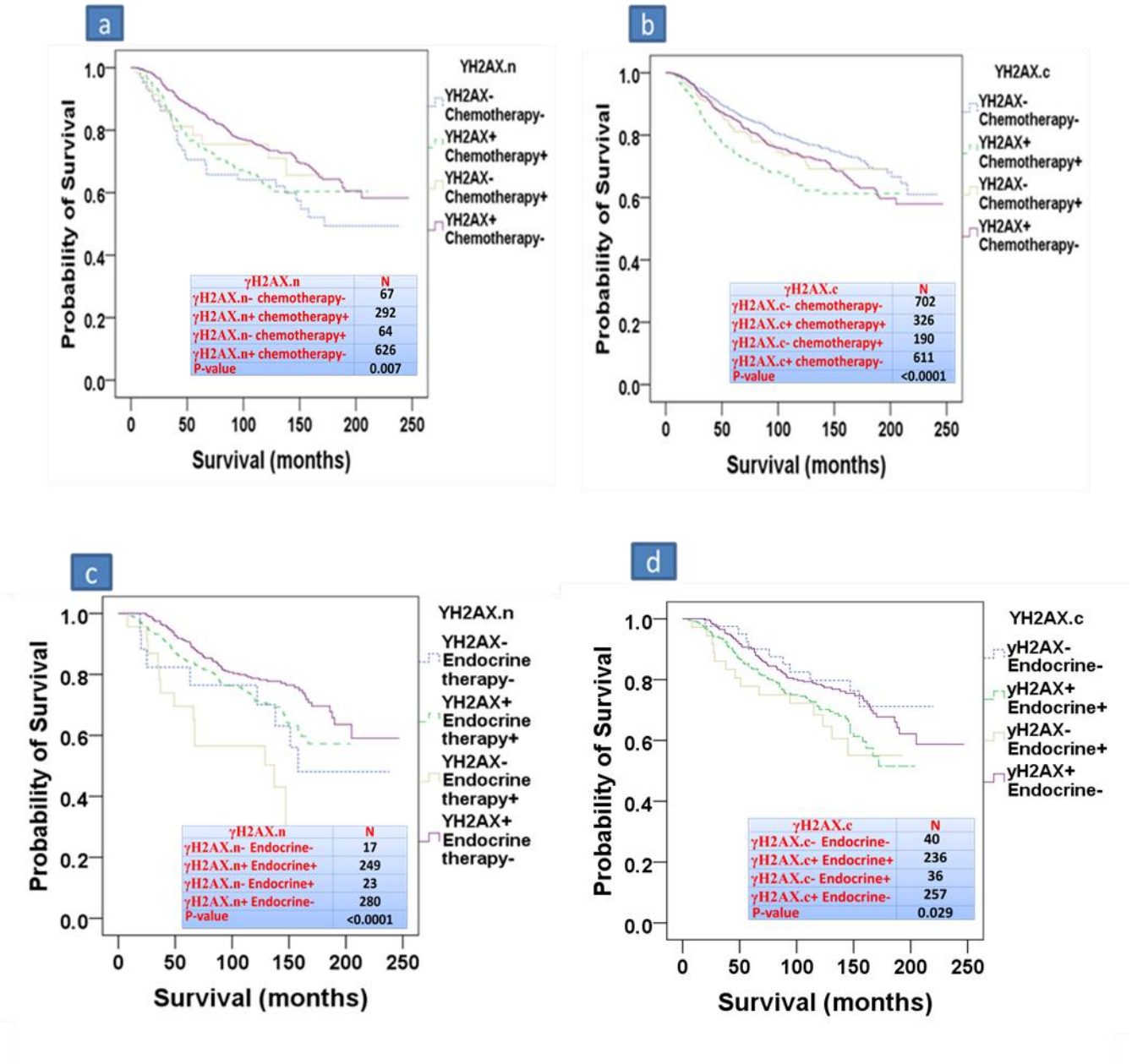

Figure 3.12 The associations between γH2AX with BCSS and the effect of treatment on patient's outcome. N; number of cases. Only patients who died from breast cancer were considered. a; shows association between nuclear expression of γH2AX and BCSS based on chemotherapy in unselected cases and b; cytoplasmic expression of γH2AX and BCSS based on chemotherapy in unselected cases. c; nuclear expression of γH2AX and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. d; cytoplasmic expression of γH2AX and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. P<0.01 was considered significant. Some ER-positive tumours did not receive endocrine therapy, because the treatment plan for those cases was as patients with a score of NPI≤ 3.4 received no adjuvant therapy.

3.6.6 Relationship between DNA Damage Sensors and Signal Transducers Proteins and Patients' Outcomes by Multivariate Analysis

Table 3.11 and Figure 3.13 show Cox-regression analyses for predictors of BCSS for ATM and ATR. Only markers showed significant association in univariate analysis were considered. It seems that ATM, but not ATR is independent prognostic marker for breast cancer.

Multivariate analysis for combinations (such as ATM with chemotherapy or ATM with endocrine therapy) does not usually perform, multivariate analysis applies for the main marker results (+ vs. -) as combination effect may be biased by the other factors and not by the target marker.

Table 3.11 Cox-Regression Analyses for Predictors of BCSS

Parameters	P-value	95.0% CI		Parameters	P-value	95.0% CI	
		Lower	Upper			Lower	Upper
ATM	0.007	0.529	0.906	**ATR**	0.7	0.744	1.234
NPI	0.963	0.659	1.549	**NPI**	0.264	0.885	1.559
Tumour Size	<0.0001	1.373	2.956	**Tumour Size**	0.033	1.035	2.186
Tumour Stage	<0.0001	1.331	2.345	**Tumour Stage**	0.001	1.246	2.533
Tumour Grade	0.014	1.091	2.190	**Tumour Grade**	0.019	1.081	2.360
BLBC	0.208	0.588	1.123	**BLBC**	0.289	0.584	1.174
Ki-67	0.009	1.116	2.184	**Ki-67**	0.040	1.015	1.965

NPI=Nottingham prognostic index. Basal Like BC (BLBC) as defined by Triple negative +positive expression of CK5 and CK14 and CK17. Only markers in univariate analysis that were statistically significant with patients' outcomes were applied for Cox regression (IBM SPSS 21.0) for multivariate analyses. ATR showed a trend for poor BCSS in univariate analysis.

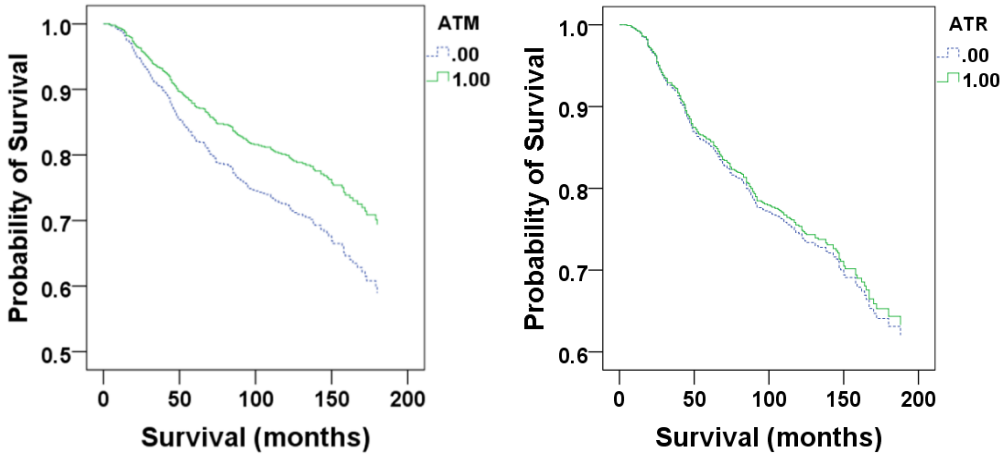

Figure 3.13 Multivariate Cox-regression analysis for ATM and ATR. In order to test for confounders and prognostic or predictive independency of the investigated biomarker from standard prognostic/predictive factors; tumour grade, stage and size, NPI, BLBC, and Ki-67 were included. 0= negative expression, and 1=positive expression.

3.6.7 Expression of DNA Damage Sensors and Signal Transducers Proteins in Cell Lines by Reverse Phase Protein Microarray

RPPA was used to evaluate the expression level of DNA damage sensors and signal transducers markers in different cell lines (BRCA1 deficient HeLasilenciX® cells and its control (proficient BRCA1)), MCF-7 (proficient BRCA1 and ER^+) and MDA-MB-436 (deficient BRCA1 and ER^-) cells. Figure 3.14 shows the expression of ATM, ATR, CHK1, CHK2 and γH2AX in different cell lines using RPPA.

RPPA confirmed the IHC results (especially nuclear expression, but not cytoplasmic for CHK1 and γH2AX), with the exception of ATR, and demonstrated a higher level of expression of DNA damage sensors and signal transducers markers in HeLa BRCA1 control or MCF-7 cell lines than BRCA1 deficient HeLa or MDA-MB-436 cell lines. All the markers showed similar expression amongst the cell lines.

In regards to statistics; there was a high significant difference in the expression of CHK1 between; HeLa BRCA1 control vs. MDA-MB-436 (P=0.0002), HeLa BRCA1 vs. MDA-MB-436 (P=0.008), and finally MDA-MB-436 vs. MCF-7 (P=0.0008). For ATM; HeLa BRCA1 control vs. MDA-MB-436 (P=0.007). For ATR; HeLa BRCA1 vs. HeLa BRCA1 control (P<0.0001), HeLa BRCA1 control vs. MDA-MB-436 (P<0.0001), HeLa BRCA1 control vs. MCF-7 (P<0.0001) and finally MDA-MB-436 vs. MCF-7 (P=0.007). Finally for γH2AX; MDA-MB-436 vs. MCF-7 (P=0.007).

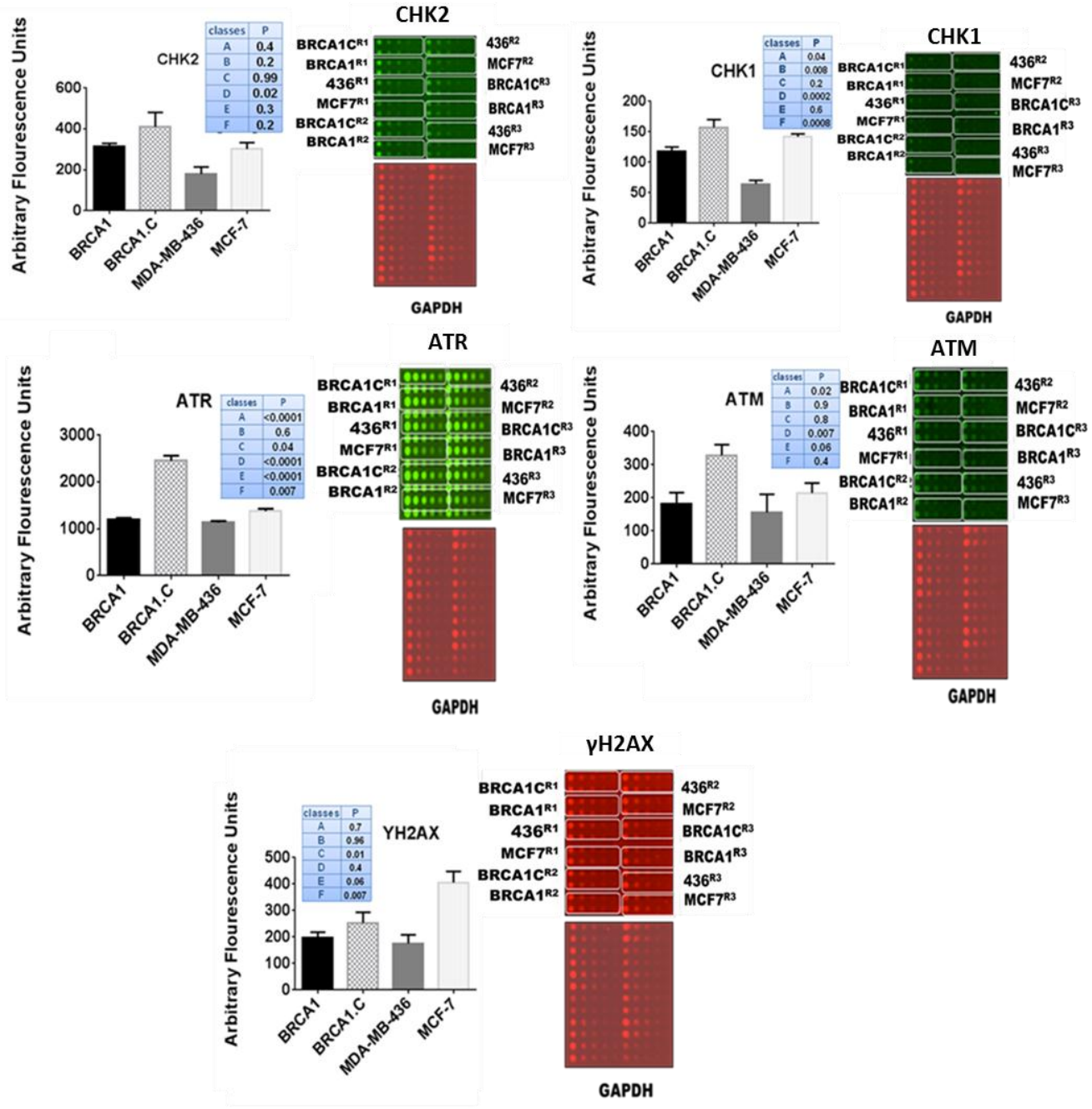

Figure 3.14 The DNA-damage sensors and signal transducers protein levels detected by RPPA in different cell lines (BRCA1 deficient HeLaSilenciX® cells and its control [BRCA1 and BRCA1.C respectively], MCF-7 and MDA-MB-436 cells). For image of nitrocellulose slide spotted with different cell lysates; the red square represents the 700 channel for detection of mouse antibody while green square the 800 channel for rabbit antibody. Images of scanned nitrocellulose slides printed with extracted protein from cell lines and probed with the antibodies against the target proteins. Five 2-fold dilutions of each sample were printed in duplicate. Background was subtracted and the intensity of each spot was normalised to its corresponding GAPDH level. Each (^{R}) represents different passage of each sample, therefore, three different passages of each sample were used. Error bars represent Mean (SD). HeLa BRCA1; between passage21 and 30, HeLa BRCA1 control; between passage 15 and 20, MCF-7; between passage 25 and 32, and MDA-MB-436; between passage 12 and 20. A= BRCA1 vs. BRCA1.C, B= BRCA1 vs. MDA-MB-436, C= BRCA1 vs. MCF-7, D= BRCA1.C vs. MDA-MB-436, E= BRCA1.C vs. MCF-7, and F= MDA-MB-436 vs. MCF-7. One way ANOVA test was used.

3.7 Discussion

Cellular response to any genotoxic stress is a highly complex process, and it usually begins with one of two systems; the "sensing" or "detection" of the damage of DNA, then a number of events that include signal transduction and activation of transcription factors. The activated transcription factors stimulate expressions of several genes which usually have roles in different cellular functions such as DNA repair, cell cycle arrest, and apoptosis[328]. Focusing on how the repair of DNA is modulated in accordance with the responding to the damage provides important applications in the treatment of cancer [341].

In this Chapter, the expression of DNA damage sensors and signal transducers proteins (ATM, ATR, CHK1, CHK2 and γH2AX) was examined in breast tumours (using IHC, TMA) and in different cell lines (using RPPA). These markers were correlated with clinical outcome, tumour pathological features and the expression of other tumour biomarkers, in order to demonstrate their expression patterns in BC cases or the cell lines. Figure 3.15 summarises the finding in this chapter and pathway involved in DNA damage sensors and signal transducers in BC.

All the findings by IHC, particularly nuclear expression, were similar to those observed with RPPA in different cell lines, with the exception of ATR. In RPPA, the HeLa BRCA1 or MDA-MB-436 cell lines (ATM, CHK1, CHK2 and γH2AX) or, in the case of IHC, *BRCA1* hereditary [known germline mutation cases], or sporadic $BRCA1^-/ER^-$ cases) showed lower levels of all DNA damage sensors and signal transducers proteins (than the HeLa BRCA1control cell line or MCF-7 (ER^+ and $BRCA1^+$) and for IHC; sporadic $BRCA1^+/ER^+$ cases. Current progress in identifying the function of *BRCA1* suggests that this gene has an important role in a common pathway that is involved in response to DNA-damage or even cell-cycle control and repair of DNA-damage [341, 342]. It is therefore expected that any defect of BRCA1 will have an impact on response to DNA-damage [341, 342]. Wiltshire et al have used BC cell line HCC1937 and found that BRCA1 deficiency results in increased chromosome damage and was involved in controlling both G2/M checkpoints [342]. In this study the influence of BRCA1 deficiency as in HeLa BRCA1/ MDA-MB-436 cell lines or a mutation as in hereditary BRCA1 BC tissue turns out to respond to DNA-damage. This is illustrated by observing the low level of the basic markers of DNA-damage such as ATM, ATR and signal transducer such as CHK1, and CHK2, in addition to the DNA damage repair factor protein γH2AX. However, further studies are warranted to observe the functional behaviour of the cells, and to investigate the effect of BRCA1 on the level of these markers (ATM, ATR, CHK1, CHK2 and γH2AX). The quantitative assessment of DNA damage can performed by Comet assay through single gel electrophoresis and analysed by fluorescent microscope. In addition, cell cycle analysis employing flow cytometry would help to distinguish cells in different phases of the cell cycle [343].

In IHC, the expression of ATR in known *BRCA1* germline mutations cases was higher than in sporadic cases, unlike the findings in regards to the cell line, which showed the opposite effect. Interestingly, this finding is in agreement with the cytoplasmic expression of both CHK1 and γH2AX. Although the RPPA results reflect most of the findings pertaining to IHC, the assessment of the markers presented here highlights the variations in using cellular pathways to investigate the use of *in vivo* tumour cells and cancer cell lines. Other reports confirm the variations between cell lines and tissue by studying the function of different genes, such as cell-cycle progression genes, protein processing and protein turnover, and have stated that cell lines differed most significantly from tumours based on meta-analysis using Gene Ontology [344, 345]. Such behaviour, however, may possibly have arisen as a result of different factors relating to the cell culture environment [345]. The noticeable difference between IHC and RPPA used for the study of ATR expression could be due to the difference in the samples used, for example using cervical cell lines (HeLa-BRCA1 cell lines). In addition, the composition of the cell culture medium may be precisely why patterns of protein expression differentiate cancer cell lines from breast tumour tissue. However, variations in environmental selection pressures may also clarify the differential patterns of the expression of protein in tumour tissue and the cell lines. Compounds may have a significant and diverse effect on the altered pathways between cell lines and tumour tissue. In addition, the result of the IHC was considered both nuclear and cytoplasmic expression of the marker, whereas in cell lines the expression was investigated regardless cellular localisation.

ATM/ATR activation and "sensor" proteins recruit CHK1/CHK2 at damage sites, where the latter are activated [346, 347]. DSBs activate ATM, leading to the phosphorylation of both P53 and CHK2: this step then leads to the accumulation of P53 and the activation of its downstream target genes [348, 349]. Transcriptional activation will direct into two mechanisms, whether it is apoptosis or G1-cell cycle arrest, and enforce the S- and G2- cell cycle arrests regulated by CHK1. ATR (predominantly) or ATM (to a lesser extent) phosphorylates CHK1 [346, 347]. Interestingly, the existence of crosstalk between these pathways is expected, as stalled replication forks can cause DSB leading to ATM activation and DSB repair can produce RPA- coated ssDNA, which triggers the pathway of ATR [350]. A number of siRNA studies have investigated the importance of CHK1 and CHK2 and these showed that CHK1 is essential for both S and G2 arrest within damaged cells, whereas CHK2 is not able to influence arrest [351-355]. It is expected that the arrest of the S phase will halt overt DNA damage, but if cells are not able to arrest in this phase, due to the absence of CHK1, more DNA breaks will arise. This increased damage may stimulate another checkpoint mechanism, which helps prevent progression through the G2 phase [355]. A study of the MDA-MB-231 cell line, by Zhang et al showed that suppression of CHK2 had no effect on the arrest of the cell cycle, while cells simultaneously suppressed for both CHK1 and CHK2 continued to be arrested, mainly in the G2 phase [355]. Based on the finding by Zhang et al [355], it's expected that high CHK1 and CHK2 should lead to cell cycle arrest in order to induce repair or cell death. In the present study, CHK2, CHK1.n, ATR and ATM were rarely expressed, whereas CHK1.c and γH2AXn.c were highly expressed. It may thus be suggested that the high cytoplasmic expression of CHK1 may have an adverse effect of

cell cycle arrest of the DNA damaged cancerous cell and the active cellular localisation is in the nucleus for repair. As both ATM and ATR were rarely expressed, it is possible that the repair may induce by an alternate incorrect checkpoint regulator, and could not sensors the damage correctly. In addition, the cytoplasmic expression of CHK1 may play some role in tumorigenesis through the DNA damage sensors and signal transducers pathway and takes this action as a survival pathway for the tumour cell [356, 357]. However this hypothesis needs further studies to be confirmed, cell fractionation and cell cycle analysis with employing flow cytometry may help here.

In cervical cancer cell line, inhibition of ATM severely decreased clonogenic survival, suggesting that cervical cancer cells seriously rely on signalling axis of ATM for survival after irradiation, whereas the high level of ATM increased the survival [358]. In addition, Bueno et al [359] have showed that low protein expression of ATM was associated with distant metastasis and reduced DFI in BC. The present study supports these findings where the low protein expression of ATM was highly associated with poor BCSS and shorter time to induce distant metastasis. In addition, ATM protein expression was an independent prognostic marker in sporadic BC. Therefore, the results can lead to suggest that ATM may be a clinically applicable marker of BC outcome.

The subcellular localisation of phosphorylated CHK1 at S345 (used in the present study) was mainly observed in the cytoplasmic fraction, indicating that this particular site of phosphorylation promoted the cytoplasmic localisation of CHK1. In terms of phosphorylation at S317, CHK1 was detected in the nucleus towards the late S phase. It is thus possible that CHK1 de-phosphorylation at S345 induced the movement of CHK1 from the cytoplasm, in addition to its accumulation in the nucleus. According to this concept, the mobility shift in CHK1, upon damage of DNA, was observed only in the cytoplasm [339]. The phosphorylations might be critical for transducing signals to downstream targets [339]. Thus, it would help to address these two phosphorylation sites and to clarify the mechanisms related to how these different phosphorylation sites regulate function of CHK1. Mass spectrometry offers a rapid and highly sensitive method to mapping different phosphorylation sites [360]. The subcellular localisation of CHK1 and the aggressive feature (such as high histologic grade, high nuclear pleomorphism and tumour size more than 1.5cm) of cytoplasmic CHK1 in this study may be explained by a defect of CHK1 in BC. Puc et al [361] studied the effect of cytoplasmic CHK1 on genomic instability using a mouse mammary tumour model. They found that at the G2 phase of the cell cycle, endogenous CHK1 could be observed leaving from the nucleus at the time of peak AKT kinase phosphorylation. The reduction of nuclear CHK1 may possibly explain the raise of Cdc25A protein noticed in G2/M phases [362]. In addition, the PTEN reduction in U2OS cells with RNA interference (RNAi) resulted in increased cytoplasmic expression of CHK1, and then serine 280 phosphorylation seems to be a trigger for CHK1 monoubiquitination, which have been proved affect CHK1 cellular localisation. In general, their study suggests that increased serine 280 phosphorylation and ubiquitination of CHK1 in $PTEN^{-/-}$ cells improved cytoplasmic localisation, therefore inhibiting its checkpoint function [361]. This study in some points may help to explain the

high association between PTEN and high cytoplasmic/low nuclear CHK1 noticed in the present study. Further studies regarding the effect of PTEN on cytoplasmic CHK1 are warranted. Introducing a CHK1-null allele into mice with mammary epithelium-specific inactivation of PTEN, in order to investigate the effect of PTEN disruption on genetic instability and if it has a role in inactivation of nuclear/cytoplasmic CHK1 that lead to tumour formation. Southern-blot analysis can be used for detecting the amount of PTEN.

In this Chapter, the expression of the DNA damage sensors and signal transducers proteins CHK2^{-}, γH2AX.n^{+}.c^{+}, CHK1.n^{-}c^{+}, ATM^{-} and ATR^{+} were associated with aggressive clinicpathological features. However, low or high levels of CHK1 have been reported in certain human or mouse tumours [363-369] and mutation of the *CHK2* or *ATM* has also been associated with an increased risk of BC [370, 371]. Heikkinen et al have hypothesised that germline mutations in *ATR* may account for some BC families [372]. In the present study, although few cases showed primary nuclear localisation, a high amount of CHK1 was detected in the cytoplasm of the cells. I can hypothesise that the cytoplasmic localisation of CHK1 may make it inaccessible, in terms of beginning its role by ATM/ATR, in response to DNA damage. The absence of the expression of CHK1 from the nuclei and high expression in the cytoplasm enhanced the aggressive characteristics of BC, such as high histologic grade 3 tumours, larger tumour size and the occurrence of TN and BLBC. Ganzinelli et al supported the findings here, highlighting a low expression of nuclear , but not cytoplasmic CHK1 in TNBCs [373]. Further study is warranted to confirm the importance of CHK1 in BLBC or TNBC. HRM analysis with employing real-time PCR will help in mutation detection. This will help in investigating CHK1 inhibitors for treatment in TNBC or BLBC, where strategies of treatment are limited at present.

In BC, a strong correlation has been found between the positive Ki-67 and tumour grade, age, and rate of mitotic indices [374-376]. In the present study, there was a strong positive association between the protein expression levels of CHK1n^{-}.c^{+}, and the proliferation marker, Ki-67, [377] and the tumour suppressor P53. This finding is consistent with the data of Lundgren [332], suggesting that CHK1 is a marker for tumour aggressiveness and proliferative activities in addition, Gottifredi et al stated that P53 and CHK1 play interdependent and complementary roles in regulating both the arrest and resumption of G_2 after DNA damage [378]. In addition γH2AXn^{+}.c^{+}, ATM^{-}, and ATR^{+} showed similar association with Ki-67, thus, these markers may possibly influence the aggressive nature of breast tumours.

Histone H2AX is phosphorylated on Ser-139, in response to DNA damage after exposure to genetic agents. In addition, histone H2AX is phosphorylated in untreated normal cells as well as tumour cells; this occurs throughout the cell cycle and, predominantly, in the S and G2/M phases [379]. In the present study, the observed significant negative correlation between γH2AX.n and the protein P53 may thus indicate that the tumour protein P53 has an important role in facilitating phosphorylation of histone H2AX, an essential step in the mobilisation of the DNA repair machinery at the site of a DNA-DSB [379]. Further studies

are warranted to investigate the effect of P53 on phosphorylation of γH2AX. However, the known cellular localisation for γH2AX is the nucleus and chromosome [380-382] and the mislocalisation of protein to the cytoplasm has been reported in different studies [383, 384]. Overexpression of Tyrosine Kinase receptor type 1(TrkA), (cell differentiation kinase), causes accumulation of γH2AX proteins in the cytoplasm, leading to considerable cell death in U2OS cells [385]. In addition, Jung et al [386] suggested that cytoplasmic expression of γH2AX could be an important factor in TrkA-mediated cell death by controlling TrkA upon the damage of DNA. γH2AX is a marker of activated DNA damage and its overexpression in cancer indicating a role in oncogenic transformation [387]. Although γH2AX was overexpressed in this study, there is no evidence showing this was due to DNA damage, therefore, genetics- and genomics-based methods that have confirmed functions for conserved DNA damage response and DNA repair proteins will help to discover novel components of these response pathways. Comet assay or FLARE^{TM} Assay (Fragment Length Analysis using Repair Enzymes) will provide the ability to detect DNA damage in cells.

The Development of DSB is subsequently followed by H2AX phosphorylation, leading to the formation of γH2AX, which is the first step in recruiting and localising DNA repair proteins [388]. The inhibition or depletion of CHK1 in cells activates the immediate phosphorylation of ATR targets and H2AX induction and thus DNA breaks accumulate [389]. The results here show that $\gamma H2AXn^{+}.c^{+}$ is negatively associated with ATR and ATM. However, ATR^{-} had a significant association with $CHK1n^{-}.c^{+}$ and $CHK2^{-}$. It can be hypothesised that the high level of γH2AX could be the result of defect and unchecked cell cycles or a defect in DNA damage sensors pathway. Banath et al have demonstrated that the H2AX gene is frequently altered in cervical cancer cell lines, which can induce a 3-fold increase in the formation of γH2AX [390]. Other studies have also demonstrated a high level of γH2AX in various types of cancers, such as lung, breast, colon and ovarian carcinomas [2, 391].

Regardless of significant developments in cancer therapy in recent years, resistance towards chemotherapy remains the most important challenge to address amongst patients suffering from solid tumours [392]. Endocrine resistance is also commonly found in BC therapies, with around one-third of ER-α positive BC resistant to targeted drugs [332]. For this reason, clarification of the resistance mechanisms may prove vital in enhancing strategies of treatment. In general, all the markers in this chapter, which were associated with poor features and received endocrine therapy had worse patient's outcome than who did not receive the treatment. Endocrine responsiveness mechanisms in BC are controlled by sophisticated interactions between steroid hormones and several signalling pathways, which are more than likely influenced by various genetic changes [393]. The identification of the genes responsible for playing some role in resistance to Tamoxifen is one strategy in understanding the main mechanisms within this complex series of events. A small number of studies have dealt with genetic alterations and resistance to anti-oestrogen; it may therefore be of great benefit to continue the investigation into potential markers associated with

resistance. Lundgren et al and other studies are in some way in agreement with the findings of this study, in highlighting that CHK1 is associated with an impaired Tamoxifen response [332, 394]. However, it is too early to suggest the impact of CHK1 or any marker in this study on response to chemotherapy or endocrine therapy, therefore further studies are warranted to investigate it. Genome /exome sequencing can be performed on BC patients who received treatment, and then evaluate the response to the therapy. After sequencing, the data can be analysed and compared with the clinical data for each patient (object response to therapy).

Based on the findings here, the subcellular localisation of γH2AX or CHK1 may have some roles in poor patients' outcomes even after receiving chemotherapy or endocrine treatment; it would have been interesting to have assessed the cellular fractionation in the current cohort – this could be carried out through further work/investigations by others continuing the study. The cytotoxicity of DNA-damaging agents, which normally produce cell-cycle arrest in tumour cells, may be improved by combining them with checkpoint inhibitors. In addition, any mutation or defect in the pathways of the DNA damage sensors proteins may cause mislocalisation in the cytoplasm and thus affect the function of treatment. Nuclear and cytoplasmic fractions of the targeted proteins such as CHK1 and γH2AX can be used and treated separately with Tamoxifen or with chemotherapy such as CMF, therefore flow cytometry will help to observe how the different compounds affected Tamoxifen/chemotherapy-induced cell cycle arrest and apoptosis. However, nuclear and cytoplasmic fractions can be used to detect CHK1 or γH2AX by Western blotting. And finally Immunofluorescence may help to visualise $CHK1^{+}$ or $\gamma H2AX^{+}$ cells upon Tamoxifen/chemotherapy stimulation.

The markers CHK1.c and γH2AX.c when they were analysed as continuous variables, some correlations lost the significance in comparison to categorical data. It is more logic to consider the continuous variable as correlation is biological and certainly the cut-off points will reflect these correlations, but it can be argued that from a clinical point of view, using markers as a dichotomised data may be more helpful for patient management such as HER-2, ER and Ki67 status in BC which are used as positive/ negative and not as continuous variables.

Knowing the subcellular location of a protein is important for understanding its functions [395]. However, regardless co-expression of cellular localisation, CHK1.n (regardless cytoplasmic expression) showed no correlation with P53, but was statistically significant with cytoplasmic CHK1 without considering the nuclear expression, similarly was seen with CK5 (Tables 3.8 and 3.9). In addition, Pearson's correlation (continuous data analysis) of γH2AX.c had no correlation with ER or PgR and CHK1.c with PgR, but was highly significant with their nuclear expression. Therefore, it can be hypothesised that cytoplasmic, but not nuclear, expression of CHK1 may have the main role in association with P53 in BC. In contrast, nuclear expression of γH2AX may have the main role with ER and PgR and similarly nuclear expression may be the main localisation in the association between

CHK1 and PgR. Further studies are warranted to investigate the effect of cellular localisation of CHK1 and γH2AX on their function. However, Amino acid composition-based methods, these methods use machine learning approaches, which include neural networks [396]and support vector machines (SVM), is usually used to predict subcellular localisation of a protein. Improvement in understanding the molecular details in the cytoplasmic/nuclear actions of CHK1 or γH2AX may help to find a target for BC, especially the two cellular compartments showed different features of BC.

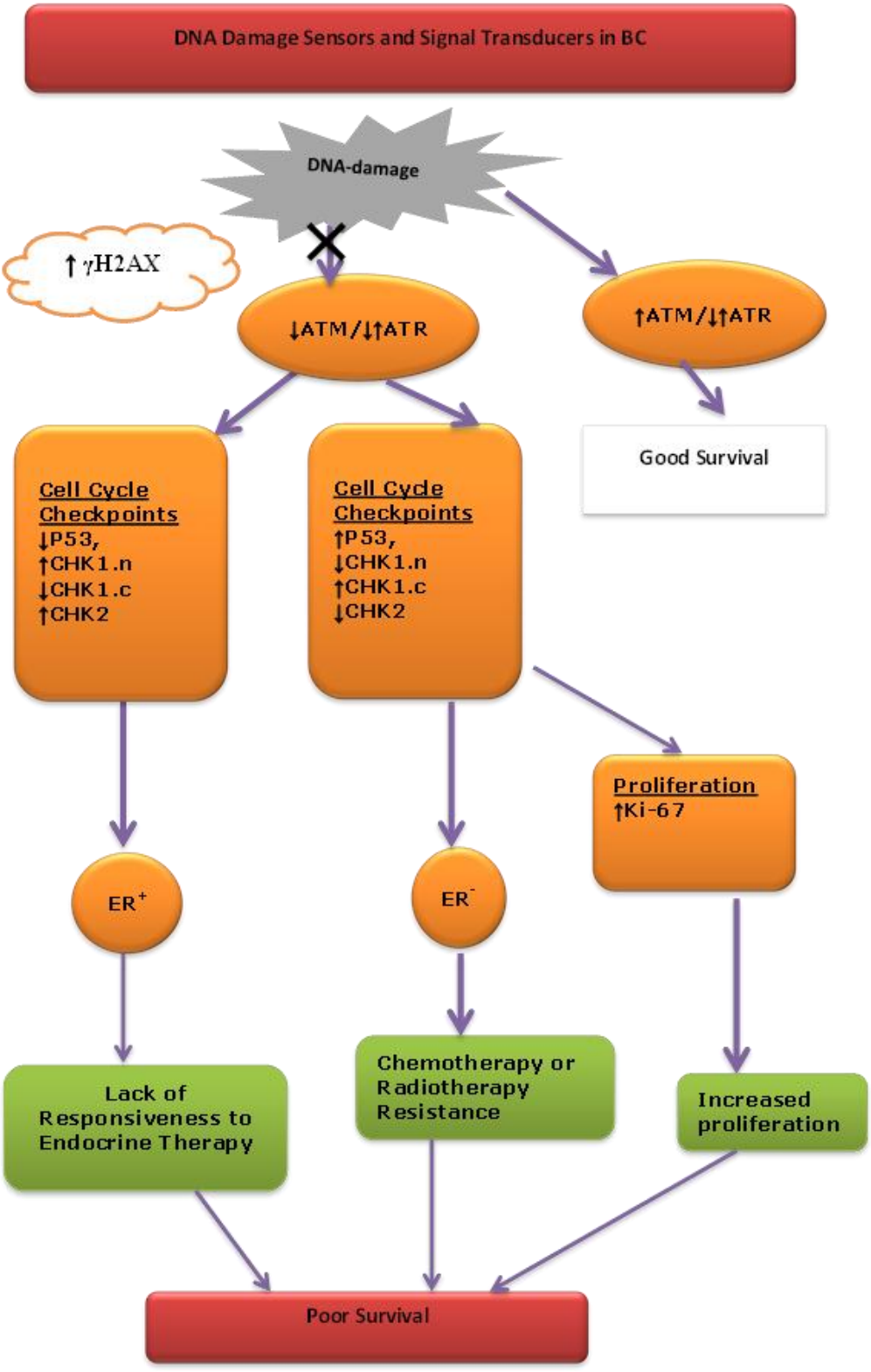

Figure 3.15 A summary of the finding in this chapter and pathway involved in DNA damage sensors and signal transducers in breast cancer. **X** represents a defect in the response to DNA damage by showing low level expression of ATM. As response to DNA-damage the level of ATM should be increased. Here the low level of ATM may explain a defect in the response to the damage or the cancer itself has other causes than a defect in DNA repair.

Chapter 4

4.1 Introduction

4.1.1 DNA Double Strand Break Repair

Increasing evidence proposes that *BRCA1* plays a role in many essential functions, such as DNA repair, transcription and control of the cell cycle. Of the various types of DNA damage that can occur within a mammalian cell, the DSB is understood to be the most lethal [262, 397]. Many studies have indicated a link between DSB, genomic instability and cancer and this relationship is based upon the fact that syndromes representing a predisposition to cancer are characterised by genome instability and arise from a mutation in DSB-responsive genes [262, 398]. DSB induction in the early stage of tumorigenesis, in addition to concomitant activation of damage responsive proteins, gives rise to cellular apoptosis. In the continuous presence of DSB and in the advanced stages of cancer, proteins that respond to the breaks, such as P53, became mutated, leading to a defect in apoptosis or senescence: this will result in the development of carcinogenesis [2, 399].

DSB can be generated by extrinsic sources, such as IR or radiomimetic drugs used for chemotherapy; additionally, intrinsic sources, such as ROS, are believed to induce DSB [400]. Unlike single strand nicks or base modification, DSB can initiate gross chromosomal aberrations, if not repaired quickly. However, the repair process may be error-prone or, in some cases, may be detrimental to the organism. It is recognised that mammalian cells have the ability to quickly transfer the damage signal to the cell cycle arrest or machineries of apoptosis and DNA repair mechanisms [399]. Damaged cells require time for repair and this is achieved by the action of cell cycle arrest. Occasionally, apoptosis may be more prudent for cells where the DNA remains unrepaired or has excessive damage. Both these mechanisms are strongly believed to work as barriers to carcinogenesis [399]. It has been determined that the molecular mechanisms of DSBR can be divided into two pathways of repair: HR and NHEJ [341].

4.1.1.1 Homologous Recombination

Figure 4.1A outlines the mechanisms of the HR pathway. The main difference between the HR and NHEJ pathways lies in their requirement for a homologous DNA template during the repair process [262]. The HR pathway is a more accurate method of repair: the sister chromatid is used as a template, in order to copy the missing information into the broken locus; however, as sister chromatids are identical to each other, the repair process for damaged DNA can be initiated without genetic sequence [401]. Thus, this pathway only functions in the S/G2 phases of the cell cycle [402].

BRCA1 has a role in DSB: it initiates the response to DNA damage, thereby maintaining genomic integrity [403, 404]. BARD1 is known as the main protein-binding partner to BRCA1. BARD1 and BRCA1 are co-ordinately expressed; however, BARD1 is apparently expressed independently of BRCA1 in some tissues that respond to hormonal regulation, particularly in the uterus [405] and testis [406]. BARD1 and BRCA1 develop a constant heterodimer via their respective RING domains. The heterodimer, which is more resistant to proteolysis than the respective homodimers, is the efficient domain for ligase activity of ubiquitin, the repair of DNA and transcriptional regulation [407-410]. A tumour-associated mutation of BRCA1, C61G within the RING finger domain, can interrupt the interaction of BRCA1/BARD1 [410], which implies that the heterodimer of BARD1/BRCA1 has a significant function in BRCA1-mediated tumour suppression. Genomic integrity is maintained by the suppressor gene *BRCA1* by protecting cells from the harmful effects of DSBs and primarily stimulates HR, which is typically known to be an error-free repair method [341].

In HR, BRCA1 co-localises with Rad51 to form a complex [403, 404]. A single strand of DNA is coated by Rad51 to form a nucleoprotein filament that penetrates and makes pairs with a homologous region in duplex DNA, leading to the activation of strand exchange and the creation of a crossover between the juxtaposed DNA [411, 412]. This action suggests that BRCA1 has a role in the detection and repair of DSBs [403]. The formation of Rad51 is decreased after treatment with DNA-damaging agents and during HR in *BRCA1*-deficient cells [413] and thus HR is defective in *BRCA1*-deficient cells [414]. It remains unclear whether Rad51 and BRCA1 can bind directly [413]. Rad51 is not the only protein that interacts with BRCA1 to modulate DNA repair; Rad50, with its partners Mer11 and NBS1, has a similar function [415, 416].

Bau et al proposed that Rad51 may be required for NHEJ for DSB repair [417]. In terms of an *in-vitro* end-joining assay, some studies have shown that cells from mice or humans that expressed deficient *BRCA1* had more reduced end-joining activity than *BRCA1* proficient cells [36, 418]. Additionally, *in vivo BRCA1* increases precise end-joining activity [36] (the ATM/CHK2 mediated signalling pathway is also necessary for precise NHEJ [419, 420]). *BRCA1* also plays a role in NHEJ, as it is able to protect DNA ends from excess trimming by exonucleases (e.g., Mer11-dependent nucleolytic activity). This was confirmed by Paull et al who demonstrated the strong binding of *BRCA1* to the ends of DNA and the inhibition of Mer11 activity of the MRN complex [421]. Thus, it is suggested that *BRCA1* prevents familial BC; not only by stimulating HR, but also by decreasing error-prone NHEJ and promoting error-free NHEJ methods [413].

In eukaryotes, there are six conserved structural maintenance of chromosome (SMC) protein family members, SMC1–6. These participate in different functions, including condensation of chromosomes, the cohesion of sister-chromatids and the repair of DNA damage [422]. The precise mechanisms of how these proteins enhance such distinct functions have not yet been determined. Insights directly into the mode-of-action of the SMC family

proteins are suggested by their unique organisation, in addition to their pattern of association with various other proteins. As they develop their particular mature configuration, SMC proteins fold into highly elongated structures containing an asymmetrically-localised ATPase activity [423, 424]. SMC6L1 is a key component of the SMC5-SMC6 complex, which is involved in DNA-DSB through HR [425, 426]. It has been suggested that this complex may possibly enhance the homologous recombination of sister chromatids, simply by enrolling the complex of SMC1-SMC3 to DSBs. The association between precatenanes (intertwined replicated DNA duplexes) and the linkages of sister chromatid that accumulate in SMC6 mutants within the existence of DNA damage remains to be identified [427].

4.1.1.2 Non Homologous End Joining

Figure 4.1B highlights the mechanisms of the NHEJ pathway. Unlike HR, NHEJ combines the two broken DNA strand ends with little or no homology sequence, leading to the deletion or insertion of filler DNA, which is operative in all phases of the cell cycle [399, 428]. Genetic studies featuring radiosensitive mammalian cell lines defective in the re-joining of DSBs and also mutations in the genes that encode the components of NHEJ have been helpful in identifying a variety of proteins involved in the DSBR process [429, 430]. The main two proteins that mediate NHEJ are KU70/KU80 and DNA-PK. The DNA binding subunit of DNA-PK, KU, is a heterodimer that includes 70 and 80 kDa subunits (KU70 and KU80 respectively). The first step in NHEJ is the detection of DSB by the KU70/KU80 heterodimer. Cells defective in either KU70 or KU80 are IR-sensitive and do not complete V(D)J recombination (combines Variable, Diverse, and Joining gene). Although NHEJ is considered the predominant repair pathway in mammalian cells, it is an error-prone method, due to the fact that it does not base repair on an intact homologous template (sister chromatid) [399, 428].

It has been demonstrated that the XRCC5, XRCC6 and XRCC7 genes encode the DNA-PK; XRCC5 and XRCC6 encode the KU70/KU80 heterodimer (the DNA-binding subunit of DNA-PK), while XRCC7 encodes the 470 kDa DNA-catalytic subunit of protein kinase DNA-PK [431-433]. Despite the fact that the actual role of these proteins in the DSBR process remains to be identified, three possible steps have already been indicated in the repair of DSBs via NHEJ: (a) end-binding and bridging, (b) terminal processing and, finally, (c) ligation. In the first step, KU binds the ends of the DNA (the activity of the end-binding of KU indicates that it could be the initial detector of damage in NHEJ), aligns them and prepares for ligation; additionally, it protects from degradation [434]. DNA-PK is recruited towards DSBs by KU, leading to the activation of its kinase function [435]. Even though the target proteins of DNA-PK remain unidentified, many experts have proposed that DNA-PK is able to (a) phosphorylate XRCC4 and eliminate or transfer the ligase IV/XRCC4 complex from KU-bound DNA ends and thus enable essential processing actions to occur [436, 437], (b) manage the accessibility of DNA ends to processing in its inactivation through auto-phosphorylation and/or by means of enabling the translocation of KU away from the DSB and, finally, [438] (c) phosphorylate both KU70 and KU80, with as-yet-undetermined effects.

In mammals, DNA polymerases assist in increasing the efficiency of NHEJ by enabling gaps to be filled throughout the re-joining of the two non-complementary ends. A physical interaction between KU, XRCC4 and the ligase IV–polymerase complex has also been documented [439]. Finally, throughout the final step of NHEJ, the DNA ligase IV tight complex and XRCC4, which has been demonstrated to form a tetramer, are recruited to ligate the DSBs [440, 441]. Several studies have aimed to identify other factors involved in NHEJ. Recent studies using human radiosensitive SCID (severe combined immunodeficiency) cells determined mutations in a novel protein of DNA-DSBR , known as Artemis, and revealed the value of this protein in lymphoid differentiation in bone marrow [264, 442]. Artemis is involved in DNA end processing, as a result of the $5^{'}$ exonuclease activity [443].

4.2 Hypothesis

Of the various types of DNA damage that can occur within a mammalian cell, DSB is understood to be the most lethal [262, 397]. Many studies have indicated a link between DSB, genomic instability and cancer and this relationship is based upon the fact that syndromes representing a predisposition to cancer are characterised by genome instability and arise from a mutation in DSB-responsive genes [262, 398]. Women with reduced or aberrant BRCA1 and show defect in DNA-DSBR pathway are generally TNBC and basal phenotype, with aggressive features [444]. It is thus hypothesised that alterations of the repair pathways of DNA-DSBs may also contribute to the development and progression of at least a proportion of sporadic breast carcinoma particularly those associated with features similar to breast carcinoma arising in patients with *BRCA*1 germline mutations and particularly with respect to development of forms of ER- negative BC.

4.3 Aim

The aim of this chapter is to investigate the roles of DNA-DSBR pathways using different markers in the repair pathways of HR and NHEJ in BC series using IHC in TMAs and RPPA, in order to determine the association between the DNA-DSBR markers, pathological features, tumour phenotypes and clinical outcomes.

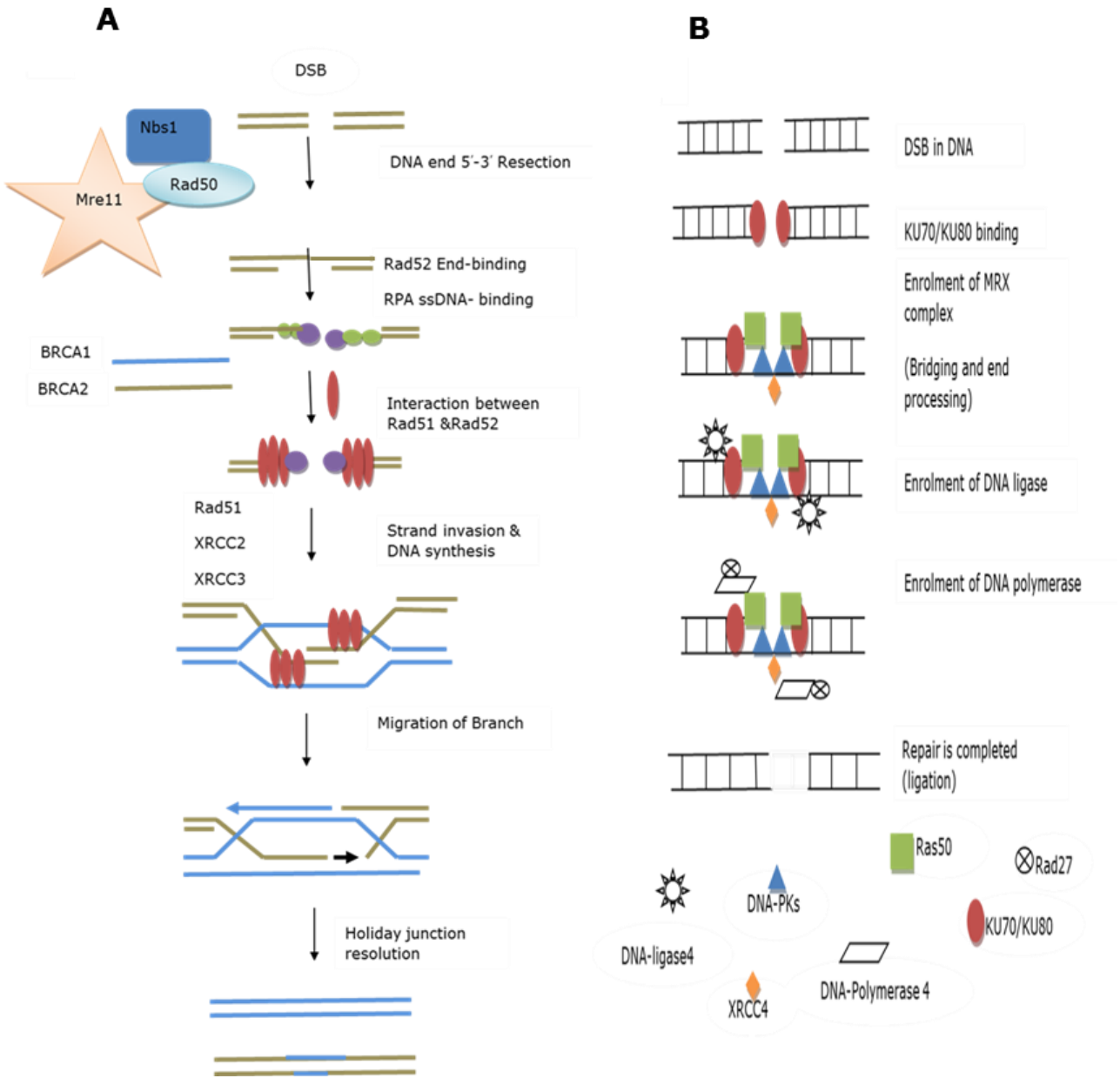

Figure 4.1 Outline of DNA-DSB repair: A: HR, B: NHEJ. In HR; Nbs1, Mer11 and Rad51 form complex which is called MRN. Homology search is started by Rad51 and BRCA1. Holliday junction is a mobile junction between 4 strands of DNA and it is importance in maintaining genomic integrity. In NHEJ; KU70/KU80 binds to the DNA ends, align, and protect them from degradation.

4.4 Materials and Methods

A previously described in Chapter 2

4.4.1 Patient Samples

All data are as previously described in Chapter 2 Section 2.1.1. Three cohorts were used: A) 1904 unselected cases of female primary operable invasive tumours between 1986 and 1998, B) 386 cases selected from a consecutive series of primary operable ER negative tumours between 1998 and 2007 and C) 24 well-characterised series of breast tumours from patients with known *BRCA1* germline mutations. However, High Resolution Melting Analysis (HRMA) with employing PCR was used for *BRCA1* mutation detection in group C (this was performed by Dr Ahmed Benhasouna). All cases were obtained from the well-characterised Nottingham Tenovus primary breast carcinoma series.

4.4.2 Available Biomarkers' Data

Data on a wide range of biomarkers of known clinical and biological relevance to BC were accessible and saved on a web-based interface (Distiller; Slidepath Ltd, Dublin, Ireland). These include, ER, PgR, HER2, CK5, CK17, CK14, tumour suppressor proteins (P53 and PTEN), BRCA1 down regulators proteins such as and MTA1 and ID4 and cell proliferative marker (Ki-67) [270-274, 331].

4.4.3 Immunohistochemistry

As previously described in Chapter 2 Section 2.1.3. Six markers from HR and NHEJ repair pathways were investigated in this chapter; BRCA1, BARD1, Rad51, SMC6L1, KU70/KU80 and DNA-PK.

4.4.3.1 Immunohistochemical Antibody Labelling Using the Novolink Detection Method

As previously described in Chapter 2 Section 2.1.3.1. All the markers were stained by the author except BRCA1 which was previously stained by our research group (with thanks to Dr Ahmed Benhasouna). All these markers have already been previously successfully stained on TMA [287, 335, 336, 445-448].

4.4.3.2 Optimisation of Antibodies used for IHC

As previously described in Chapter 2 Section 2.1.3.2. In addition to Western blotting, specificity of staining was confirmed by application of negative (with omission of the primary antibody) and positive controls. Positive controls were used according to the manufacturer's datasheet and/or from the human protein atlas available at http://www.proteinatlas.org/. This helped not only to test the specificity of staining but also to assess the pattern and intensity of protein expressions in the appropriate tissue. Details of the negative and positive controls used are summarised in Table 4.1. Moreover, some control TMA slides containing a variety of BC cases with some containing cores from different areas of the same cases in addition to normal parenchymal elements were used during optimisation to assess the degree of expression heterogeneity.

Table 4.1 Immunohistochemistry Positive and Negative Controls of Antibodies Used in this Chapter.

Antibody	Positive control	Negative control	Reference
Homologous Recombination Repair Markers			
Rad51	Normal kidney or BC tissue.	BC tissue	Abcam/ human protein atlas available at http://www.proteinatlas.org/
BRCA1	Normal kidney or BC tissues	BC tissue	Calbiochem / human protein atlas available at http://www.proteinatlas.org/
BARD1	Normal liver or BC tissue.	BC tissue	Novus Biologicals/ human protein atlas available at http://www.proteinatlas.org/
SMC6L1	Normal kidney or BC tissue.	BC tissue	Abcam/ human protein atlas available at http://www.proteinatlas.org/
Non Homologous End Joining Repair Markers			
DNA-PK	BC tissue	BC tissue	Cell signalling/ human protein atlas available at http://www.proteinatlas.org/
KU70/KU80	BC tissue	BC tissue	Abcam/ human protein atlas available at http://www.proteinatlas.org/
Staining of positive and negative controls was performed together in the same run. Negative staining was performed without adding the antibody and showed no staining. All were stained on TMA. All these markers have already been previously successfully stained on TMA [287, 335, 336, 445-448].			

4.4.3.3 Immunohistochemistry Scoring

As previously described in Chapter 2 Section 2.1.3.3. For evaluation of IHC of the TMA, a modified H-score was used [282]. For H-score, both the intensity of staining and the percentage of stained cells were considered within each tissue core. Staining intensity was scored as 0, 1, 2 or 3 for negative, weak, moderate and strong, respectively. The proportion (percentage) of positive cells for each intensity was subjectively estimated. Multiplication of the two indices (intensity and percentage positive cells) provided final scores that range from 0 to 300.

The author re-scored each marker with at least 30% of a randomly chosen subset of cases. A statistical agreement test was performed (Kappa value) for each marker, where there

was good agreement (≥0.5), and an average was taken. If there were discrepancies, the highest scoring was taken. Kappa values are summarised in Table 4.2. Rad51, KU70/KU80 and BRCA1 were scored using light microscopy, whereas the others were scored visually on high-resolution monitors using a web-based interface (Distiller, Slidepath Ltd., Dublin, Ireland). Scoring of all markers was performed by the author except BRCA1 was performed by Dr Ahmed Benhasouna and rescored by the author.

Table 4.2 The Statistical Agreement between Different Scoring of Antibodies Used in this Chapter.

Markers	Percentage of re-scoring	Kappa value
Homologous Recombination repair Markers		
BRCA1.n	30%	0.57
BRCA1.c	30%	0.69
BARD1.c	30%	0.97
BARD1.n	30%	1
SMC6L1.n	30%	0.62
SMC6L1.c	30%	0.75
Rad51.n	30%	0.8
Rad51.c	30%	0.6
Non Homologous End Joining repair Markers		
KU70/KU80	100%	0.98
DNA-PK	30%	0.7
Kappa test was performed on IBM SPSS 21.0. An average was taken after re-scoring.		

4.4.4 Specificity of the Antibodies by Western Blot (W.B)

As previously described in Chapter 2 Section 2.1.4.4. Western blot was used on all markers except BRCA1 and BARD1, because these markers were stained before applying W.B. A mixture of different cell lysates to detect only the specificity of an antibody has been applied in different studies and showed its reliability [283, 284]. In the present study, all the markers were detected in a mixture of different lysates (MCF-7, MDA-MB-231, HeLa BRCA1 and its control), in addition, Rad51 and KU70/KU80 was done on single cell line (MCF-7) and showed similar finding to the mixture cell lines (data in Appendix 2). All the antibodies tested in this chapter share the same positive controls which were MCF-7, HeLa BRCA1 cell lines. The pre-stained marker 'full range rainbow marker' (Invitrogen Life Technologies) was used as a molecular weight standard. http://www.proteinatlas.org/ provides profile data for positive controls of all the markers used in this study. Table 4.3 summarises the details of W.B for each marker.

Table 4.3 List of Antibodies Tested by Western Blot on Different Cell Lines.

Antibody	Cell lines	Specific positive cell lines*
Homologous Recombination Repair Markers		
Rad51	MCF-7	MCF-7 or HeLa BRCA1 cell lines
SMC6L1	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
Non Homologous End Joining Repair Markers		
KU70/KU80	MCF-7	MCF-7 or HeLa BRCA1 cell lines
DNA-PK	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
Cell lines and reagents were obtained from the group of Dr Madhusudan Srinivasan, thawing and freezing procedures were done by Nada Albarakati. Passages, Bradford assay and gel electrophoresis were done by the author. Passages used in W.B were as follows; HeLa BRCA1; passages 29&30, HeLa BRCA1 control; passages 15&16, MCF-7; passages 25&26, MDA-MB-231; passages 15&16. *Data available at http://www.proteinatlas.org/.		

4.4.5 Reverse Phase Protein Microarray

RPPA was carried out as previously described in Chapter 2 Section 2.1.5. Cell lines preparation and protein extraction and interpretation of the results were carried out by the author. However, RPPA run and analysis was carried out by Dr Ola Nejm (Immunology, School of Life Sciences, University Hospital, Nottingham, UK) as a collaborative project.

In addition to HR and NHEJ repair markers, PTEN was analysed using RPPA (details of the antibody dilution is presented in Chapter 2, Table 2.6).

4.5 Statistical Analysis

All statistical analyses were done by the author using IBM SPSS statistic 21.0 software. For all statistical tests, a two-sided *P-value* of <0.01 was considered statistically significant.

4.5.1 The Determination of the Optimal Cut-offs

As described previously in Chapter 2 Section 2.1.6.1. The expression of the biomarkers investigate in this study were dichotomised using two different approaches: a) using the mean or median of the H-score of the staining according to distribution pattern whether normally or not normally distributed, or b) using x-tile software (version 3.6.1, 2003-2005, Yale University, USA). If the cut-off by mean or median was very high (e.g H-score >200) then x-tile was considered, all the markers in this study used x-tile except SMC6L1 and BRCA1. However, for SMC6L1, regardless cellular localisation, both x-tile cut-points and mean or/median were high so a more reasonable cut-off based on frequency distribution, therefore, mean was for nuclear and median for cytoplasmic expression of SMC6L1. Some markers showed similar cut-off by x-tile and mean/median such as BARD1.n, and BRCA1.n. The means of DNA-PK and Rad51 were high so x-tile was considered. If the mean/median of Rad51 was taken then more than three quarter of the cases will be considered negative

Table 4.4 shows the details of the antibodies used in this chapter. Details of H-score histograms of all markers are presented in Appendix 2.

Table 4.4 Sources, Dilution, Cut-offs Point and Pre-Treatment Conditions of the Antibodies Used in this Chapter.

Antibody	Clone	Source	Dilution IHC	Dilution W.B RPPA	[+]Distribution	Cut-offs	IHC kit
Homologous Recombination Repair							
BARD1	NBP1-19636	Novus Biologicals	1:50 1h	NT 1:200	Nuclear/cytoplasmic	≥130 H-score cytoplasm, >0 H-score [*] nuclear, x-tile.	Novolink
BRCA1	Ab-1 (MS110)	Calbiochem	1:150 1h	NT 1:200	Nuclear/cytoplasmic	≥93 H-score [*] nuclear, ≥40 H-score cytoplasmic, means.	ABC
Rad51	Ab88572	Abcam	1:70 1h	1:1,000 1:100	Nuclear/cytoplasmic	Nuclear ≥8 H-score Cytoplasm ≥80 H-score, x-tile.	Novolink
SMC6L1	AB57759	Abcam	1:100 1h	1:1,000 1:250	Nuclear/cytoplasmic	Nuclear >240 H-score, mean Cytoplasm ≥230 H-score, median.	Novolink
Non Homologous End Joining Repair							
KU70/KU80	Ab3108	Abcam	1:2500 1h	1:1,000 1:500	Nuclear	≥90 H-score, x-tile.	Novolink
DNA-PK	3H6	Cell signalling	1:28 1h	1:2,000 1:150	Nuclear	Nuclear ≥150 H-score, x-tile.	Novolink

All the antibodies were pre-treated in citrate antigen retrieval pH=6.0 in microwave for 20 minutes and stained on TMA using IHC. [+] Cellular localisation. BRCA1 was stained/scored by Dr Ahmed Benhasouna. All the antibodies were incubated at room temperature for 1 hour. [*]BARD1.n and BRCA1.n showed similar cut-off by x-tile and mean/median. IHC= Immunohistochemistry. W.B= western blotting. NT= not tested. W.B was not run on BRCA1 and BARD1, because these markers were stained before applying W.B. 1h= 1 hour incubation with the primary antibody at room temperature.

4.5.2 Univariate Analysis with Clinico-Pathological Parameters and Tumour Markers

The differences between all markers, with regards to clinico-pathological features, or with other tumour markers were analysed using the Pearson Chi-Squared test (X^2). Consequently, X^2 was also used in order to examine the inter-relations between markers themselves. In addition analysis of continuous variables was performed using Pearson's correlation and ANOVA. One way ANOVA was used to find out which of different BC classes (by IHC or RPPA) were significantly different from each other (post hoc test; Tukey).

4.5.3 Univariate Analysis with Patients' Outcome

Patients who were alive or those who died for any reason other than BC were not included. The Kaplan-Meier method was used to generate a univariate survival curve and the differences in survival among the biomarkers were evaluated using the log-rank test.

4.5.4 Multivariate Analysis with Patients' Outcome

If a marker in univariate analysis was statistically significant with patient's outcome, then Cox regression was applied for multivariate analyses to test for confounders and prognostic or predictive independency of the investigated biomarker from standard prognostic/predictive factors such as tumour grade, tumour stage, and tumour size.

4.6 Results

4.6.1 Expression of DNA-DSB Repair Markers in Invasive Breast Cancer

Western blotting validated the specificity of antibodies in DNA-DSB repair, which was deemed validated by a single band at the correct protein size (see Figure 4.2). However, some antibodies, such as BARD1 and BRCA1, were stained previously before applying W.B, however, positive and negative tissue controls were used to validate the specificity of the antibodies. Normal liver tissue was used as positive control of BARD1 (Figure 4.3), and normal kidney tissue for BRCA1 (Figure 4.4) (http://www.proteinatlas.org/). However BRCA1 was stained previously by Dr Ahmed Benhasouna [449] and BARD1 by the author.

In invasive tumours, the markers for HR repair (Rad51, BRCA1, BARD1 and SMC6L1) showed both nuclear and cytoplasmic staining (see Figures 4.3-4.6), whereas, the markers of NHEJ repair pathway (DNA-PK and KU70/KU80) demonstrated only nuclear staining, with no cytoplasmic or membranous staining observed (Figure 4.7). Although BRCA1 and Rad51 are localised in the nucleus, cytoplasmic expression has been mentioned previously (in breast and human soft tissue sarcoma cells) [450-452]. In addition, BARD1 and is expected to express in cytoplasm of the cell [453, 454] as well as SMC6L1, from Human Protein-Protein Interaction Mining Tool available at (http://liweilab.genetics.ac.cn/).

Table 4.5 displays the frequencies of HR and NHEJ repair proteins in sporadic and known *BRCA1* germline mutations BCs (hereditary), while Figure 4.8 represents the distribution of DNA-DSB repair markers (mean) in different classes of BC by IHC. The four classes included were classified based on BRCA1 and ER proteins status. Class 1; sporadic BRCA1 negative and ER negative, class 2; sporadic BRCA1 positive and ER positive, class 3; known *BRCA1* germline mutations BC that showing ER negativity, and finally; known *BRCA1* germline mutations BC and showing ER positivity.

There was a strong expression of nuclear BRCA1, nuclear Rad51, or BARD1.c (Figure 4.8) in sporadic $BRCA1^{+}/ER^{+}$ cancers, compared to sporadic $BRCA1^{-}/ER^{-}$ (for all $P<0.0001$) or in comparison to known *BRCA1* germline mutations that showing ER negativity (BRCA1.n and BARD1.c; $P<0.0001$ and for Rad51.n; $P=0.0001$). In contrast, cytoplasmic expression of BRCA1 or SMC6L1 in terms of sporadic $BRCA1^{+}/ER^{+}$ cancers was the weakest amongst the other classes.

The expression of the NHEJ markers (KU70/KU80 and DNA-PK) showed similar levels in sporadic classes, however, DNA-PK showed a significant difference between known *BRCA1* germline mutations tumours that expressing ER^{-} and sporadic $BRCA1^{+}/ER^{+}$ ($P=0.009$).

The large error bars of Figure 4.8 are expected, the mean of H-score does not explain repeated observation, it shows the distribution of different cases share ER and BRCA1 status

but with other factors such as grade, stage, and size of the tumour which may have some effects on the expression of the markers.

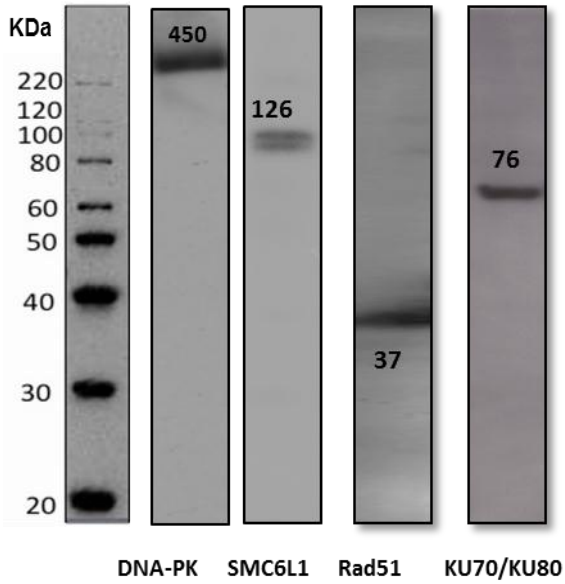

Figure 4.2 Detection of different DNA-DSB repair proteins level by Western blot. Detection of DNA-PK and SMC6L1 levels by Western blot in a mixture of cell lines, MDA-MB-231, MCF-7, HeLa BRCA1 and its control, whereas KU70/KU80 and Rad51 by Western blot in MCF-7 cell line. The predicted size of each protein is labelled on the band. Passages used in W.B were as follows; HeLa BRCA1; passages29&30, HeLa BRCA1 control; passages 15&16, MCF-7; passages 25&26, and MDA-MB-231; passages 15&16.

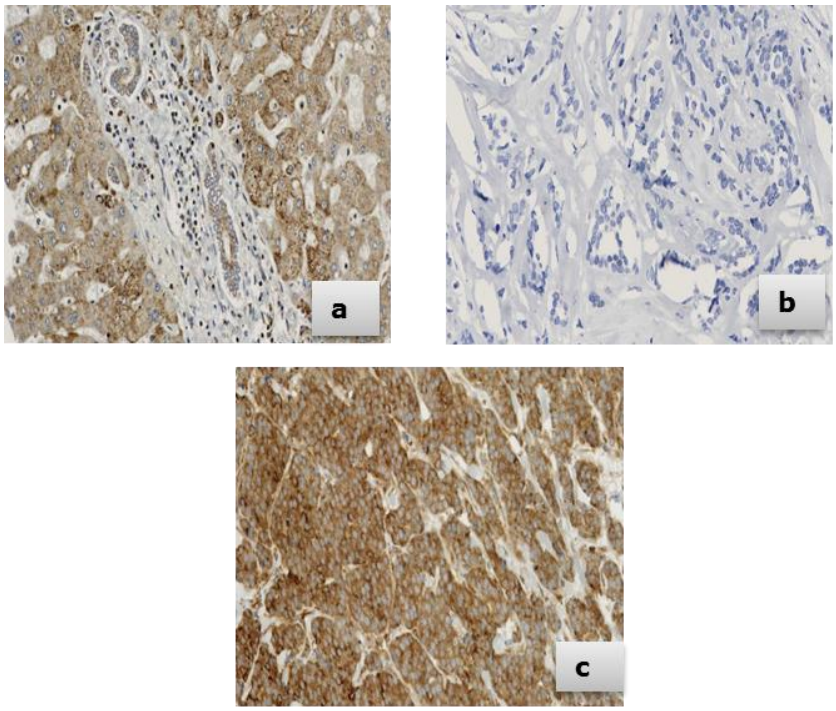

Figure 4.3 Expression of BARD1 on TMA. Where a; positive control in normal liver tissue, b; negative control in invasive ductal/NST breast cancer; stage 2 and grade 3, and c; cytoplasmic expression of BARD1 in invasive ductal carcinoma/NST; stage 1, and grade 3. Magnification x20.

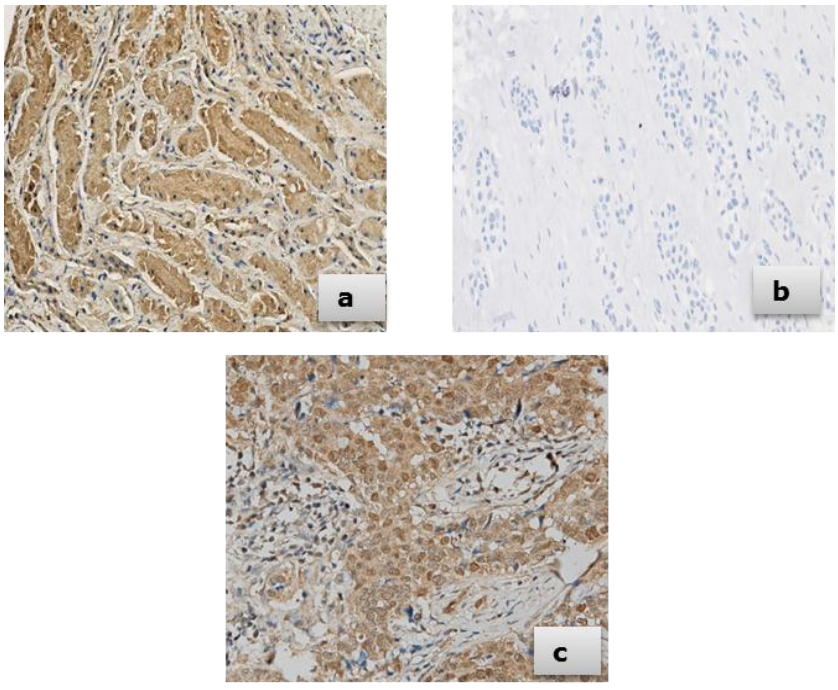

Figure 4.4 Expression of BRCA1 on TMA. Where a; positive control in normal kidney tissue, b; negative control in invasive ductal/NST breast cancer; stage 1 and grade 3, and c; expression of nuclear and cytoplasmic BRCA1 in invasive ductal carcinoma/NST; stage 1 and grade 3. Magnification x20

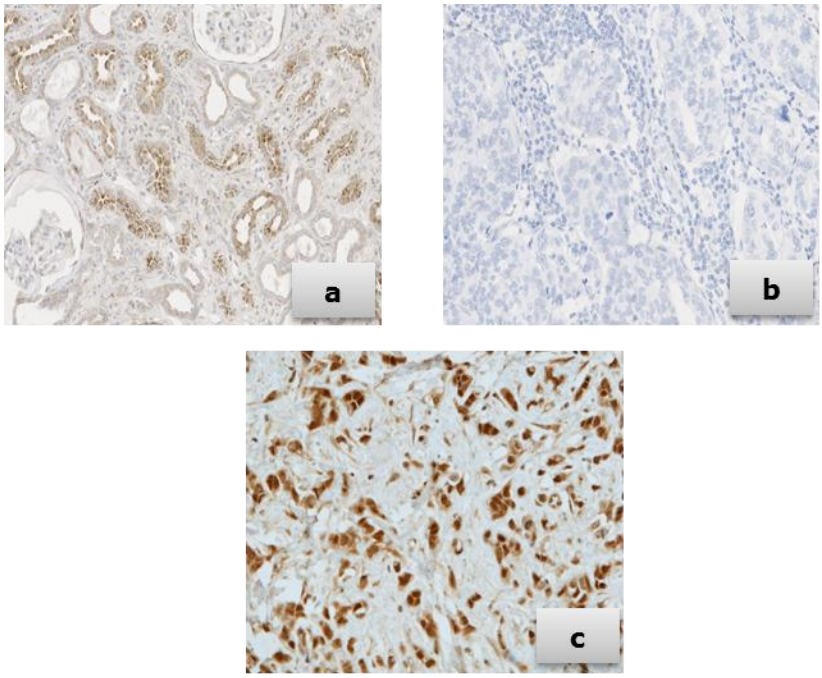

Figure 4.5 Expression of Rad51 on TMA. Where a; positive control in normal kidney tissue, b; negative control in invasive ductal /NST breast cancer; grade 3 and stage 1, and c; expression of nuclear and cytoplasmic Rad51 in invasive ductal carcinoma /NST; grade 3 and stage 1. Magnification x20

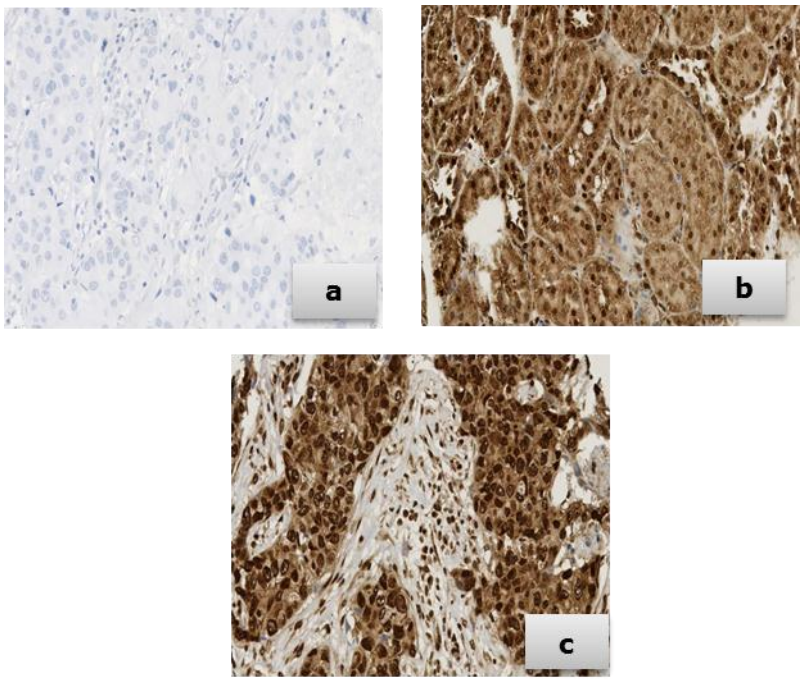

Figure 4.6 Expression of SMC6L1 on TMA. a; negative control for SMC6L1 in breast cancer tissue, b; positive control in normal kidney tissue and c; SMC6L1.nuclear and cytoplasmic expression in breast cancer tissue. a and c are invasive ductal carcinoma/NST; stage 1, and grade 3. Magnification x20

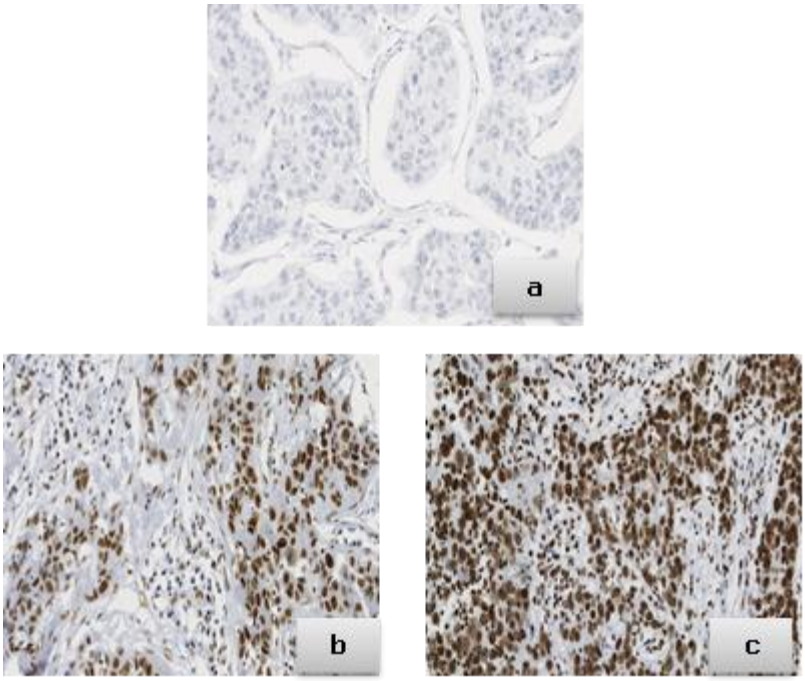

Figure 4.7 Expression of DNA-PK and KU70/KU80 on TMA. a; negative control for both markers in breast cancer tissue, b; DNA-PK. nuclear, and c; KU70/KU80.nuclear. All are invasive ductal carcinoma/NST; stage 1, and grade 3. Positive control for these two proteins is breast cancer tissue. Magnification x20

Table 4.5 Frequency of DNA-DSB Repair proteins Expression in Breast Cancer

Markers	Sporadic BC		known *BRCA1* germline mutations BC	
	%	Frequency	%	Frequency
Non Homologous End Joining Repair				
KU70/KU80	86.9%	1130/1301	100%	22/22
DNA-PK	85.2%	1142/1341	78.9%	15/19
Homologous Recombination Repair				
Rad51				
Cytoplasmic	89.2%	1051/1184	100%	18/18
Nuclear	34.7%	411/1184	0%	0/18
BRCA1				
Cytoplasmic	41.8%	732/1758	0%	0/23
Nuclear	72.8%	1280/1758	13%	3/23
BARD1				
Cytoplasmic	79.8%	1179/1477	26.3%	5/19
Nuclear	9.1%	135/1477	5.3%	1/19
SMC6L1				
Cytoplasmic	67.9%	942/1387	65%	13/20
Nuclear	65.9%	914/1387	75%	15/20
Sporadic BC includes both unselected and ER-negative BC cases. The number of cases may be reduced due to loss cases during preparation of tissue for staining (TMA sectioning or IHC procedure).				

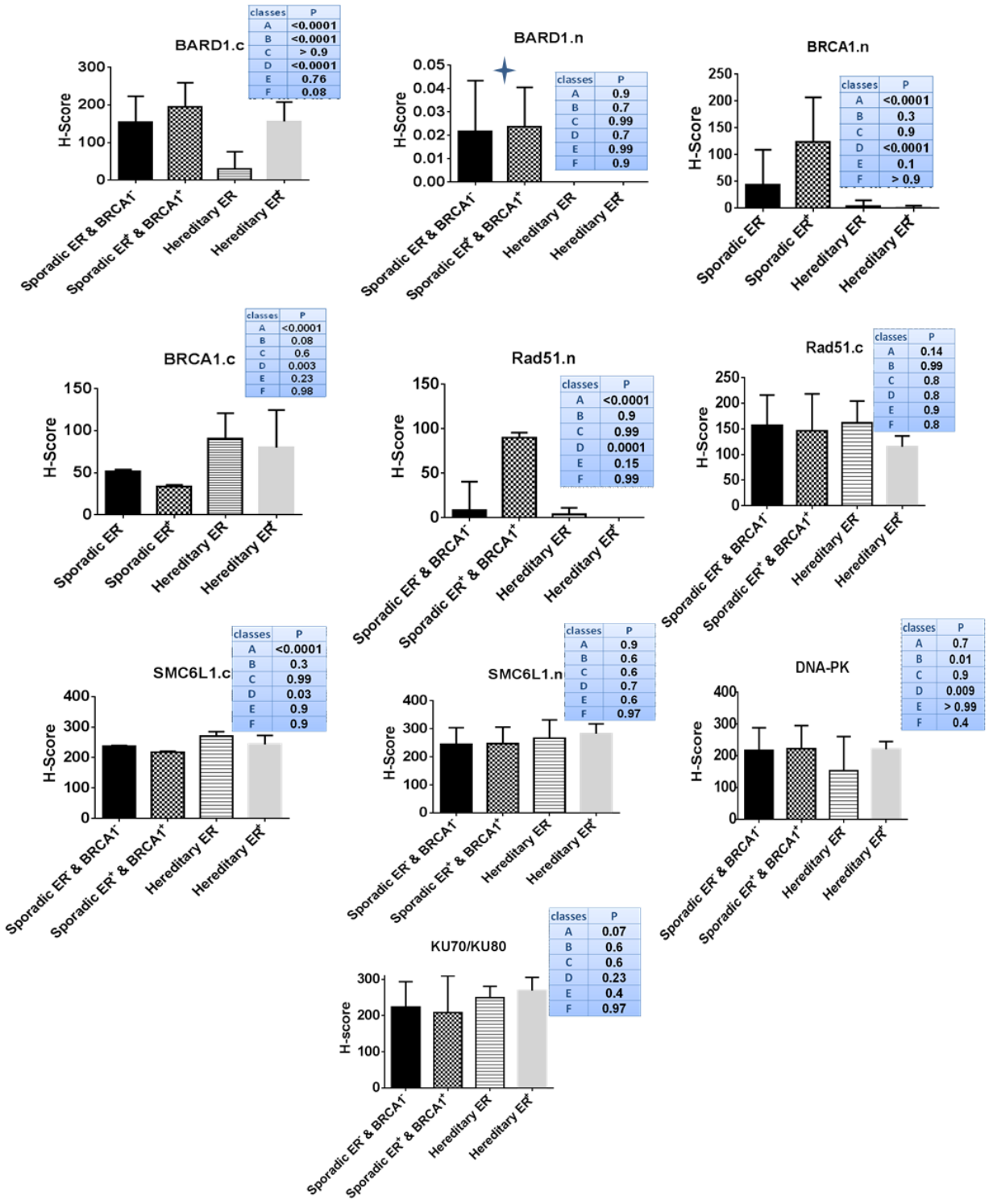

Figure 4.8 DNA-DSB repair protein levels detected by IHC in breast cancer on TMA. Each bar represents different class based on hereditary or sporadic BRCA1 and ER status. n= nuclear and c= cytoplasmic expression. Error bars represent Mean (SD) and was created on H-score (ranges 0-300). A= sporadic cases [ER$^-$ & BRCA1$^-$] vs. sporadic cases [ER$^+$ & BRCA1$^+$], B= sporadic cases [ER$^-$ & BRCA1$^-$] vs. Hereditary cases [ER$^-$], C= sporadic cases [ER$^-$ & BRCA1$^-$]vs. Hereditary cases [ER$^+$], D= sporadic cases [ER$^+$ & BRCA1$^+$]vs. Hereditary cases [ER$^-$], E= sporadic cases [ER$^+$ & BRCA1$^+$] vs. Hereditary cases [ER$^+$], and F= Hereditary cases [ER$^-$] vs. Hereditary cases [ER$^+$]. ANOVA test was used for each marker within the classes. ✦Very few cases showed nuclear staining of BARD1.

4.6.2 Correlation of DNA-DSB Repair Markers

Table 4.6a summarises the associations between the DNA-DSB repair markers (Pearson X^2). There was a high association between both the HR and NHEJ markers. Rad51.n^-.c^+ showed a strong association with KU70/KU80$^+$ (P<0.0001), DNA-PK$^+$ (P=0.002), SMC6L1.c^+ and BARD1.c^+ (both P<0.0001). In addition, BRCA1.n^-.c^+ had a significant association with KU70/KU80$^+$ and DNA-PK$^+$, Rad51.c^+, Rad51.n^-, and BARD1.c^+ (all P<0.0001). SMC6L1.n^+.c^+ was highly associated with KU70/KU80$^+$, DNA-PK$^+$, BRCA1.n^- (all P<0.0001), BRCA1.c^- and BARD1.c^+ (both P=0.006). In terms of NHEJ markers, KU70/KU80 and DNA-PK were positively associated with each other (P<0.0001). DNA-PK$^+$ was highly associated with BARD1.c (P<0.0001), and however, BARD1nuclear and its cytoplasmic expression showed a negative association with each other (P<0.0001).

BRCA1 is combined with BARD 1 as they form a complex in the process of DNA repair. Thus the functional component should demonstrate double nuclear positivity while other combinations may not be fully functional [455]. Table 4.6b shows complex of BRCA1/BARD1 and their subcellular localisation. BARD1n^-c^+ and BRCA1n^+c^- were mainly expressed in the complex. In addition, Table 4.6c shows a high correlation between BRCA1.n^+/BARD1.c^+ complex and the apoptotic marker P53$^-$ (P<0.0001). Details of significant and non-significant associations are summarised in Appendix 2.

The high value of X^2 of all the results in this section can be referred to, i) a bias in the population of patients, ii) based on the data here, it does not seem to have any assumption issues, the main problem is normally when one of the expectation values is 5 or less, but in the results presented here a large chi squared value is resulted when the expectations are not less than 5. However, the data just seem to show that it is very likely that the association is not due to chance.

Table 4.6a Correlation between DNA -DSB Repair Markers.

SMC6L1							
Parameters		**c^-n^- N (%)**	**c^+n^+ N (%)**	**c^+n^- N (%)**	**c^-n^+ N (%)**	**X^2**	**P**
KU70/KU80	**Negative**	38(26.8)	41(8.9)	18(12.3)	18(15.3)	31	<0.0001
	Positive	104(73.2)	422(91.1)	128(87.7)	100(84.7)		
DNA-PK	**Negative**	66 (34.6)	34 (5.5)	48 (24)	23 (15.2)	114	<0.0001
	Positive	125 (65.4)	580 (94.5)	152 (76)	128 (84.8)		
BARD1.c	**Negative**	58(28.3)	114(17.5)	39(18.6)	40(23.4)	13	0.006
	Positive	147(71.7)	537(82.5)	171(81.4)	131(76.6)		
BRCA1.c	**Negative**	110 (61.8)	292 (51)	115 (57.8)	100 (64.1)	12.5	0.006
	Positive	68 (38.2)	280 (49)	84 (42.2)	56 (35.9)		
BRCA1.n	**Negative**	86 (48.3)	344 (59.9)	133 (66.8)	72 (46.2)	22.8	<0.0001
	Positive	92 (51.7)	230 (40.1)	66 (33.2)	84 (53.8)		
Rad51							
Parameters		**c^-n^- N (%)**	**c^+n^+ N (%)**	**c^+n^- N (%)**	**c^-n^+ N (%)**	**X^2**	**P**
KU70/KU80	**Negative**	24(36.9)	24(7.5)	58(9.7)	11(23.4)	55	<0.0001
	Positive	41(63.1)	294(92.5)	542(90.3)	36(76.6)		
DNA-PK	**Negative**	13(31)	24(10.5)	82(16.5)	1(4)	15	0.002
	Positive	29(69)	205(89.5)	414(83.5)	24(96)		
SMC6L1.c	**Negative**	26(55.3)	84(34.7)	131(26.4)	8(28.6)	19.5	<0.0001
	Positive	21(44.7)	158(65.3)	365(73.6)	20(71.4)		
BARD1.c	**Negative**	12(26.7)	23(9.5)	148(27.9)	3(8.8)	37	<0.0001
	Positive	33(73.3)	218(90.5)	383(72.1)	31(91.2)		
N= number of cases. c. = cytoplasmic, and n. = nuclear expression. . The cut off points of positivity are presented in the next tables.							

Table 4.6a Correlation between DNA -DSB Repair Markers Continued.

Markers		BRCA1					
		$c^- n^-$ N (%)	$c^+ n^+$ N (%)	$c^+ n^-$ N (%)	$c^- n^+$ N (%)	X^2	P
Rad51.c	Negative	20 (7.3)	16 (11.7)	11 (4.2)	47 (16.8)	27	<0.0001
	Positive	253 (92.7)	121 (88.3)	252 (95.8)	233 (83.2)		
Rad51.n	Negative	228 (83.2)	75 (54)	210 (79.5)	120 (42.6)	135.8	<0.0001
	Positive	46 (16.8)	64 (46)	54 (20.5)	162 (57.4)		
KU70/KU80	Negative	32 (10.1)	29 (18.6)	6 (2.3)	58 (18.4)	43.7	<0.0001
	Positive	284 (89.9)	127 (81.4)	259 (97.7)	258 (81.6)		
DNA-PK	Negative	68 (20.9)	22 (13.8)	22 (7.1)	37 (12.6)	25.7	<0.0001
	Positive	258 (79.1)	137 (86.2)	287 (92.9)	257 (87.4)		
BARD1.c	Negative	97 (28.5)	22 (11.9)	83 (25.3)	36 (11.1)	44	<0.0001
	Positive	243 (71.5)	163 (88.1)	245 (74.7)	287 (88.9)		

BARD1.c					
Markers		Negative N (%)	positive N (%)	X^2	P
BARD1.n	Negative	297(99.7)	1043(88.6)	35	<0.0001
	Positive	1(0.3)	134(11.4)		
Rad51.n	Negative	160(85.6)	417(62.2)	36	<0.0001
	Positive	27(14.4)	253(37.8)		

DNA-PK					
Markers		Negative N (%)	positive N (%)		
BARD1.c	Negative	67(36.2)	176(16.9)	37	<0.0001
	Positive	118(63.8)	864(83.1)		
KU70/KU80	Negative	37(28.2)	68(9.5)	35.5	<0.0001
	Positive	94(71.8)	645(90.5)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. The cut off points of positivity were as follows: ≥ 40 H-score for BRCA1.c, and ≥93 for BRCA1.n, : ≥ 130 H-score for BARD1.c and > 0 H-score for BARD1.n, ≥8 H-score for Rad51.n and ≥80H-score for Rad51.c, >240 H-score for SMC6L1.n, and ≥ 230 H-score for SMC6L1.c, ≥90 H-score for KU70/KU80 and, ≥ 150 H-score for DNA-PK.

Table 4.6b BRCA1/BARD1 and their Subcellular Localisation.

Markers		BRCA1/BARD1 complex any localisation		
		Negative N (%)	positive N (%)	P X^2
BARD1	BARD1n-c-	96 (100)	141(10.7)	<0.0001
	BARD1n+c+	0	134(10.2)	
	BARD1n-c+	0	1043(79.1)	512
	BARD1n+c-	0	1(0.1)	
BRCA1	BRCA1n-c-	96 (100)	244(16)	<0.0001
	BRCA1n+c+	0	283(18.6)	
	BRCA1n+c-	0	566(37.2)	384
	BRCA1n-c+	0	430(28.2)	

BRCA1 is combined with BARD 1 as they form a complex in the process of DNA repair. Thus the functional component should demonstrate double nuclear positivity while other combinations may not be fully functional. N= number of cases. c. = cytoplasmic, n. = nuclear expression. The cut off points of positivity; ≥ 40 H-score for BRCA1.c, and ≥93 for BRCA1.n, ≥ 130 H-score for BARD1.c and > 0 H-score for BARD1.n.

Table 4.6c Correlation between BRCA1/BARD1 Complex and the Apoptotic Marker P53.

BRCA1.n/BARD1.c	P53		
	Negative N (%)	positive N (%)	P X^2
BRCA1.n- BARD1.c-	72 (10.1)	107 (23.7)	<0.0001
BRCA1.n+ BARD1.c+	334 (46.8)	112 (24.8)	
BRCA1.n- BARD1.c+	261 (36.6)	222 (49.2)	89
BRCA1.n+ BARD1.c-	46 (6.5)	10(2.2)	

BARD1 location in the cell cytoplasm correlates with its apoptotic function. N= number of cases. c. = cytoplasmic, n. = nuclear expression. ≥ 40 H-score for BRCA1.c, and ≥93 for BRCA1.n, ≥ 130 H-score for BARD1.c and > 0 H-score for BARD1.n, and for P53; ≥5%.

4.6.3 Correlation of DNA-DSB Repair Markers with Other Tumour Biomarkers

The association between categorical (X^2) DNA-DSB repair markers (regarding subcellular localisation) and other tumour biomarkers are summarised in Table 4.7a-d.

Hormone receptors; ER^-, PgR^- and TN tumours showed high significant associations with Rad51.n^-.c^+ (all P<0.0001, Table 4.7a), BRCA1.n^-.c^+ (all P<0.0001, Table 4.7b), and BARD1.c^+ (all P<0.0001, Table 4.7c), but SMC6L1.n^+.c^+ was only significant with TN (P<0.0001, Table 4.7c) and KU70/KU80 with ER (P=0.005, Table 4.7d).

There was a highly significant association between the HR markers, but not the NHEJ markers, with at least one of the basal cytokeratins (CK5, CK14 and/or CK17) or BLBC (Tables4.7a-c).

There was a high significant association between negative PTEN and all HR repair markers; Rad51n^-.c^+ (P<0.0001, Table 4.7a), BRCA1n^+.c^- (P<0.0001, Table 4.7b), SMC6L1n^+.c^+ (P=0.001, Table 4.7c), and finally BARD1.c^+ (P=0.005, Table 4.7c). However, regarding NHEJ repair markers; only DNA-PK showed such an association with PTEN (P<0.0001, Table 4.7d).

Two markers that acted as down regulators for BRCA1 were investigated here: these were ID4 and MTA1 (data for these markers are saved on a web-based interface (Distiller; Slidepath Ltd, Dublin, Ireland))). All HR and NHEJ markers were significantly associated with at least one of BRCA1 down regulators (ID4 or MTA1), regardless of cellular localisation (Tables4.7a-d).

$P53^-$ was significantly associated with HR, but not NHEJ repair markers or SMC6L1, (for both Rad51 and BRCA1; P<0.0001, and for BARD1; P=0.0001, Tables 4.7a-c). There was a high significant association with all HR/NHEJ repair proteins with at least one of DNA-damage sensors and signal transducers proteins such as CHK1, CHK2, ATM, ATR, and γH2AX. For HR repair; Rad51 (CHK1.n, CHK1.c, CHK2, ATM, γH2AX.n; all P<0.0001, except γH2AX.c; P=0.001, Table 4.7a), BRCA1 (CHK1.n, CHK1.c, CHK2, ATM, ATR, γH2AX.n, and γH2AX.c; all P<0.0001, Table 4.7b), SMC6L1 (CHK1.c; P=0.001, γH2AX.n, γH2AX.c, ATR and CHK2; P<0.0001, Table 4.7c), BARD1.c (γH2AX.n; P<0.0001, γH2AX.c; P=0.001, Table 4.7c), for NHEJ repair markers, a high significant association was seen between KU70/KU80 and DNA-damage sensors and signal transducer such as (CHK1.c; P<0.0001, and ATR; P=0.007, Table 4.7d), in addition DNA-PK had a significant association with CHK1.c, CHK2, ATR, γH2AX.c and γH2AX.n; all P<0.0001, (Table 4.7d).

Cell cycle progression/arrest regulator marker $P27^-$ showed a significant association with Rad51, BRCA1, BARD1.c (all P<0.0001, Tables 4.7a-c), and KU70/KU80 (P=0.002,

Table 4.7d). Details of significant and non-significant associations are summarised in Appendix 2.

Table 4.8 shows the correlation on continuous data of DNA- DSB repair proteins with other tumour markers (Pearson's correlation). Some correlations between categorical and continuous data were different therefore, only these categorical correlations were re-analysed, but regardless co-expression of cellular localisation, in order to compare each cellular localisation separately. Therefore, Table 4.9 shows the association between categorical (X^2) DNA-DSB repair markers and other tumour biomarkers but regardless co-expression of subcellular localisation to make the comparison with continuous data (Pearson's correlation, Table 4.8) easier.

Table 4.8 is confirming the categorical data (Tables 4.7 and 4.9) in the area of statistical significant and direction of the correlation. However, when Rad51.n was analysed as a continuous variable, some correlations showed different results. A trend for significant correlation was observed with DNA-PK (P=0.031, continuous data, Table 4.8) while a significant correlation was found with categorical data (P=0.004, Table 4.9). Similarly was seen in the correlation between BRCA1.c and P27 (P=0.2, continuous data; Table 4.8 and P=0.005, categorical data; Table 4.9), PgR (P=0.37, continuous data; Table 4.8 and P=0.001, categorical data; Table 4.9) ER (P=0.759, continuous data; Table 4.8 and P<0.0001, categorical data; Table 4.9), Rad51.n where the correlation lost significance in continuous data(P=0.047, continuous data; Table 4.8 and P=0.009, categorical data; Table 4.9), but the correlation was changed to significant with SMC6L1.n (P=0.002, continuous data; Table 4.8 and P=0.06, categorical data; Table 4.9).

The high value of X^2 of all the results in this section can be referred to, i) a bias in the population of patients, ii) based on the data here, it does not seem to have any assumption issues, the main problem is normally when one of the expectation values is 5 or less, but in the results presented here a large chi squared value is resulted when the expectations are not less than 5. However, the data just seem to show that it is very likely that the association is not due to chance.

Table 4.7a Correlation between Rad51 with other Tumour Markers.

Markers		Rad51					
		$c^- n^-$ N (%)	$c^+ n^+$ N (%)	$c^+ n^-$ N (%)	$c^- n^+$ N (%)	X^2	P
ER	**Negative**	23(33.3)	81(24.4)	387(56.7)	5(9.4)	126	<0.0001
	Positive	46(66.7)	251(75.6)	295(43.3)	48(90.6)		
PgR	**Negative**	30(46.9)	127(38.7)	426(65.4)	6(12.5)	100	<0.0001
	Positive	34(53.1)	201(61.3)	225(34.6)	42(87.5)		
Triple Negative	**Negative**	52(78.8)	284(86.3)	397(60.5)	49(94.2)	88	<0.0001
	Positive	14(21.2)	45(13.7)	259(39.5)	3(5.8)		
CK5	**Negative**	46(86.8)	235(83.9)	376(67.5)	41(95.3)	42	<0.0001
	Positive	7(13.2)	45(16.1)	181(32.5)	2(4.7)		
CK17	**Negative**	51(94.4)	215(84.6)	463(79.3)	36(90)	11.5	0.009
	Positive	3(5.6)	39(15.4)	121(20.7)	4(10)		
BLBC	**Negative**	49(87.5)	278(88)	406(68.1)	45(93.8)	58	<0.0001
	Positive	7(12.5)	38(12)	190(31.9)	3(6.2)		
P53	**Negative**	47(75.8)	233(71)	370(55.1)	40(80)	37	<0.0001
	Positive	15(24.2)	95(29)	302(44.9)	10(20)		
ID4.c	**Negative**	41(57.7)	113(32.3)	236(34.3)	24(43.6)	19	<0.0001
	Positive	30(42.3)	237(67.7)	452(65.7)	31(56.4)		
PTEN	**Negative**	27 (69.2)	151 (76.3)	250 (89)	14 (53.8)	31	<0.0001
	Positive	12 (30.8)	47 (23.7)	31 (11)	12 (46.2)		
CHK1.n	**Negative**	50(73.5)	190(60.5)	518(85.6)	18(35.3)	114	<0.0001
	Positive	18(26.5)	124(39.5)	87(14.4)	33(64.7)		
CHK1.c	**Negative**	18(26.5)	21(6.7)	37(6.1)	15(29.4)	61	<0.0001
	Positive	50(73.5)	292(93.3)	568(93.9)	36(70.6)		
CHK2	**Negative**	20 (52.6)	88 (43.3)	191 (60.8)	8 (32)	20	<0.0001
	Positive	18 (47.4)	115 (56.7)	123 (39.2)	17 (68)		
Ki-67	**Negative**	32(55.2)	139(50)	149(24.1)	22(53.7)	77	<0.0001
	Positive	26(44.8)	139(50)	469(75.9)	19(46.3)		
ATM	**Negative**	20 (64.5)	98 (44.7)	311 (62.8)	13 (37.1)	26.5	<0.0001
	Positive	11 (35.5)	121 (55.3)	184 (37.2)	22 (62.9)		
γH2AX.n	**Negative**	4(11.1)	8(3.6)	93(19.2)	1(3.7)	34	<0.0001
	Positive	32(88.9)	217(96.4)	392(80.8)	26(96.3)		
γH2AX.c	**Negative**	8(22.2)	24(10.7)	37(7.6)	7(25.9)	17	0.001
	Positive	28(77.8)	201(89.3)	448(92.4)	20(74.1)		
P27	**Negative**	12(44.4)	34(28.6)	203(59.2)	8(34.8)	36	<0.0001
	Positive	15(55.6)	85(71.4)	140(40.8)	15(65.2)		
MTA1.n	**Negative**	19(46.3)	82(34)	250(48.2)	10(30.3)	16	0.001
	Positive	22(53.7)	159(66)	269(51.8)	23(69.7)		
MTA1.c	**Negative**	15(35.7)	26(10.8)	63(12.1)	9(27.3)	25	<0.0001
	Positive	27(64.3)	215(89.2)	456(87.9)	24(72.7)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. The cut off points of positivity were the same as previously published [273], and were as follows: ≥1% for ER, PgR, ≥1 H-score for PTEN, ≥75 for ATM, ≥10% for CK5, >34% for Ki-67, and ≥70 H-score for P27, ≥120 H-score for MTA1.c, ≥50 H-score for MTA1.n, ≥20 H-score for CHK1.n and ≥80 H-score for CHK1.c, ≥105 H-score for CHK2, ≥ 40 and ≥ 120 H-score for γH2AX nuclear and cytoplasmic respectively, ≥ 8 and ≥ 80 H-score for Rad51 nuclear and cytoplasmic respectively. ≥ 100 H-score for ID4.c. Triple negative (ER, PgR and HER-2). BLBC= Triple negative +positive expression of CK5 and CK14 and CK17

Table 4.7b Correlation between Co-expression of Cellular Localisation of BRCA1 with other Tumour Markers.

Markers		BRCA1					
		c^-n^- N (%)	c^+n^+ N (%)	c^-n^+ N (%)	c^+n^- N (%)	X^2	P
ER	**Negative**	253 (53.9)	68 (24.2)	76 (13.7)	270 (63.2)	325	<0.0001
	Positive	216 (46.1)	213 (75.8)	478 (86.3)	157 (36.8)		
PgR	**Negative**	301 (65.6)	96 (34.3)	155 (28)	269 (67.1)	225	<0.0001
	Positive	158 (34.4)	184 (65.7)	399 (72)	132 (32.9)		
Triple Negative	**Negative**	276 (60)	239 (84.8)	498 (90.2)	240 (60)	179.8	<0.0001
	Positive	184 (40)	43 (15.2)	54 (9.8)	160 (40)		
HER-2	**Negative**	391 (84.6)	237 (85.3)	515 (92.1)	307 (75.2)	52.6	<0.0001
	Positive	71 (15.4)	41 (14.7)	44 (7.9)	101 (24.8)		
CK5	**Negative**	247 (67.5)	194 (87.4)	363 (90.1)	190 (65.3)	95	<0.0001
	Positive	119 (32.5)	28 (12.6)	40 (9.9)	101 (34.7)		
CK17	**Negative**	292 (78.5)	171 (85.9)	319 (91.1)	265 (78.4)	28	<0.0001
	Positive	80 (21.5)	28 (14.1)	31 (8.9)	73 (21.6)		
CK14	**Negative**	396 (86.8)	249 (89.2)	498 (92.1)	330 (81.7)	24	<0.0001
	Positive	60 (13.2)	30 (10.8)	43 (7.9)	74 (18.3)		
BLBC	**Negative**	277 (67.7)	232 (88.5)	491 (94.6)	237 (67.7)	154	<0.0001
	Positive	132 (32.3)	30 (11.5)	28 (5.4)	113 (32.3)		
P53	**Negative**	277 (59.1)	185 (66.1)	449 (81.3)	204 (47.8)	126.9	<0.0001
	Positive	192 (40.9)	95 (33.9)	103 (18.7)	223 (52.2)		
ID4.n	**Negative**	354 (83.3)	194 (85.1)	419 (90.3)	308 (80.8)	16	0.0001
	Positive	71 (16.7)	34 (14.9)	45 (9.7)	73 (19.2)		
PTEN	**Negative**	186 (90.3)	126 (81.3)	198 (71.5)	102 (79.7)	26	<0.0001
	Positive	20 (9.7)	29 (18.7)	79 (28.5)	26 (20.3)		
CHK1.n	**Negative**	259 (80.9)	111 (67.3)	214 (65.6)	210 (78.1)	25.5	<0.0001
	Positive	61 (19.1)	54 (32.7)	112 (34.4)	59 (21.9)		
CHK1.c	**Negative**	32 (10)	12 (7.3)	53 (16.3)	7 (2.6)	33	<0.0001
	Positive	288 (90)	153 (92.7)	272 (83.7)	262 (97.4)		
CHK2	**Negative**	159 (62.6)	69 (40.8)	117 (41.5)	90 (58.8)	34.5	<0.0001
	Positive	95 (37.4)	100 (59.2)	165 (58.5)	63 (41.2)		
Ki-67	**Negative**	109 (26.2)	93 (41.7)	255 (57.7)	102 (25.6)	125	<0.0001
	Positive	307 (73.8)	130 (58.3)	187 (42.3)	297 (74.4)		
ATR	**Negative**	150 (55.8)	97 (51.1)	213 (57.6)	66 (38.8)	17.8	<0.0001
	Positive	119 (44.2)	93 (48.9)	157 (42.4)	104 (61.2)		
ATM	**Negative**	233 (71)	72 (38.3)	159 (47.5)	168 (55.3)	62.8	<0.0001
	Positive	95 (29)	116 (61.7)	176 (52.5)	136 (44.7)		
MTA1.n	**Negative**	159 (48.2)	64 (36.8)	108 (34.7)	126 (39.7)	13	0.004
	Positive	171 (51.8)	110 (63.2)	203 (65.3)	191 (60.3)		
MTA1.c	**Negative**	41 (12.4)	25 (14.3)	68 (21.8)	26 (8.2)	25	<0.0001
	Positive	289 (87.6)	150 (85.7)	244 (78.2)	291 (91.8)		
P27	**Negative**	103 (57.5)	21 (27.6)	45 (25.4)	131 (61.5)	70	<0.0001
	Positive	76 (42.5)	55 (72.4)	132 (74.6)	82 (38.5)		
γH2AX.n	**Negative**	74 (23.4)	7 (4.5)	17 (6.2)	40 (13.2)	51	<0.0001
	Positive	242 (76.6)	148 (95.5)	258 (93.8)	264 (86.8)		
γH2AX.c	**Negative**	33 (10.4)	20 (12.9)	46 (16.7)	15 (4.9)	21.6	<0.0001
	Positive	283 (89.6)	135 (87.1)	229 (83.3)	290 (95.1)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. The cut off points of positivity were the same as previously published [273], and were as follows: ≥1% for ER, PgR and CK17; 3+ of HercepTest for HER2; ≥10% for CK5 and CK14, >34% for Ki-67, ≥70 H-score for P27, and ≥5% for P53. PTEN; ≥1 H-score, .≥50 for MTA1.n and ≥120 H-score for cytoplasmic, ≥20 H-score for CHK1.n and ≥80 H-score for CHK1.c, ≥105 H-score for CHK2, ≥ 40 and ≥ 120 H-score for γH2AX nuclear and cytoplasmic respectively.≥75% for ATM, ≥18 H-score for ATR. ID4.n ≥12 H-score. ≥ 40 H-score for BRCA1.c, and ≥93 for BRCA1.n. Triple negative (ER, PgR and HER-2). BLBC= Triple negative +positive expression of CK5 and CK14 and CK17

Table 4.7c Correlation between HR Repair Markers with other Tumour Markers.

Markers		SMC6L1					
		$c^- n^-$ N (%)	$c^+ n^+$ N (%)	$c^+ n^-$ N (%)	$c^- n^+$ N (%)	X^2	P
TN	Negative	169(75.4)	498(74.2)	128(59)	132(72.1)	21	<0.0001
	Positive	55(24.6)	173(25.8)	89(41)	51(27.9)		
CK5	Negative	128(77.1)	397(75.5)	107(63.3)	114(83.8)	18	<0.0001
	Positive	38(22.9)	129(24.5)	62(36.7)	22(16.2)		
CK17	Negative	146(86.4)	422(80.2)	129(72.9)	113(86.9)	14	0.003
	Positive	23(13.6)	104(19.8)	48(27.1)	17(13.1)		
BLBC	Negative	160(82.5)	476(79.2)	127(62.6)	135(80.8)	30	<0.0001
	Positive	34(17.5)	125(20.8)	76(37.4)	32(19.2)		
PTEN	Negative	120 (81.6)	284 (79.8)	111 (91)	78 (70.3)	16	0.001
	Positive	27 (18.4)	72 (20.2)	11 (9)	33 (29.7)		
CHK1.c	Negative	27(18.4)	31(6.6)	16(10.5)	13(10.5)	18	0.001
	Positive	120(81.6)	436(93.4)	136(89.5)	111(89.5)		
CHK2	Negative	93 (60)	160 (40.2)	84 (64.6)	51 (47.7)	32.6	<0.0001
	Positive	62 (40)	238 (59.8)	46 (35.4)	56 (52.3)		
ATR	Negative	132 (67.7)	207 (42.2)	79 (49.1)	106 (73.6)	65	<0.0001
	Positive	63 (32.3)	283 (57.8)	82 (50.9)	38 (26.4)		
Ki-67	Negative	83(40.5)	194(31.3)	52(27.4)	73(45.6)	19	<0.0001
	Positive	122(59.5)	426(68.7)	138(72.6)	87(54.4)		
MTA1.n	Negative	126(58.1)	181(27.3)	150(68.8)	40(23.4)	170	<0.0001
	Positive	91(41.9)	482(72.7)	68(31.2)	131(76.6)		
MTA1.c	Negative	78(35.9)	34(5.1)	18(8.3)	59(34.5)	185	<0.0001
	Positive	139(64.1)	630(94.9)	200(91.7)	112(65.5)		
γH2AX.n	Negative	47(25.3)	28(4.4)	71(37.8)	6(3.7)	180	<0.0001
	Positive	139(74.7)	603(95.6)	117(62.2)	156(96.3)		
γH2AX.c	Negative	36(19.3)	29(4.6)	7(3.7)	44(27.2)	100	<0.0001
	Positive	151(80.7)	602(95.4)	181(96.3)	118(72.8)		

Markers		BARD1.c			
		Negative N (%)	Positive N (%)	X^2	P
ER	Negative	188(65.7)	437(38.6)	68	<0.0001
	Positive	98(34.3)	696(61.4)		
PgR	Negative	180(69)	552(50.7)	28	<0.0001
	Positive	81(31)	536(49.3)		
TN	Negative	137(52.7)	830(75.2)	52	<0.0001
	Positive	123(47.3)	273(24.8)		
CK5	Negative	132(60.3)	676(78.5)	31	<0.0001
	Positive	87(39.7)	185(21.5)		
BLBC	Negative	138(59.5)	799(80.3)	45	<0.0001
	Positive	94(40.5)	196(19.7)		
P53	Negative	149(53.4)	706(64.2)	11	0.0001
	Positive	130(46.6)	394(35.8)		
ID4.n	Negative	214(77)	882(85.9)	13	<0.0001
	Positive	64(23)	145(14.1)		
ID4.c	Negative	147(51.9)	389(33.4)	33	<0.0001
	Positive	136(48.1)	774(66.6)		
MTA1.n	Negative	141(54)	365(35.9)	28	<0.0001
	Positive	120(46)	651(64.1)		
MTA1.c	Negative	74(28.4)	111(10.9)	51	<0.0001
	Positive	187(71.6)	906(89.1)		
PTEN	Negative	87 (91.6)	558 (79.5)	8	0.005
	Positive	8 (8.4)	144 (20.5)		
P27	Negative	114(68.3)	170(39.8)	39	<0.0001
	Positive	53(31.7)	257(60.2)		
CHK2	Negative	62 (60.8)	333 (47.2)	6.6	0.01
	Positive	40 (39.2)	373 (52.8)		
γH2AX.n	Negative	59(24.5)	90(9.9)	35.5	<0.0001
	Positive	182(75.5)	815(90.1)		
γH2AX.c	Negative	38(15.8)	76(8.4)	11.6	0.001
	Positive	203(84.2)	830(91.6)		

N= number of cases. c. = cytoplasmic and n. = nuclear expression. Co-expression of cellular localisation of SMC6L1 is considered. BARD1 shows very few cases of nuclear expression 5.3% so, only cytoplasmic was considered. The cut off points of positivity were the same as Tables 4.7a-b. >240 H-score for SMC6L1.n, and ≥ 230 H-score for SMC6L1.c, ≥ 130 H-score for BARD1.c .Triple negative (ER, PgR and HER-2). BLBC= Triple negative +positive expression of CK5 and CK14 and CK17

Table 4.7d Correlation between NHEJ Repair Markers with other Tumour Markers.

Markers		KU70/KU80			
		Negative N (%)	**Positive N (%)**	X^2	**P**
ER	**Negative**	51(32.7)	489(44.6)	8	0.005
	Positive	105(67.3)	607(55.4)		
P53	**Negative**	114(74.5)	653(60.9)	11	0.001
	Positive	39(25.5)	420(39.1)		
ID4.c	**Negative**	82(48)	402(36.2)	9	0.003
	Positive	89(52)	709(63.8)		
CHK1.c	**Negative**	40(26)	83(8.1)	45	<0.0001
	Positive	114(74)	936(91.9)		
ATR	**Negative**	84 (65.1)	325 (52.2)	7	0.007
	Positive	45 (34.9)	298 (47.8)		
MTA1.c	**Negative**	37(33)	88(11.3)	38	<0.0001
	Positive	75(67)	689(88.7)		
P27	**Negative**	24(32)	233(51.2)	9.5	0.002
	Positive	51(68)	222(48.8)		
Markers		**DNA-PK**			
		Negative N (%)	**Positive N (%)**	X^2	**P**
PTEN	**Negative**	124 (93.9)	469 (78.7)	16.6	<0.0001
	Positive	8 (6.1)	127 (21.3)		
CHK1.c	**Negative**	26(20.2)	53(7.2)	22	<0.0001
	Positive	103(79.8)	685(92.8)		
CHK2	**Negative**	104 (77.6)	274 (43.9)	50	<0.0001
	Positive	30 (22.4)	350 (56.1)		
ATR	**Negative**	106 (67.9)	360 (45.7)	25.8	<0.0001
	Positive	50 (32.1)	428 (54.3)		
MTA1.n	**Negative**	129(69)	340(33.8)	82	<0.0001
	Positive	58(31)	667(66.2)		
MTA1.c	**Negative**	73(39)	88(8.7)	124	<0.0001
	Positive	114(61)	920(91.3)		
γH2AX .n	**Negative**	53(32.9)	92(9.8)	64	<0.0001
	Positive	108(67.1)	843(90.2)		
γH2AX .c	**Negative**	29(18)	76(8.1)	15.5	<0.0001
	Positive	132(82)	860(91.9)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. The cut off points of positivity were the same as previously published [273], and were as follows: ≥90 for KU70/KU80, ≥150 for DNA-PK, ≥1% for ER, ≥70 H-score for P27, and ≥5% for P53. PTEN; ≥1 H-score, .≥50 for MTA1.n and ≥120 H-score for cytoplasmic, ≥80 H-score for CHK1.c, ≥105 H-score for CHK2, ≥ 40 and ≥ 120 H-score for γH2AX nuclear and cytoplasmic respectively. ≥18 H-score for ATR. ID4.n ≥12 and ID4.c ≥100 H-score. Triple negative (ER, PgR and HER-2). NHEJ repair markers only expressed in the nucleus with no cytoplasmic expression.

Table 4.8 Pearson's Correlations between DNA-DSB Repair Markers with other Tumour Markers.

Markers		BRCA1 .c	BRCA1 .n	BARD1 .n	BARD1 .c	Rad51 .c	Rad51 .n	SMC6L1 .c	SMC6L1 .n	KU70/ KU80	DNA/PK
BRCA1.c	**R**	*	-0.129	-0.009	0.009	0.174	-0.064	0.090	0.093	0.119	0.140
	P		<0.0001	0.764	0.748	<0.0001	0.047	0.003	0.002	<0.0001	<0.0001
	N		1756	1178	1178	955	955	1106	1106	1055	1090
BRCA1.n	**R**	-0.129	*	0.044	0.218	-0.088	0.449	-0.122	0.002	-0.108	0.070
	P	<0.0001		0.129	<0.0001	0.007	<0.0001	<0.0001	0.953	<0.0001	0.020
	N	1756		1178	1178	955	955	1106	1106	1055	1090
BARD1.n	**R**	-0.009	0.044	*	0.169	0.052	-0.024	0.037	0.051	0.011	0.054
	P	0.764	0.129		<0.0001	0.132	0.493	0.195	0.072	0.734	0.061
	N	1178	1178		1477	851	851	1237	1237	918	1225
BARD1.c	**R**	*				0.085	0.131	0.212	0.201	0.063	0.280
	P					0.014	<0.0001	<0.0001	<0.0001	0.058	<0.0001
	N					851	851	1237	1237	918	1225
Rad51.c	**R**					*	0.031	0.119	-0.027	0.164	0.021
	P						0.283	0.001	0.436	<0.0001	0.551
	N						1178	813	813	1030	792
Rad51.n	**R**						*			0.159	0.077
	P									<0.0001	0.031
	N									1030	792
SMC6L1.c	**R**						-0.084	*	0.416	0.143	0.208
	P						0.016		<0.0001	<0.0001	<0.0001
	N						813		1387	869	1156
SMC6L1.n	**R**						0.031	*		0.280	0.504
	P						0.382			<0.0001	<0.0001
	N						813			869	1156
P53	**R**	0.099	-0.172	0.044	0.059	0.111	-0.113	0.037	0.013	0.004	0.043
	P	<0.0001	<0.0001	0.149	0.054	0.001	0.001	0.228	0.685	0.904	0.175
	N	1433	1433	1088	1088	865	865	1042	1042	978	999
PgR	**R**	0.024	0.251	-0.004	0.021	-0.081	0.192	0.009	0.052	0.080	0.037
	P	0.372	<0.0001	0.906	0.481	0.017	<0.0001	0.771	0.094	0.013	0.239
	N	1433	1433	1085	1085	865	865	1039	1039	971	999
ER	**R**	-0.008	0.300	-0.021	-0.035	-0.109	0.231	-0.048	0.090	0.119	0.048
	P	0.759	<0.0001	0.482	0.244	0.001	<0.0001	0.123	0.003	<0.0001	0.130
	N	1440	1440	1103	1103	875	875	1052	1052	982	1015
Ki-67	**R**	0.049	-0.279	0.007	-0.008	0.175	-0.226	0.132	-0.052	-0.095	0.045
	P	0.061	<0.0001	0.815	0.768	<0.0001	<0.0001	<0.0001	0.077	0.002	0.131
	N	1483	1483	1248	1248	995	995	1175	1175	1075	1144
CHK1.c	**R**	0.240	-0.052	0.019	0.202	0.300	0.011	0.125	0.194	0.161	0.205
	P	<0.0001	0.090	0.558	<0.0001	<0.0001	0.722	<0.0001	<0.0001	<0.0001	<0.0001
	N	1081	1081	939	939	1037	1037	890	890	1173	867
CHK1.n	**R**	0.010	0.352	-0.007	0.071	-0.150	0.457	-0.135	-0.004	-0.039	0.078
	P	0.747	<0.0001	0.841	0.030	<0.0001	<0.0001	<0.0001	0.900	0.181	0.021
	N	1081	1081	939	939	1037	1037	890	890	1173	867
CHK2	**R**	0.013	0.317	0.116	0.124	-0.081	0.208	0.129	0.256	0.027	0.340
	P	0.701	<0.0001	0.001	<0.0001	0.051	<0.0001	<0.0001	<0.0001	0.485	<0.0001
	N	858	858	808	808	580	580	790	790	650	758
ATR	**R**	0.091	-0.085	-0.032	0.055	-0.011	-0.042	0.235	0.098	0.076	0.202
	P	0.004	0.007	0.304	0.078	0.783	0.272	<0.0001	0.002	0.037	<0.0001
	N	1000	1000	1011	1011	673	673	991	991	753	946
ATM	**R**	0.119	0.234	0.016	0.038	0.022	0.157	-0.028	0.044	0.026	0.051
	P	<0.0001	<0.0001	0.616	0.250	0.535	<0.0001	0.394	0.187	0.446	0.134
	N	1158	1158	940	940	780	780	906	906	863	871

*Repeated analysis or analysis between the marker itself. N= number of cases. R= Pearson's correlation, P=Probability value. This table shows the correlation on continuous data of DNA- DSB repair proteins with other tumour markers. The cut off points of positivity were the same as Tables 4.7.

Table 4.8 Pearson's Correlations between DNA-DSB Repair Markers with other Tumour Markers Continued.

Markers		BRCA1 .c	BRCA1 .n	BARD1 .n	BARD1 .c	Rad51 .c	Rad51 .n	SMC6L1 .c	SMC6L1 .n	KU70/ KU80	DNA/PK
P27	R	-0.049	0.251	-0.043	0.181	0.044	0.187	-0.066	-0.030	-0.058	0.022
	P	0.215	<0.0001	0.293	<0.0001	0.322	<0.0001	0.125	0.489	0.179	0.607
	N	647	647	594	594	512	512	538	538	530	527
γH2AX.c	R	0.103	-0.033	-0.020	0.250	0.224	0.027	0.391	0.202	0.072	0.167
	P	0.001	0.281	0.500	<0.0001	<0.0001	0.459	<0.0001	<0.0001	0.039	<0.0001
	N	1052	1052	1146	1146	773	773	1167	1167	818	1096
γH2AX.n	R	0.018	0.293	0.068	0.284	-0.038	0.263	0.042	0.439	0.173	0.376
	P	0.561	<0.0001	0.021	<0.0001	0.298	<0.0001	0.151	<0.0001	<0.0001	<0.0001
	N	1052	1052	1146	1146	773	773	1167	1167	818	1096
MTA1.n	R	0.016	0.187	0.085	0.280	-0.061	0.132	0.095	0.388	0.146	0.366
	P	0.600	<0.0001	0.002	<0.0001	0.076	<0.0001	0.001	<0.0001	<0.0001	<0.0001
	N	1135	1135	1277	1277	834	834	1269	1269	888	1194
MTA1.c	R	0.093	-0.069	0.061	0.415	0.209	-0.016	0.454	0.308	0.161	0.428
	P	0.002	0.021	0.030	<0.0001	<0.0001	0.644	<0.0001	<0.0001	<0.0001	<0.0001
	N	1135	1135	1277	1277	834	834	1269	1269	888	1194
ID4.n	R	0.073	-0.063	0	-0.099	-0.059	-0.068	-0.036	-0.005	-0.034	-0.052
	P	0.005	0.015	0.994	<0.0001	0.055	0.026	0.217	0.866	0.241	0.072
	N	1501	1501	1305	1305	1074	1074	1209	1209	1170	1181
ID4.c	R	0.074	-0.003	0.061	0.336	0.145	0.017	0.116	0.087	0.039	0.153
	P	0.004	0.910	0.030	<0.0001	<0.0001	0.587	<0.0001	0.003	0.189	<0.0001
	N	1496	1496	1274	1274	1060	1060	1185	1185	1151	1159
CK17	R	0.067	-0.137	0.017	-0.024	0.071	-0.070	0.072	0.013	0.028	0.025
	P	0.018	<0.0001	0.583	0.433	0.030	0.034	0.023	0.680	0.365	0.427
	N	1261	1261	1073	1073	932	932	1002	1002	1026	983
CK5	R	-0.004	-0.174	0.008	0.003	0.134	-0.128	0.020	-0.116	-0.042	-0.029
	P	0.887	<0.0001	0.801	0.926	<0.0001	<0.0001	0.563	0.001	0.224	0.409
	N	1081	1081	882	882	756	756	817	817	848	803
PTEN	R	-0.035	0.214	0.150	0.094	-0.195	0.158	-0.134	0.104	0.054	0.161
	P	0.332	<0.0001	<0.0001	0.008	<.0001	<0.0001	<0.0001	0.005	0.184	<0.0001
	N	766	766	797	797	544	544	736	736	606	728

* Repeated analysis or analysis between the marker itself. N= number of cases. R= Pearson's correlation, P=Probability value. This table shows the correlation on continuous data of DNA- DSB repair proteins with other tumour markers. The cut off points of positivity were the same as Tables 4.7.

Table 4.9 Correlation between HR Repair Proteins with other Tumour Markers Regardless Co-expression of Cellular Localisation

Markers		BRCA1.n			BRCA1.c		
		Negative N (%)	**Positive N (%)**	**P X^2**	**Negative N (%)**	**Positive N (%)**	**P X^2**
ER	**Negative**	526(58.5)	144 (17.2)	<0.0001 312	329(32.2)	338(47.7)	<0.0001 43
	Positive	373(41.5)	693 (82.8)		694(67.8)	370(52.3)	
PgR	**Negative**	572(66.4)	252 (30.1)	<0.0001 223	456(45)	365(53.6)	0.001 12
	Positive	290(33.6)	584 (69.9)		557 (55)	316(46.4)	
CK5	**Negative**	438(66.5)	559 (89.2)	<0.0001 95	610(79.3)	384(74.9)	0.06 3
	Positive	221(33.5)	68 (10.8)		159(20.7)	129(25.1)	
P27	**Negative**	235(59.6)	66(26.1)	<0.0001 70	148(41.6)	152(52.6)	0.005 7.9
	Positive	159(40.4)	187 (73.9)		20 (58.4)	137(47.4)	
SMC6L1.n	**Negative**	219(34.5)	158 (33.5)	0.7 0.1	225(36.5)	152(31.1)	0.06 3
	Positive	416(65.5)	314 (66.5)		392(63.5)	336(68.9)	
MTA1.n	**Negative**	286(44.1)	172 (35.4)	0.003 8.6	267(41.7)	190(38.7)	0.3 1
	Positive	363(55.9)	314 (64.6)		374(58.3)	301(61.3)	
CHK1.c	**Negative**	40 (6.8)	65 (13.2)	<0.0001 12.7	85 (13.2)	19 (4.4)	<0.0001 23
	Positive	551(93.2)	427 (86.8)		560(86.8)	415(95.6)	
CHK1.n	**Negative**	470(79.5)	326 (66.1)	<0.0001 24.7	473(73.2)	321(74)	0.8 0.07
	Positive	121(20.5)	167 (33.9)		173(26.8)	113 (26)	
CHK2	**Negative**	249(61.2)	186 (41.2)	<0.0001 34	276(51.5)	159(49.4)	0.5 0.4
	Positive	158(38.8)	266 (58.8)		260(48.5)	163(50.6)	
γH2AX.n	**Negative**	114(18.3)	24 (5.6)	<0.0001 36	91(15.4)	47(10.2)	0.014 6
	Positive	508 (81.7)	407(94.4)		500(84.6)	412(89.8)	
γH2AX.c	**Negative**	49 (7.9)	66(15.3)	<0.0001 14.5	79(13.4)	35(7.6)	0.003 9
	Positive	574(92.1)	365 (84.7)		512 (86.6)	425(92.4)	
Rad51.n	**Negative**	440(81.5)	195(46.2)	<0.0001 131	348(62.6)	285(70.7)	0.009 6.9
	Positive	100(18.5)	227(53.8)		208 (37.4)	118(29.3)	
BARD1.c	**Negative**	181 (27)	58(11.4)	<0.0001 43.8	133 (20.1)	105(20.5)	0.8 0.03
	Positive	489 (73)	452 (88.6)		530(79.9)	408(79.5)	
Markers		**Rad51.n**			**Rad51.c**		
		Negative N (%)	**Positive N (%)**	**P X^2**	**Negative N (%)**	**Positive N (%)**	**P X^2**
CK17	**Negative**	514(80.4)	255 (85.3)	0.07 3	87 (92.6)	678(80.9)	0.005 7.8
	Positive	125(19.6)	44(14.7)		7 (7.4)	160(19.1)	
P27	**Negative**	216(58.2)	42 (29.6)	<0.0001 33.7	20 (40)	237(51.3)	0.1 2
	Positive	155(41.8)	100 (70.4)		30 (60)	225(48.7)	
SMC6L1.n	**Negative**	188(34.6)	83 (30.3)	0.2 1.5	22 (29.3)	246(33.3)	0.5 0.5
	Positive	356(65.4)	191 (69.7)		53 (70.7)	492(66.7)	

N= number of cases. c. = cytoplasmic, n. = nuclear expression. This table was created to make the comparison between the continuous and categorical data much easier, since this table does not consider co-expression of subcellular localisation, leading to compare each cellular localisation separately. The cut off points of positivity were the same as Tables 4.7.

Table 4.9 Correlation between HR Repair Proteins with other Tumour Markers Regardless Co-expression of Cellular Localisation Continued.

Markers		Rad51.n			Rad51.c		
		Negative N (%)	**Positive N (%)**	**P** X^2	**Negative N (%)**	**Positive N (%)**	**P** X^2
ID4.c	**Negative**	277(36.4)	139 (33.9)	0.4 0.7	65 (51.6)	349(33.6)	<0.0001 16
	Positive	483(63.6)	271 (66.1)		61 (48.4)	689(66.4)	
MTA1.n	**Negative**	269 (48)	93 (33.5)	<0.0001 16	29 (39.2)	332(43.7)	0.4 0.5
	Positive	292 (52)	185 (66.5)		45 (60.8)	428(56.3)	
CHK1.c	**Negative**	56 (8.3)	37 (10.1)	0.3 0.9	33 (27.7)	58 (6.3)	<0.0001 60
	Positive	618(91.7)	331 (89.9)		86 (72.3)	860(93.7)	
CHK2	**Negative**	212(60.1)	97 (41.8)	<0.0001 18.7	28 (44.4)	279 (54)	0.1 2
	Positive	141(39.9)	135 (58.2)		35 (55.6)	238 (46)	
ATM	**Negative**	332 (63)	112 (43.8)	<0.0001 26	33 (50)	409(57.3)	0.2 1.3
	Positive	195 (37)	144 (56.2)		33 (50)	305(42.7)	
MTA1.c	**Negative**	78 (13.9)	36 (12.9)	0.7 0.1	24 (32)	89 (11.7)	<0.0001 24
	Positive	484(86.1)	242 (87.1)		51 (68)	671(88.3)	
γH2AX.n	**Negative**	97 (18.6)	9 (3.5)	<0.0001 33	5 (7.9)	101(14.2)	0.2 2
	Positive	425(81.4)	248 (96.5)		58 (92.1)	609(85.8)	
γH2AX.c	**Negative**	45 (8.6)	33 (12.8)	0.06 3	15 (23.8)	61(8.6)	<0.0001 15
	Positive	477(91.4)	224 (87.2)		48 (76.2)	649(91.4)	
BRCA1.c	**Negative**	348 (55)	208 (63.8)	0.009 7	67 (71.3)	486(56.6)	0.006 7.5
	Positive	285 (45)	118 (36.2)		27 (28.7)	373(43.4)	
DNA-PK	**Negative**	95 (17.6)	25 (9.8)	0.004 8	14 (20.9)	106(14.6)	0.2 1.8
	Positive	444 (82.4)	231 (90.2)		53 (79.1)	619(85.4)	
Markers		**SMC6L1.n**			**SMC6L1.c**		
		Negative N (%)	**Positive N (%)**	**P** X^2	**Negative N (%)**	**Positive N (%)**	**P** X^2
CK5	**Negative**	235 (70.1)	511 (77.2)	0.01 5.8	242 (80.1)	504 (72.5)	0.01 6.5
	Positive	100 (29.9)	151 (22.8)		60 (19.9)	191 (27.5)	
CK17	**Negative**	275 (79.5)	535 (81.6)	0.4 0.6	259 (86.6)	551 (78.4)	0.002 9
	Positive	71 (20.5)	121 (18.4)		40 (13.4)	152 (21.6)	
γH2AX.n	**Negative**	118 (31.6)	34 (4.3)	<0.0001 166	53 (15.2)	99 (12.1)	0.1 2
	Positive	256 (68.4)	759(95.7)		295 (84.8)	720 (87.9)	
BRCA1.n	**Negative**	219 (58.1)	416 (57)	0.7 0.12	158 (47.3)	477 (61.7)	<0.0001 20
	Positive	158 (41.9)	314(43)		176 (52.7)	296 (38.3)	
Ki-67	**Negative**	135 (34.2)	267 (34.2)	0.098 0	156 (42.7)	246 (30.4)	<0.0001 17
	Positive	260 (65.8)	513 (65.8)		209 (57.3)	564 (69.6)	

N= number of cases. c. = cytoplasmic, n. = nuclear expression. This table was created to make the comparison between the continuous and categorical data much easier, since this table does not consider co-expression of subcellular localisation, leading to compare each cellular localisation separately. The cut off points of positivity were the same as Tables 4.7.

4.6.4 Correlation of DNA-DSB Repair Markers with Clinico-Pathological Features

Tables 4.10a to 4.10c summarise the association between DNA-DSB repair markers (BRCA1, BARD1, Rad51, SMC6L1, KU70/KU80 and DNA-PK) and the various clinico-pathological features (Pearson X^2). HR repair markers showed a significant association with poor features such as higher tumour grade (grade III), higher nuclear pleomorphism, and higher mitotic frequency, for HR repair markers (Tables 4.10a-c): BRCA1.n^-.c^+ (all P<0.0001), Rad51.n^-.c^+ (all P<0.0001), BARD1.c^+ (P<0.0001, 0.007, and <0.0001 respectively) and SMC6L1n^+.c^+ (P=0.001, P<0.0001 and P<0.0001 respectively).

In terms of NHEJ markers (Table 4.10c): KU70/KU80$^+$ was highly associated with higher tubular formation (P=0.008) and vascular invasion (P<0.0001). DNA-PK showed no significant association with any of clinico-pathological features. Details of significant and non-significant associations are summarised in Appendix 2.

As previously discussed, the high value of X^2 can be referred to, i) a bias in the population of patients, ii) based on the data here, it does not seem to have any assumption issues, the main problem is normally when one of the expectation values is 5 or less, but in the results presented here a large chi squared value is resulted when the expectations are not less than 5. However, the data just seem to show that it is very likely that the association is not due to chance.

Table 4.10a Relationship between Rad51 with Clinico-Pathological Parameters

Parameters		Rad51					
		$c^- n^-$ N (%)	$c^+ n^+$ N (%)	$c^+ n^-$ N (%)	$c^- n^+$ N (%)	X^2	P
Size	**≤ 1.5cm**	30(41.7)	138(39.8)	177(25.7)	19(34.5)	25.5	<0.0001
	>1.5cm	42(58.3)	209(60.2)	512(74.3)	36(65.5)		
Grade	**1**	14(19.4)	67(19.3)	47(6.7)	9(16.4)	140	<0.0001
	2	31(43.1)	129(37.1)	139(19.9)	32(58.2)		
	3	27(37.5)	152(43.7)	513(73.4)	14(25.5)		
Tubules	**1**	3(4.5)	21(6.2)	14(2)	1(1.9)	44.5	<0.0001
	2	29(43.3)	117(34.6)	160(23.4)	24(44.4)		
	3	35(52.2)	200(59.2)	509(74.5)	29(53.7)		
Pleomorphism	**1**	1(1.5)	9(2.7)	1(0.1)	0	104.5	<0.0001
	2	32(47.8)	142(42.1)	139(20.4)	32(59.3)		
	3	34(50.7)	186(55.2)	541(79.4)	22(40.7)		
Mitosis	**1**	28(41.8)	135(39.9)	117(17.1)	25(46.3)	104	<0.0001
	2	16(23.9)	65(19.2)	120(17.6)	16(29.6)		
	3	23(34.3)	138(40.8)	446(65.3)	13(24.1)		
NPI	**Excellent**	9(12.5)	45(13.1)	28(4)	7(12.7)	111	<0.0001
	Good	22(30.6)	80(23.3)	61(8.8)	14(25.5)		
	Moderate1	16(22.2)	94(27.3)	250(36)	17(30.9)		
	Moderae2	16(22.2)	83(24.1)	205(29.5)	16(29.1)		
	Poor	6(8.3)	33(9.6)	109(15.7)	1(1.8)		
	Very poor	3(4.2)	9(2.6)	41(5.9)	0		
Tumour Type	**Invasive Ductal/NST**	35 (49.3)	188 (54.7)	535 (77.3)	17 (31.5)	137	<0.0001
	lobular	8 (11.3)	24 (7)	20 (2.9)	13 (24.1)		
	Atypical Medullary	1 (1.4)	5 (1.5)	21 (3)	0		
	Mixed$^+$	21 (29.6)	109 (31.7)	98 (14.2)	22 (40.7)		
	Other^{++}	6 (8.5)	18 (5.2)	18 (2.6)	2 (3.7)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. $^+$ Lobular or tubular mixed BCs. $^{++}$ Mucinous, Alveolar Lobular, Miscellaneous including Metaplastic, Adenoid Cystic, Spindle, and Tubulolobular. NST= No Special Type. NPI= Nottingham Prognostic Index. Excellent NPI (2.08–2.4), good NPI (2.42 to ⩽3.4), a moderate prognostic I NPI (3.42 to ⩽4.4), moderate prognostic II NPI (4.42 to ⩽5.4), poor NPI (5.42 to ⩽6.4), and a very poor NPI (6.5–6.8). The cut-off was as follows; ≥8 H-score for Rad51.n and ≥80H-score for Rad51.c

Table 4.10b Relationship between BRCA1 with Clinico-Pathological Parameters

Parameters		BRCA1					
		$c^- n^-$ N (%)	$c^+ n^+$ N (%)	$c^- n^+$ N (%)	$c^+ n^-$ N (%)	X^2	P
Size	**≤ 1.5cm**	128 (27.1)	88 (31.1)	229 (40.5)	108 (25.9)	31	<0.0001
	>1.5cm	345 (72.9)	195 (68.9)	337 (59.5)	309 (74.1)		
Grade	**1**	41 (8.6)	57 (20.1)	128 (22.6)	24 (5.6)	285	<0.0001
	2	91 (19.2)	93 (32.9)	255 (45.1)	66 (15.3)		
	3	342 (72.2)	133 (47)	183 (32.3)	340 (79.1)		
Tubules	**1**	18 (3.9)	16 (5.8)	38 (6.9)	12 (2.8)	51	<0.0001
	2	108 (23.5)	105 (37.9)	198 (35.8)	99 (23.4)		
	3	334 (72.6)	156(56.3)	317 (57.3)	312 (73.8)		
Pleomorphism	**1**	3 (0.7)	9 (3.2)	14 (2.5)	2 (0.5)	319	<0.0001
	2	97 (21.1)	99 (35.7)	322 (58.4)	42 (10)		
	3	359 (78.2)	169 (61)	215 (39)	378 (89.6)		
Mitosis	**1**	82 (17.8)	103 (37.2)	276 (49.9)	58 (13.7)	228.6	<0.0001
	2	78 (17)	57 (20.6)	106 (19.2)	76 (18)		
	3	300 (65.2)	117 (42.2)	171 (30.9)	289 (68.3)		
NPI	**Excellent**	28 (6)	37 (13.2)	93 (16.6)	16 (3.8)	148	<0.0001
	Good	50 (10.7)	62 (22.1)	131 (23.4)	38 (9)		
	Moderate1	151 (32.2)	75 (26.8)	171 (30.5)	146 (34.5)		
	Moderae2	137 (29.2)	69 (24.6)	104 (18.6)	134 (31.7)		
	Poor	76 (16.2)	26 (9.3)	50 (8.9)	63 (14.9)		
	Very poor	27 (5.8)	11 (3.9)	11 (2)	26 (6.1)		
Tumour Type	**Invasive Ductal/NST**	355 (75.4)	167 (60.1)	236 (42.1)	353 (83.3)	286	<0.0001
	lobular	10 (2.1)	7 (2.5)	87 (15.5)	6 (1.4)		
	Atypical Medullary	18 (3.8)	8 (2.9)	6 (1.1)	10 (2.4)		
	Mixed$^+$	70 (14.9)	80 (28.8)	195 (34.8)	46 (10.8)		
	Other^{++}	18 (3.8)	16 (5.8)	36 (6.4)	9 (2.1)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. $^+$Lobular or tubular mixed BCs. $^{++}$ Mucinous, Alveolar Lobular, Miscellaneous Including Metaplastic, Adenoid Cystic, Spindle, and Tubulolobular. NST= No Special Type. NPI= Nottingham Prognostic Index. Excellent NPI (2.08–2.4), good NPI (2.42 to ⩽3.4), a moderate prognostic I NPI (3.42 to ⩽4.4), moderate prognostic II NPI (4.42 to ⩽5.4), poor NPI (5.42 to ⩽6.4), and a very poor NPI (6.5–6.8). The cut-off was as follows; ≥ 40 H-score for BRCA1.c, and ≥93 for BRCA1.n.

Table 4.10c Relationship between DNA-DSB Repair Markers with Clinico-Pathological Parameters.

BARD1					
Parameters		**Negative N (%)**	**Positive N (%)**	X^2	**P**
Grade	**1**	27(9.1)	148(12.6)	28	<0.0001
	2	50(16.8)	350(29.8)		
	3	220(74.1)	676(57.6)		
Tubules	**1**	12(4.1)	45(3.9)	33	<0.0001
	2	43(14.8)	366(31.9)		
	3	235(81)	738(64.2)		
Pleomorphism	**1**	5(1.7)	16(1.4)	10	0.007
	2	61(21)	348(30.4)		
	3	224(77.2)	780(68.2)		
Mitosis	**1**	57(19.7)	328(28.5)	16	<0.0001
	2	43(14.8)	215(18.7)		
	3	190(65.5)	606(52.7)		
Tumour Type	**Invasive Ductal/NST**	211 (72.5)	782 (67.4)	22	<0.0001
	lobular	19 (6.5)	50 (4.3)		
	Atypical Medullary	7 (2.4)	29 (2.5)		
	Mixed^{+}	36 (12.4)	265 (22.8)		
	Other^{++}	18 (6.2)	34 (2.9)		

SMC6L1							
Parameters		**c^-n^- N (%)**	**c^+n^+ N (%)**	**c^+n^- N (%)**	**c^-n^+ N (%)**	X^2	**P**
Stage	**1**	161(65.4)	384(53.9)	147(65.9)	129(65.8)	24	0.001
	2	68(27.6)	240(33.7)	61(27.4)	61(27.4)		
	3	17(6.9)	89(12.5)	15(6.7)	15(6.7)		
Grade	**1**	34(13.8)	70(9.8)	21(9.3)	26(13.3)	22	0.001
	2	72(29.3)	189(26.5)	40(17.8)	65(33.2)		
	3	140(56.9)	455(63.7)	164(72.9)	105(53.6)		
Pleomorphism	**1**	8(3.4)	6(0.8)	1(0.5)	4(2.1)	44	<0.0001
	2	83(35.2)	169(23.9)	40(18.5)	72(38.3)		
	3	145(61.4)	531(75.2)	175(81)	112(59.6)		
Mitosis	**1**	61(25.7)	172(24.3)	38(17.5)	68(36.2)	27	<0.0001
	2	48(20.3)	138(19.5)	30(13.8)	27(14.4)		
	3	128(54)	398(56.2)	149(68.7)	93(49.5)		
Vascular Invasion	**Negative**	126(66)	241(51.6)	109(66.9)	93(66)	21	<0.0001
	Positive	65(34)	226(48.4)	54(33.1)	48(34)		
Tumour Type	**Invasive Ductal/NST**	160 (66.7)	506 (71.8)	165 (75.3)	111 (57.2)	49	<0.0001
	lobular	14 (5.8)	22 (3.1)	2 (0.9)	19 (9.8)		
	Atypical Medullary	11 (4.6)	13 (1.8)	9 (4.1)	3 (1.5)		
	Mixed^{+}	45 (18.8)	151(21.4)	37 (16.9)	52 (26.8)		
	Other^{++}	10 (4.2)	13 (1.8)	6 (2.7)	9 (4.6)		

KU70/KU80					
Parameters		**Negative N (%)**	**Positive N (%)**	X^2	**P**
Grade	**1**	26(15.5)	139(12.4)	8	0.01
	2	60(35.7)	30(27.1)		
	3	82(48.8)	681(60.5)		
Tubules	**1**	6(3.7)	44(4)	9.5	0.008
	2	63(38.7)	295(26.9)		
	3	94(57.7)	756(69)		
Vascular Invasion	**Negative**	168(100)	935(83.7)	32	<0.0001
	Positive	0	182(16.3)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. $^{+}$Lobular or tubular mixed BCs. $^{++}$ Mucinous, Alveolar Lobular, Miscellaneous Including Metaplastic, Adenoid Cystic, Spindle, and Tubulolobular. NSP=no special type. The cut-off were as follows; ≥90 for KU70/KU80, >240 H-score for SMC6L1.n, and ≥ 230 H-score for SMC6L1.c, ≥ 130 H-score for BARD1.c.

4.6.5 Relationship between DNA-DSB Repair Markers and Patients' Outcomes by Univariate Analysis

For HR markers, positive BRCA1.n (but not BRCA1.c) expression was significantly associated with longer BCSS and a longer amount of time to distant metastasis (Figures 4.9a-b; P=<0.0001 for both). BRCA1.n^{+}.c^{-} had the best BCSS (P<0.0001, Figure 4.9c), however cytoplasmic expression of BRCA1 had no effect of BCSS (P=0.6, Figure 4.9d).

Chemotherapy improved patient's BCSS showing BRCA1.n^{-} , but not cytoplasmic (P<0.0001, and P=0.027, Figures 4.10a-b), however this was not seen after receiving endocrine treatment in ER-positive tumours (P<0.0001 for both nuclear and cytoplasmic expressions, Figures 4.10c-d). BARD1, irrespective of subcellular localisation, was not significantly associated with BCSS (Figure 4.11). However, endocrine therapy improved patient's BCSS showing BARD1.n^{-} (P=0.001, Figure 4.12c). The negative expression of Rad51.n demonstrated shorter BCSS than the positive expression of Rad51.n (P<0.0001, Figures 4.13a). Rad51.c^{+} showed a trend for shorter BCSS than Rad51.c^{-} (P=0.02, Figure 4.13b). However, the co-expression of nuclear and cytoplasmic Rad51 highlighted that Rad51n^{-}.c^{+} indicates a worse BCSS, in comparison with other subcellular combinations (Rad51n^{+}.c^{+}, Rad51n^{-}.c^{-} and Rad51n^{+}.c^{-}) (P<0.0001, Figure 4.13c). In regards to chemotherapy, patients showing Rad51.n^{+} who did not receive chemotherapy showed the best BCSS, whereas the other groups showed no difference ((Rad51.n^{-}/received/not received chemotherapy, and Rad51.n^{+} received chemotherapy), P=0.002, Figure 4.14a). SMC6L1 showed no effect on BCSS (Figures 4.15a-c), however, regarding to receiving chemotherapy, SMC6L1.n^{-} showed better BCSS than SMC6L1^{+} (P=0.002, Figure 4.16a).

With regards to NHEJ, both markers DNA-PK and KU70/KU80 had no effect on patients' outcomes (Figures 4.17a-b). However, endocrine treatment improved BCSS of patients showing DNA-PK^{+} (P=0.008, Figure 4.17f).

The co-expression of KU70/KU80^{-} and DNA-PK^{+} in TN tumours demonstrated the worst BCSS (P=0.001, Figure 4.18a), but showed no effect on non-TN cases (P=0.97, Figure 4.18b). In addition, in TN^{+} cases, negative expression of KU70/KU80 and BRCA1.n represented better BCCS than KU70/KU80^{+} and BRCA1^{+}, whereas in non-TN tumours that were also negative for KU70/KU80 and BRCA1.n demonstrated the worst survival (BCSS) rates (P=0.017 and P<0.0001 respectively; Figures 4.18c-d respectively). Finally, the double negative expression of SMC6L1.n and Rad51.n in unselected cases, showed shorter BCSS than the double positive expression of SMC6L1.n and Rad51.n (P=0.003, Figure 4.18e).

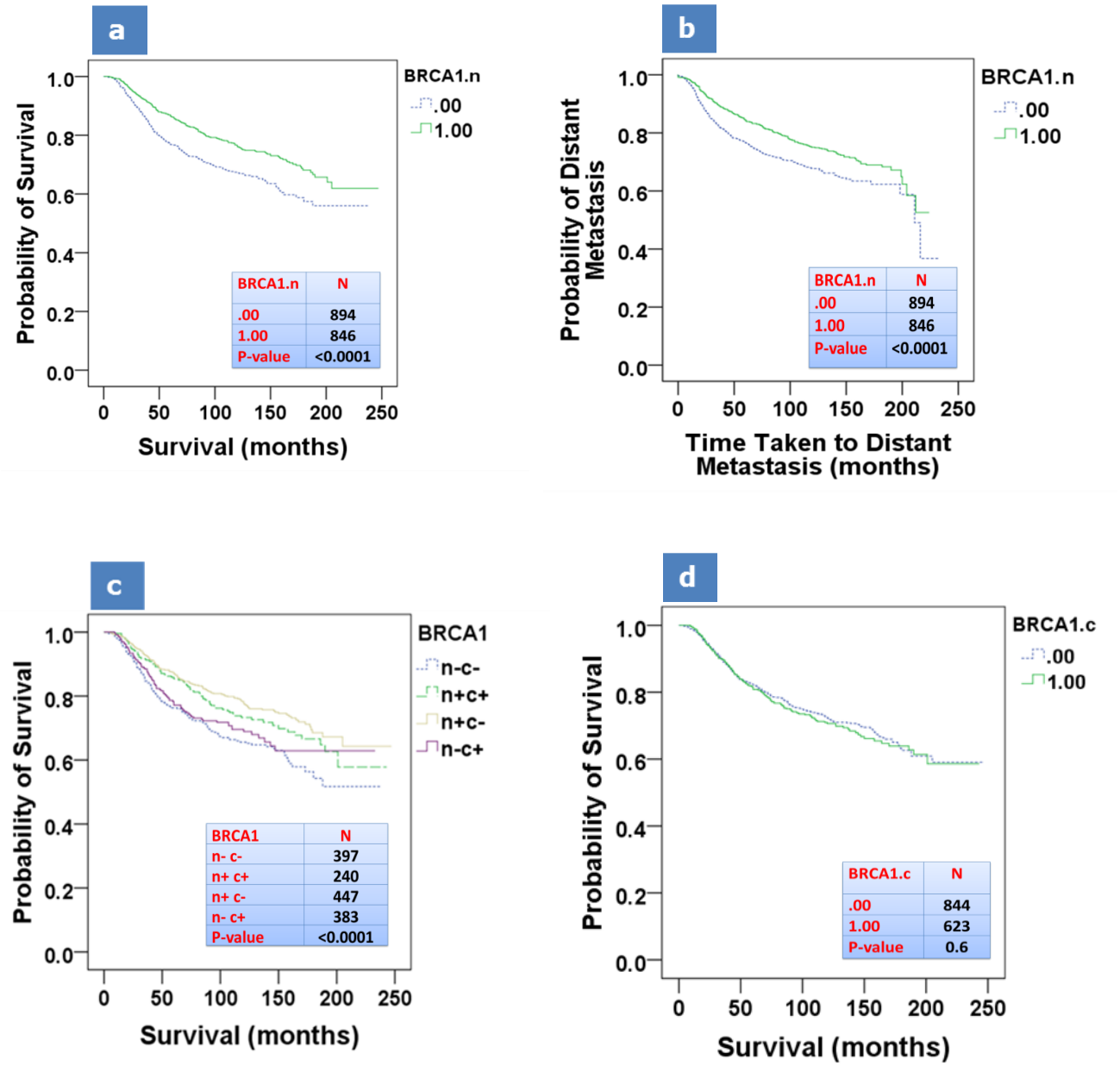

Figure 4.9 The associations between BRCA1 with patients' outcomes. 0= negative and 1= positive expression of BRCA1. Where n= nuclear expression and c= cytoplasmic expression of BRCA1 and N; number of cases. Only patients who died from breast cancer were considered. a; the association between nuclear expression of BRCA1 and BCSS, in addition b; the association between nuclear expression of BRCA1 and distant metastasis. c; co-expression of nuclear and cytoplasmic BRCA1 and its association with BCSS. d; the association between cytoplasmic expression of BRCA1 and BCSS. P<0.01 was considered significant.

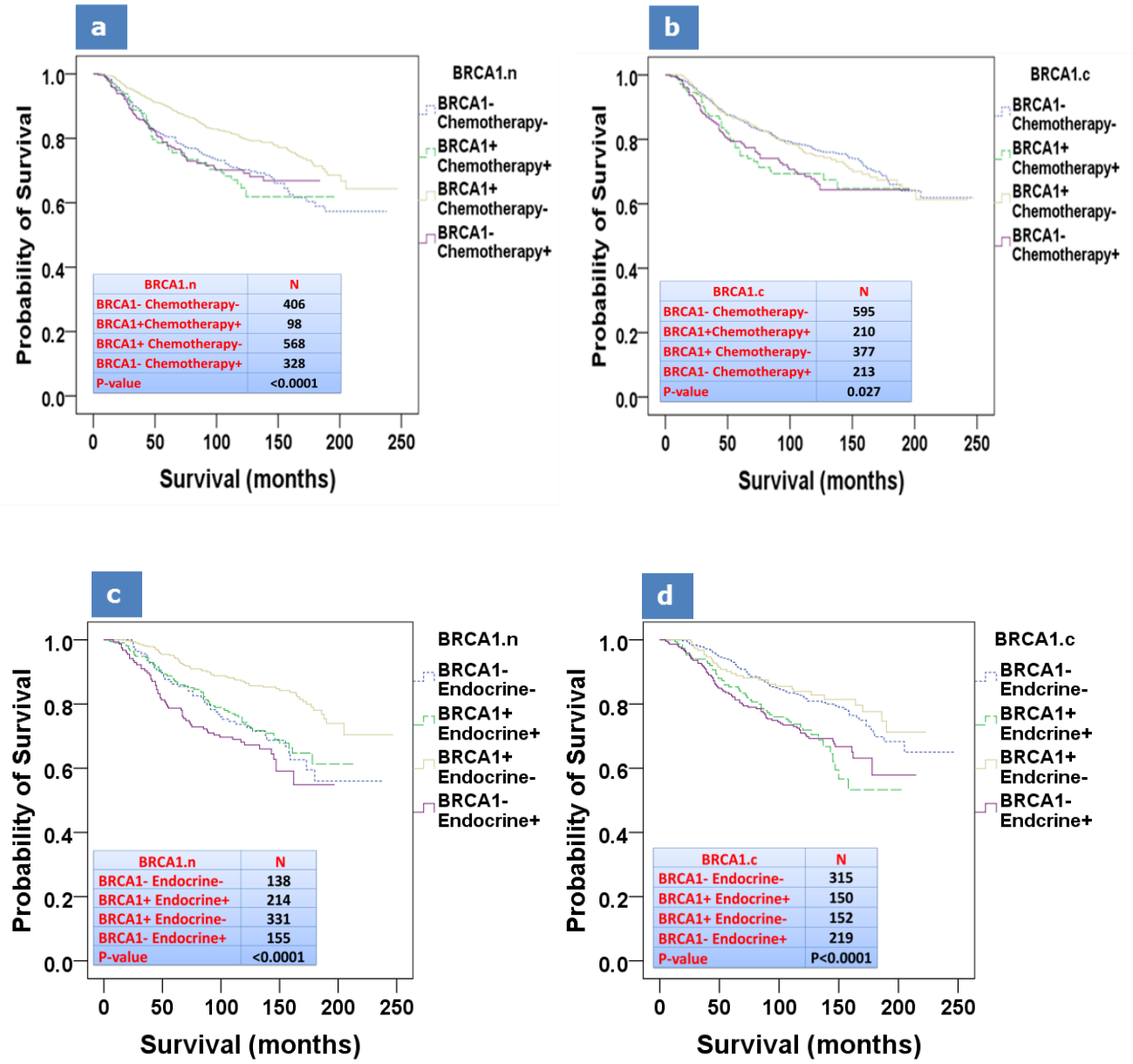

Figure 4.10 The associations between BRCA1 with BCSS and the effect of treatment on patient's outcome. N; number of cases. Only patients who died from breast cancer were considered. a; association between nuclear expression of BRCA1 and BCSS based on chemotherapy in unselected cases and b; cytoplasmic expression of BRCA1 and BCSS based on chemotherapy in unselected cases. c; nuclear expression of BRCA1 and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. d; cytoplasmic expression of BRCA1 and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. P<0.01 was considered significant. Some of ER-positive tumours did not receive endocrine therapy, because the treatment plan for those cases was as patients with a score of NPI≤ 3.4 received no adjuvant therapy.

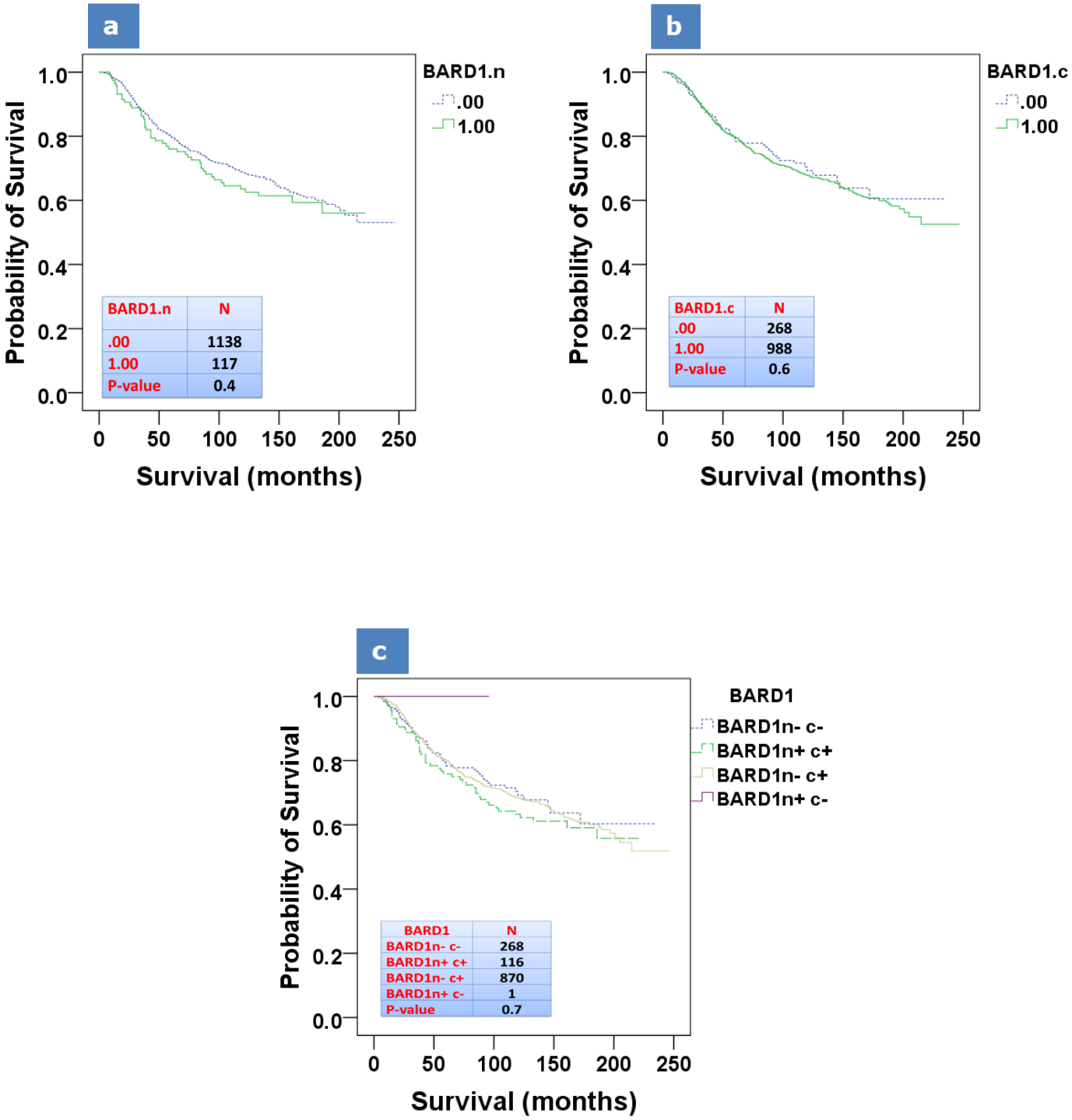

Figure 4.11 The associations between BARD1 with BCSS. 0= negative and 1= positive expression of BARD1. Where n= nuclear expression and c= cytoplasmic expression of BARD1 and N; number of cases. Only patients who died from breast cancer were considered. a; shows association between nuclear expression of BARD1 and BCSS, whereas b; shows association between cytoplasmic BARD1 and BCSS. c; co-expression of nuclear and cytoplasmic BARD1and its association with BCSS. P<0.01 was considered significant.

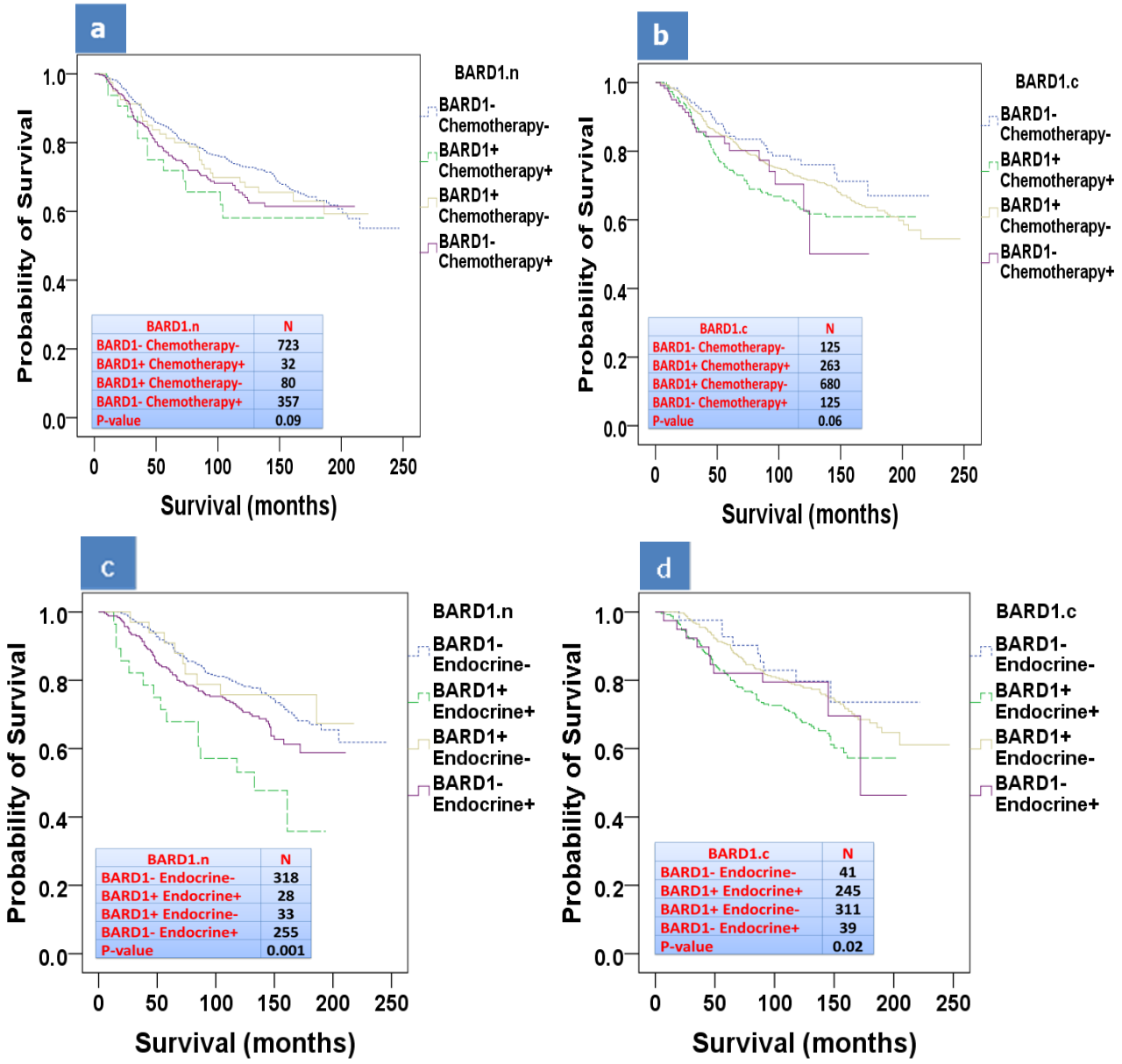

Figure 4.12 The associations between BARD1 with BCSS and the effect of treatment on patient's outcome. N; number of cases. Only patients who died from breast cancer were considered. a; association between nuclear expression of BARD1 and BCSS based on chemotherapy in unselected cases and b; cytoplasmic expression of BARD1 and BCSS based on chemotherapy in unselected cases. c; nuclear expression of BARD1 and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. d; cytoplasmic expression of BARD1 and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. P<0.01 was considered significant. Some of ER-positive tumours did not receive endocrine therapy, because the treatment plan for those cases was as patients with a score of NPI≤ 3.4 received no adjuvant therapy.

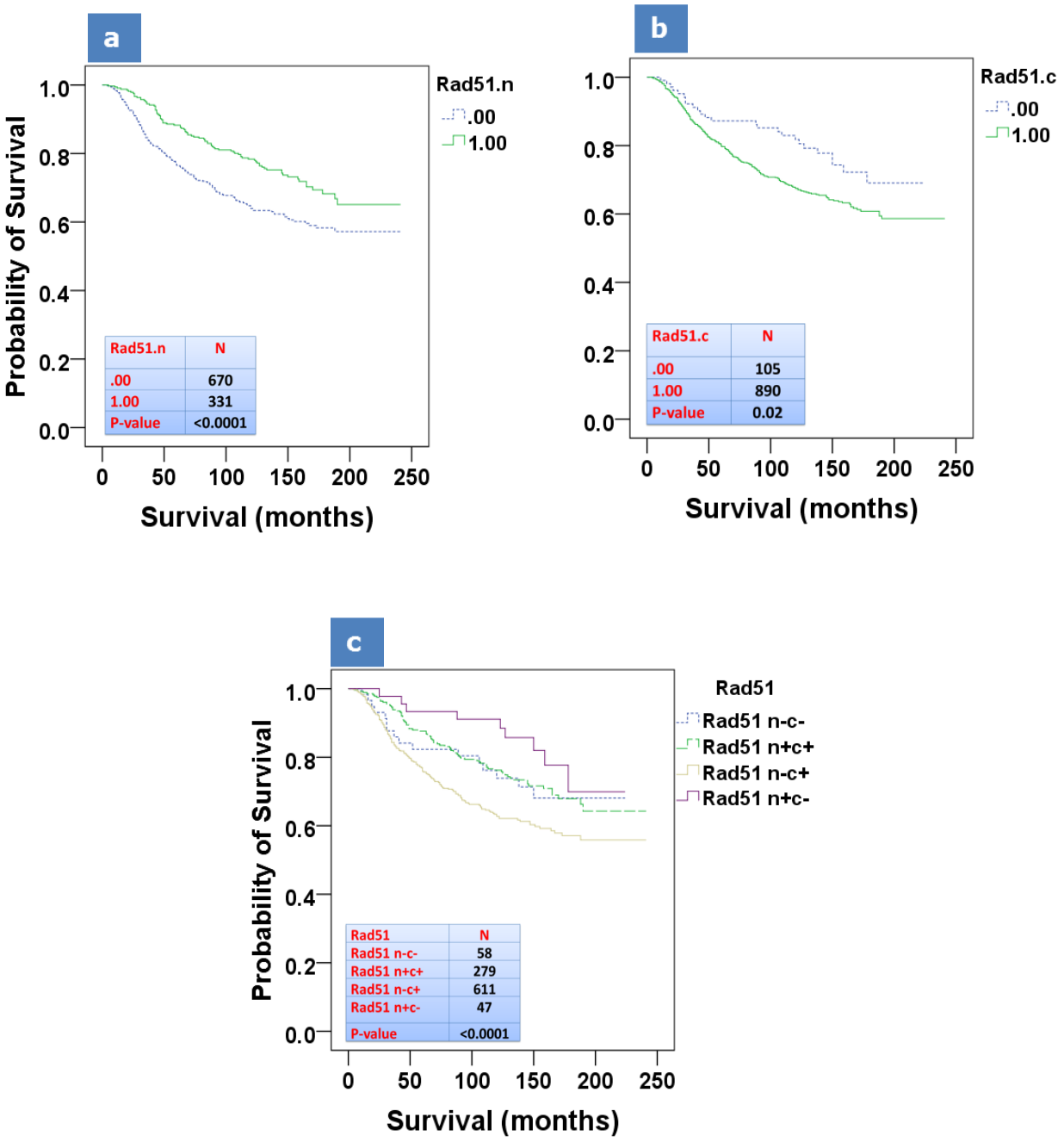

Figure 4.13 The associations between Rad51 with BCSS. 0= negative and 1= positive expression of Rad51. Where n= nuclear expression and c= cytoplasmic expression of Rad51 and N; number of cases. Only patients who died from breast cancer were considered. a; association between nuclear expression of Rad51 and BCSS, whereas b; association between cytoplasmic Rad51 and BCSS. c; co-expression of nuclear and cytoplasmic of Rad51and its association with BCSS. P<0.01 was considered significant.

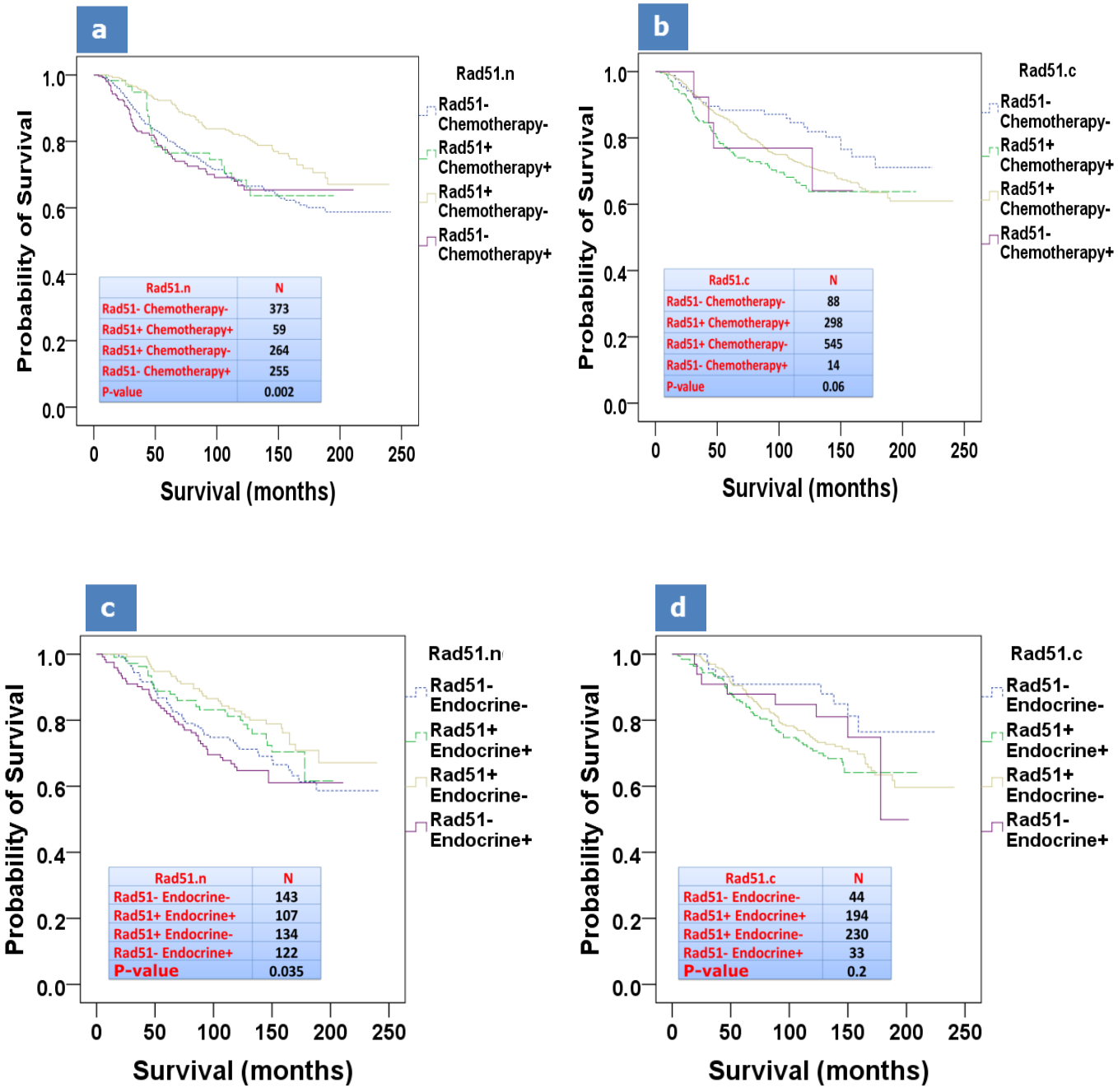

Figure 4.14 The associations between Rad51 with BCSS and the effect of treatment on patient's outcome. N; number of cases. Only patients who died from breast cancer were considered. a; association between nuclear expression of Rad51 and BCSS based on chemotherapy in unselected cases and b; cytoplasmic expression of Rad51 and BCSS based on chemotherapy in unselected cases. c; nuclear expression of Rad51 and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. d; cytoplasmic expression of Rad51 and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. P<0.01 was considered significant. Some of ER-positive tumours did not receive endocrine therapy, because the treatment plan for those cases was as patients with a score of NPI≤ 3.4 received no adjuvant therapy.

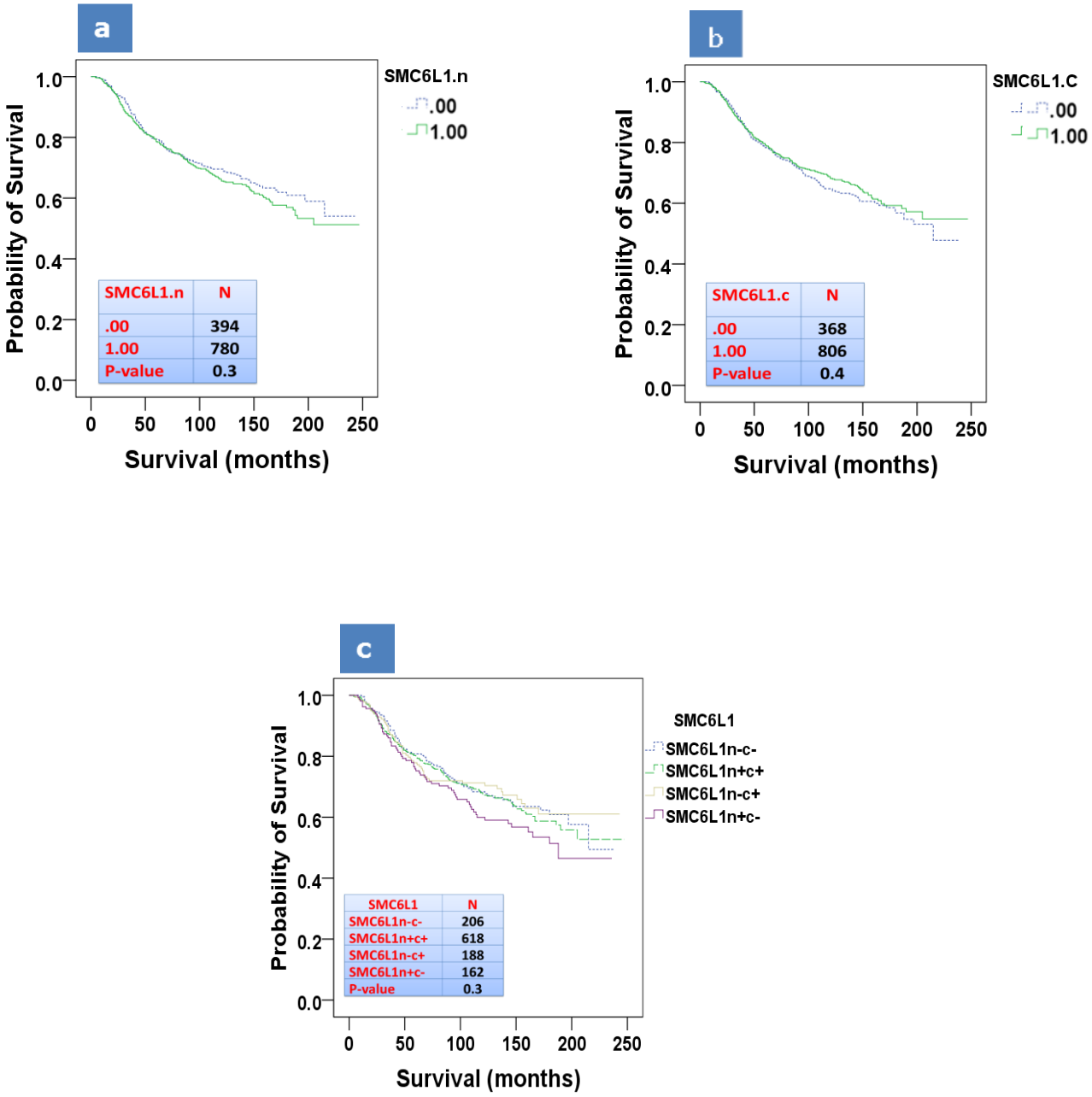

Figure 4.15 The associations between SMC6L1 with BCSS. 0= negative and 1= positive expression of SMC6L1. Where n= nuclear expression and c= cytoplasmic expression of SMC6L1 and N; number of cases. Only patients who died from breast cancer were considered. a; association between nuclear expression of SMC6L1 and BCSS, whereas b; shows association between cytoplasmic SMC6L1 and BCSS. c; co-expression of nuclear and cytoplasmic SMC6L1 and its association with BCSS. P<0.01 was considered significant.

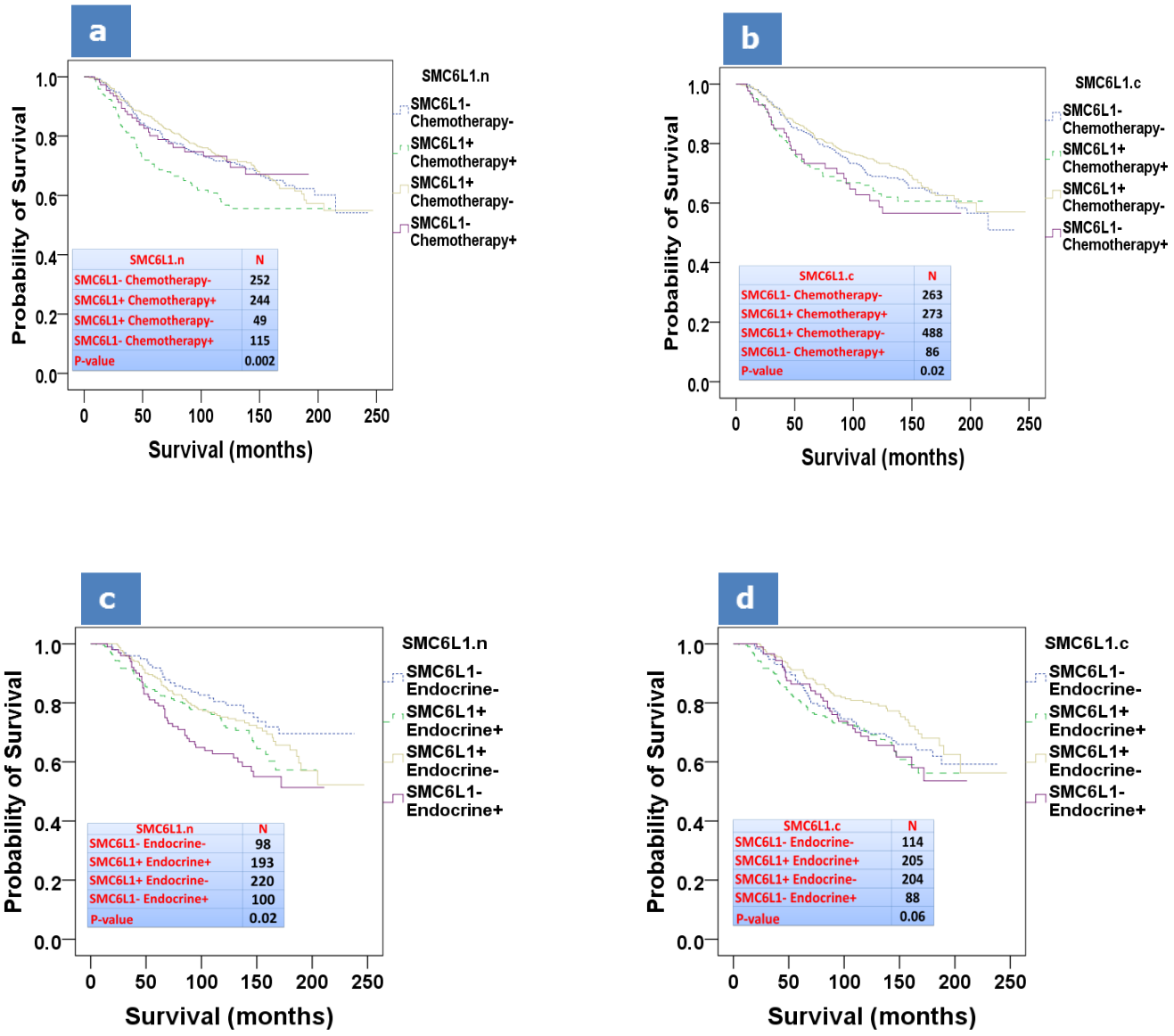

Figure 4.16 The associations between SMC6L1 with BCSS and the effect of treatment on patient's outcome. N; number of cases. Only patients who died from breast cancer were considered. a; association between nuclear expression of SMC6L1 and BCSS based on chemotherapy in unselected cases and b; cytoplasmic expression of SMC6L1 and BCSS based on chemotherapy in unselected cases. c; nuclear expression of SMC6L1 and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. d; cytoplasmic expression of SMC6L1 and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. P<0.01 was considered significant. Some of ER-positive tumours did not receive endocrine therapy, because the treatment plan for those cases was as patients with a score of NPI≤ 3.4 received no adjuvant therapy.

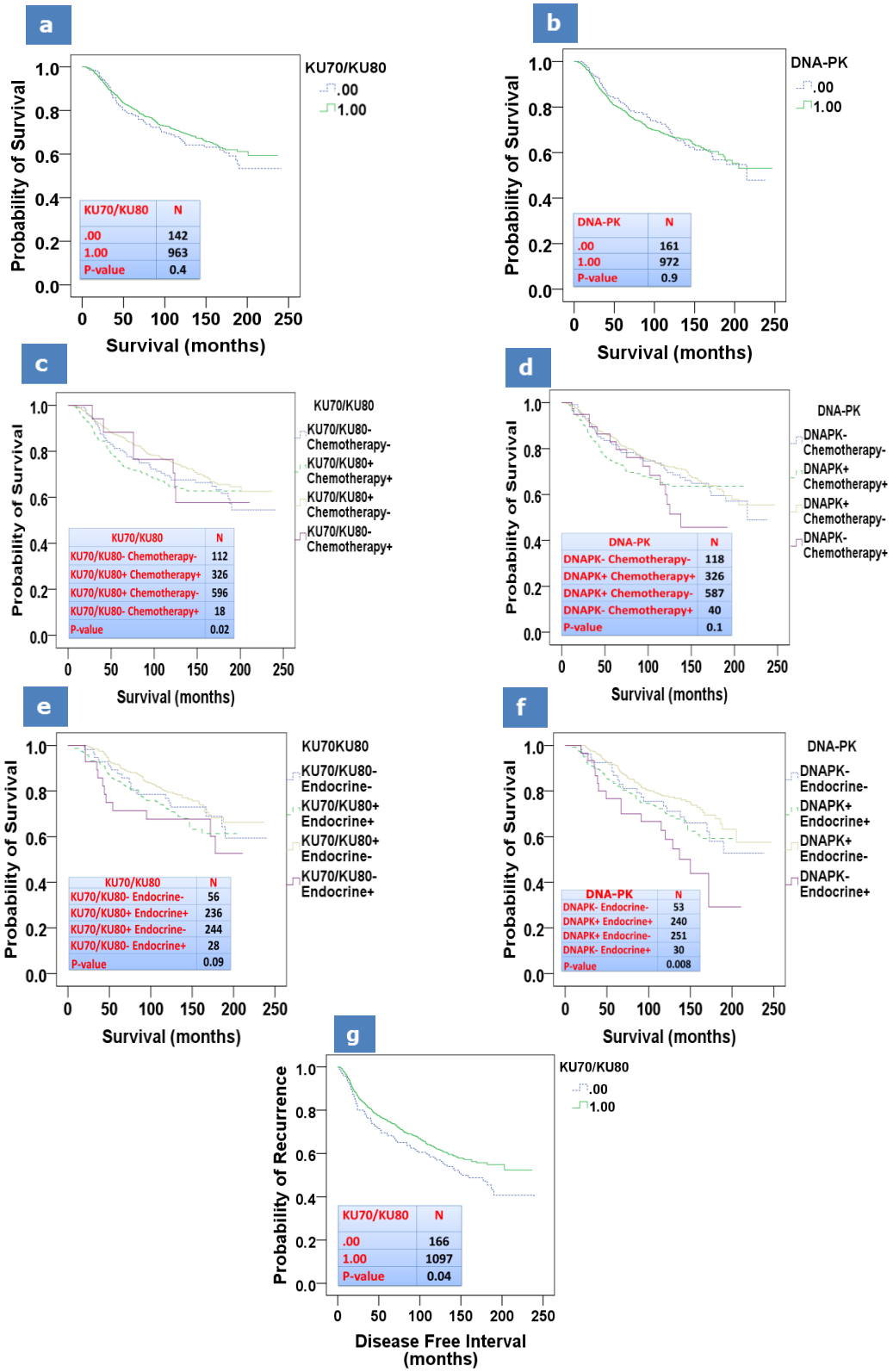

Figure 4.17 The associations between NHEJ repair markers with BCSS and the effect of treatment on patient's outcome. 0= negative and 1= positive expression. N; number of cases. Only patients who died from breast cancer were considered. a,b &g; show the association between KU70/KU80&DNA-PK and DFI/BCSS. c&d; show the association between KU70/KU80&DNA-PK and BCSS based on chemotherapy in unselected cases, respectively. e&f; show the expression of KU70/KU80& DNA-PK and BCSS based on receiving/or not endocrine therapy in only ER-positive cases, respectively. P<0.01 was significant. Some of ER-positive tumours did not receive endocrine therapy, because the treatment plan for those cases was as patients with a score of NPI≤ 3.4 received no adjuvant therapy.

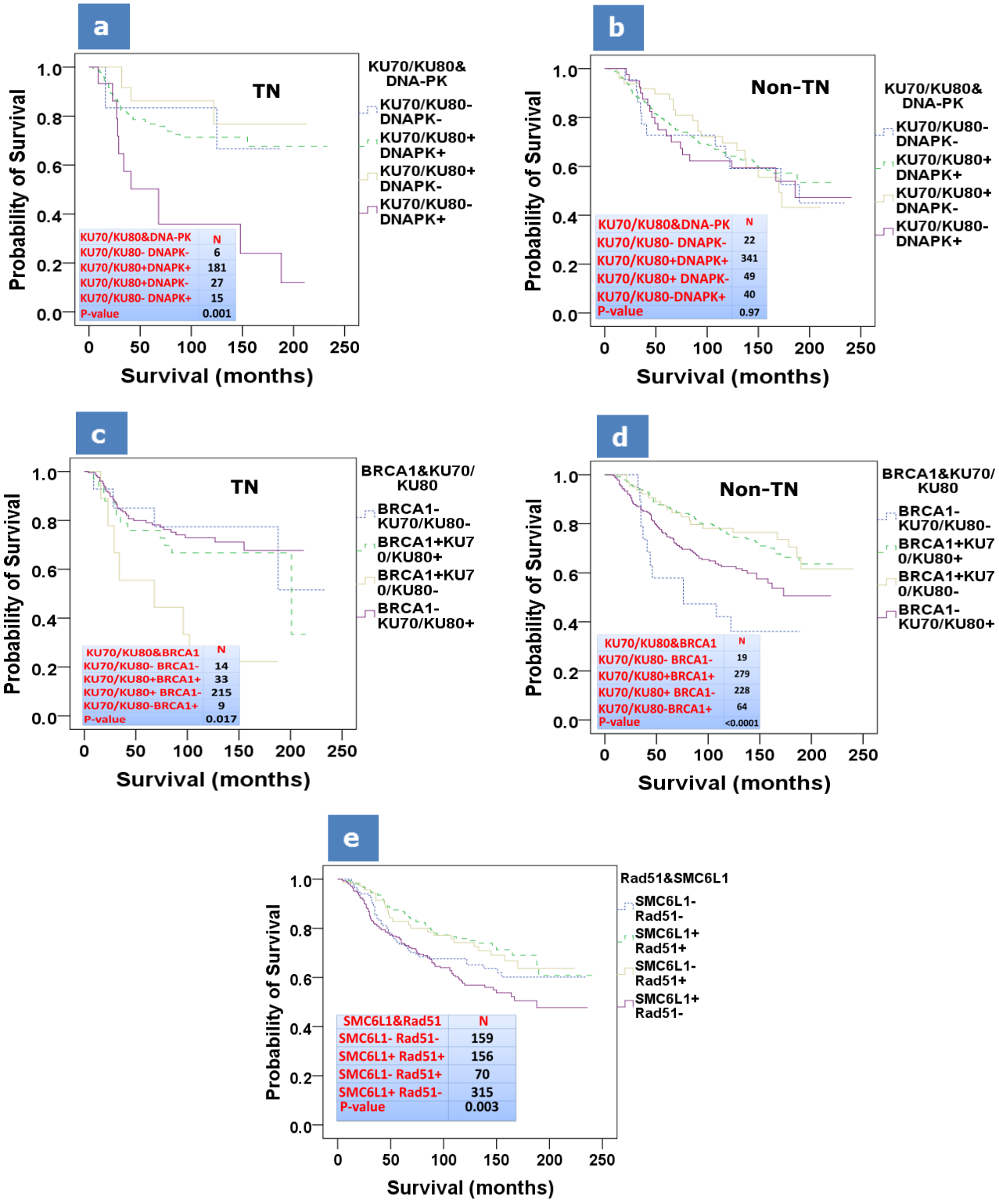

Figure 4.18 The association between co-expression of NHEJ and HR repair markers and BCSS in TN and Non-TN or unselected breast cancer. TN= triple negative breast cancer. a; shows BCSS of co-expression of KU70/KU80 and DNA-PK in TN. b, BCSS of co-expression of KU70/KU80 and DNA-PK in non-TN. c; BCSS of co-expression of KU70/KU80 and BRCA1 in TN breast cancer. d; BCSS of co-expression of KU70/KU80 and BRCA1 in non-TN. e; BCSS of co-expression of HR markers SMC6L1 and Rad51 in unselected breast cancer. All shows nuclear expression. P<0.01 was considered significant. The aim of these figures is to investigate the effect of a complex of different repair pathways on patient's outcome.

4.6.6 Expression of HR and NHEJ Repair Proteins and Patients' Outcomes by Multivariate Analysis

Table 4.11 and Figure 4.19 show Cox-regression analyses for predictors of BCSS and distant metastasis for HR and complexes of HR/NHEJ. Only markers that showed significant association in univariate analysis were considered. It shows that Rad51.n, co-expression of Rad51.n.c, KU70/KU80 &BRCA1 complex and KU70/KU80&DNA-PK complex are independent prognostic markers for BC.

Multivariate analysis for combinations (such as BRCA1 with chemotherapy or BRCA1 with endocrine therapy) does not usually perform, multivariate analysis applies for the main marker results (+ vs -) as combination effect may be biased by the other factor and not by the target marker.

Table 4.11 Cox-regression Analyses for Predictors of BCSS and Distant Metastasis.

Parameters	P-value	95.0% CI		Parameters	P-value	95.0% CI	
		Lower	Upper			Lower	Upper
BRCA1.n				**BRCA1.n.c**			
Tumour Stage	<0.000	1.510	2.375	**Tumour Stage**	<0.0001	1.511	2.377
Tumour Grade	<0.000	1.429	2.374	**Tumour Grade**	<0.0001	1.495	2.472
Tumour Size	0.002	1.184	2.134	**Tumour Size**	0.002	1.181	2.129
NPI	0.5	0.812	1.583	**NPI**	0.540	0.795	1.550
BLBC	0.2	0.638	1.116	**BLBC**	0.2	0.640	1.118
BRCA1.n	0.1	0.668	1.037	**BRCA1.n.c**	0.04	0.827	0.996
BRCA1.n*				**SMC6L1&Rad51**			
Tumour Stage	<0.0001	1.594	2.431	**Tumour Stage**	0.001	1.255	2.327
Tumour Grade	<0.0001	1.213	1.914	**Tumour Grade**	0.004	1.189	2.483
Tumour Size	0.002	1.181	2.036	**Tumour Size**	0.3	0.840	1.799
BLBC	0.1	0.618	1.058	**BLBC**	0.050	0.473	1.000
NPI	0.2	0.890	1.647	**NPI**	0.366	0.781	1.952
BRCA1.n	0.05	0.667	1.004	**SMC6L1&Rad51**	0.120	0.975	1.246
Rad51.n				**Rad51.n.c**			
Tumour Stage	<0.000	1.512	2.584	**Tumour Stage**	0.000	1.537	2.639
Tumour Grade	<0.000	1.378	2.559	**Tumour Grade**	0.000	1.402	2.620
Tumour Size	0.04	1.024	2.001	**Tumour Size**	0.026	1.045	2.045
BLBC	0.004	0.434	0.855	**BLBC**	0.003	0.425	0.839
NPI	0.94	0.661	1.466	**NPI**	0.748	0.624	1.403
Rad51.n	0.004	0.507	0.881	**Rad51.n.c**	0.01	0.557	1.991
KU70/KU80& DNA-PK+				**BRCA1&KU70/KU80^**			
Tumour Stage	0.26	0.8	2.658	**Tumour Stage**	<0.0001	1.589	3.182
Tumour Grade	0.2	0.5	34.307	**Tumour Grade**	0.001	1.257	2.588
Tumour Size	0.08	0.9	4.904	**Tumour Size**	0.09	0.9	2.288
BLBC	0.6	0.4	1.666	**BLBC**	0.96	0.7	1.527
NPI	0.5	0.5	3.798	**NPI**	0.9	0.6	1.591
KU70/KU80& DNA-PK	0.004	1.18	2.396	**BRCA1&KU70/KU80**	0.001	0.16	0.610

BLBC= as defined by TN+ positivity of CK5 and CK14 and CK17. NPI=Nottingham Prognostic Index. BCSS was considered in the analysis, except for BRCA1.n* distant metastasis was considered. Cellular localisation used for all the markers was nucleus. Only markers in univariate analysis that were statistically significant with patients' outcomes were applied for Cox regression (IBM SPSS 21.0) for multivariate analyses. + In TNBC, whereas ^ in non- TNBC.

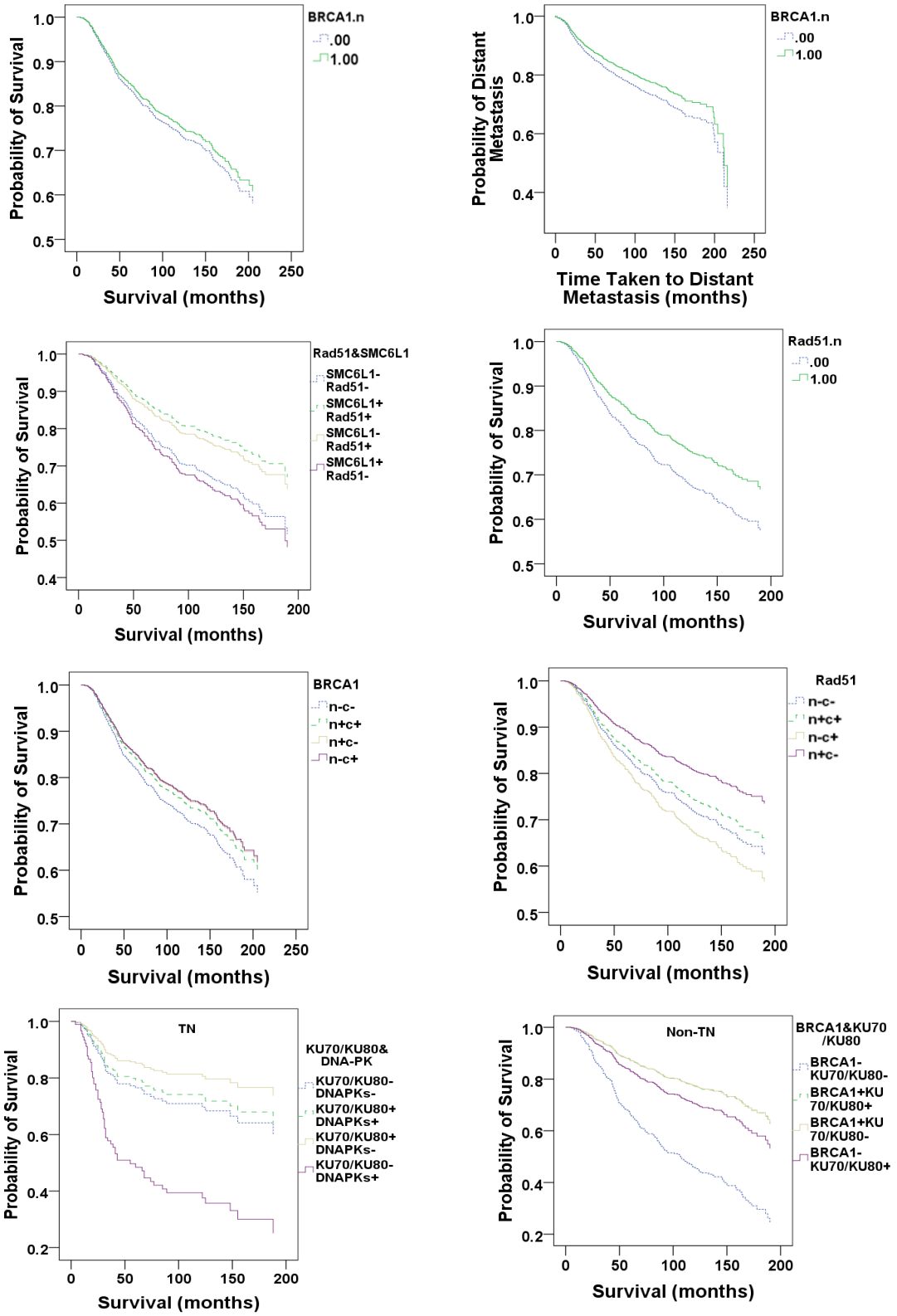

Figure 4.19 Multivariate Cox-regression analyses for HR and NHEJ markers. In order to test for confounders and prognostic or predictive independency of the investigated biomarker from standard prognostic/predictive factors; tumour grade, stage and size, NPI, and BLBC, were included. 0= negative expression, and 1=positive expression. c=cytoplasmic and n=nuclear expressions.

4.6.7 Expression of DNA-DSB Repair Markers in Cell Lines by Reverse Phase Protein Microarray

RPPA was used to evaluate the expression levels of DNA-DSB repair markers in different cell lines (BRCA1 deficient HeLaSilenciX® cells and its control, (proficient BRCA1), MCF-7, and MDA-MB-436 cells). Figure 4.20 shows the expression of HR and NHEJ repair markers in different cell lines using RPPA.

RPPA confirmed the IHC results and demonstrated a higher level of expression of DNA-DSB repair markers (Rad51, BRCA1, BARD1, SMC6L1 and DNA-PK) in the HeLa BRCA1 control or MCF-7 cell lines ($ER^{+,}$ $BRCA1^{+}$), when compared with the BRCA1 deficient HeLa, or MDA-MB-436 (ER^{-} & $BRCA1^{-}$) cell lines. In contrast, the NHEJ marker KU70/KU80 was highly expressed in BRCA1 deficient HeLa cell lines, in comparison to its control. However, only SMC6L1and KU70/KU80 showed statistical significant differences between BRCA1 deficient HeLa and MCF-7 (P=0.004 and P=0.01 respectively).

Expression of PTEN in different cell lines using RPPA is presented in Appendix 2.

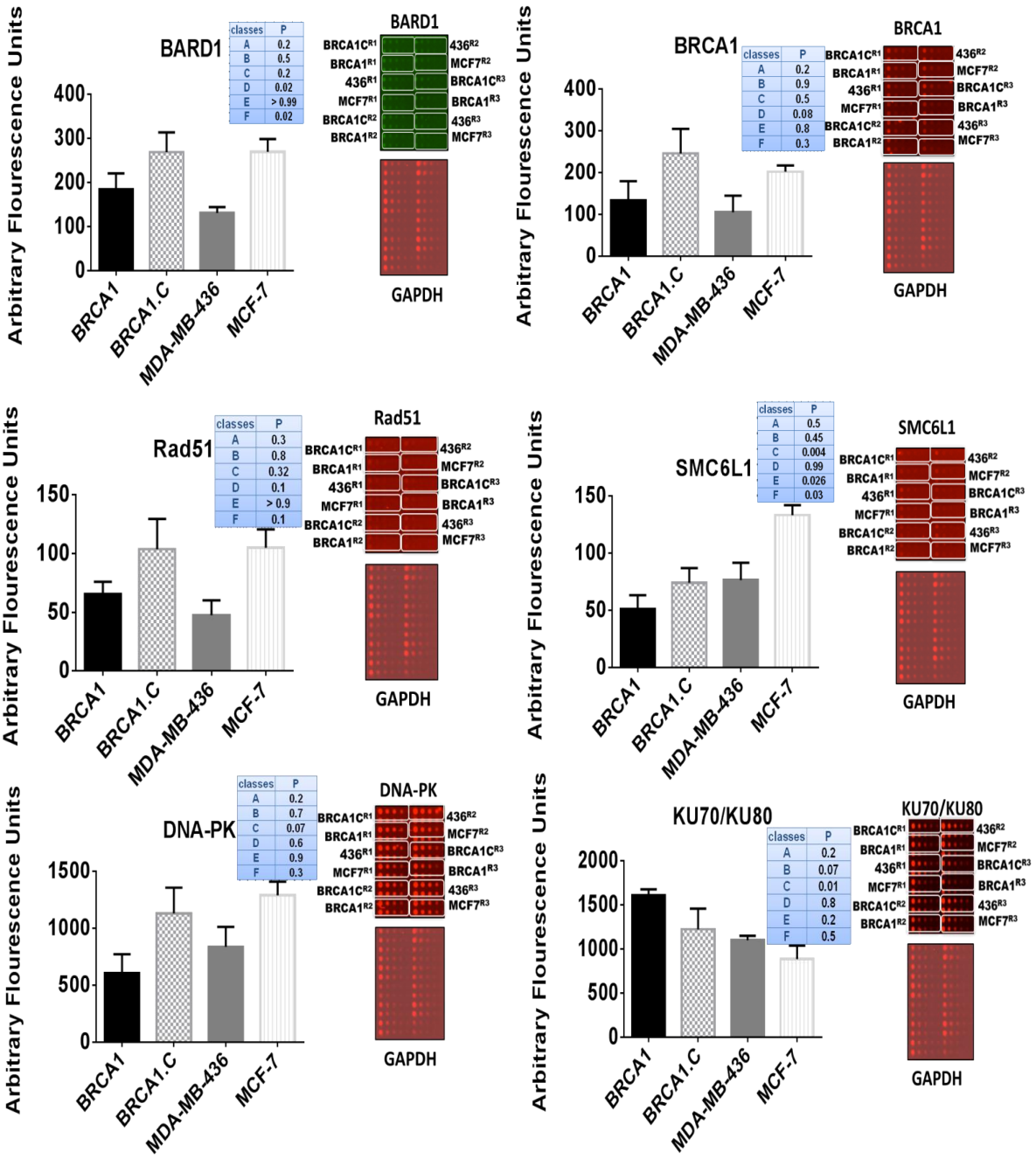

Figure 4.20 The DNA-DSB repair protein levels detected by reverse phase protein microarray in different cell lines (BRCA1 deficient HeLaSilenciX® cells and its control [BRCA1 and BRCA1.C respectively], MCF-7 and MDA-MB-436 (436) cells). For image of nitrocellulose slide spotted with different cell lysates; the red square represents the 700 channel for detection of mouse antibody while green square the 800 channel for rabbit antibody. Images of scanned nitrocellulose slides printed with extracted protein from cell lines and probed with the antibodies against the target proteins. Five 2-fold dilutions of each sample were printed in duplicate. Background was subtracted and the intensity of each spot was normalised to its corresponding GAPDH level. Each (^{R}) represents different passage of each sample, therefore, three different passages of each sample were used. Error bars represent Mean (SD). HeLa BRCA1; between passage21 and 30, HeLa BRCA1 control; between passage 15 and 20, MCF-7; between passage 25 and 32, and MDA-MB-436; between passage 12 and 20. A= BRCA1 vs. BRCA1.C, B= BRCA1 vs. MDA-MB-436, C= BRCA1 vs. MCF-7, D= BRCA1.C vs. MDA-MB-436, E= BRCA1.C vs. MCF-7, and F= MDA-MB-436 vs. MCF-7. One way ANOVA test was used.

4.7 Discussion

The mechanisms of DNA repair play a significant role in the maintenance of the integrity of DNA, in addition to protecting against DNA damage. Any deregulation of these mechanisms is related to the development of cancer, as observed in BCs, particularly in terms of *BRCA1* and *BRCA2* mutations. DNA-DSB leads to the death of cells or the loss of genetic material and is thus lethal to cells. HR and NHEJ are two distinct pathways in the repair of DSBs in mammalian cells [262, 397]. However, deficiencies in HR and NHEJ have already been associated with chromosome instability, sensitivity of DNA damage and predisposition to cancer, reflecting the threats presented by DSBs [4].

DNA-DSB repair proteins Rad51, BRCA1, BARD1, SMC6L1, KU70/KU80 and DNA-PK in a series of unselected (ER- and ER+) cases, in addition to known *BRCA1* germline mutations BC cases, were investigated, in order to further understand the involvement of BRCA1/DSB repair pathways in BC using IHC, and in different cell lines (using RPPA). The results of DNA-DSB repair markers were correlated with clinical outcomes and the expression of other biomarkers, in order to demonstrate their role in BC.

It is well known that DNA damage induces an increase in the nuclear levels of the cells [456]; thus, the markers investigated were initially expected to be expressed primarily within the nucleus of a cell. Interestingly, the main markers in the HR pathway (Rad51 and BRCA1) and other markers of HR (BARD1 and SMC6L1) (but not the NHEJ pathway markers (KU70/KU80 and DNA-PK)) were expressed in both the nucleus and cytoplasm of the cells. It is important to be aware of the characteristics of false positive staining by IHC, especially in terms of cytoplasmic expression. However, false staining can be easily recognised by the observer. If the staining is false, then it would often be somewhat indistinct, showing a lack of cell-to-cell heterogeneity and having a 'muddy' appearance. However, these features were not observed in the antibodies investigated in this study and intensity varied amongst the cases; thus, staining of cytoplasmic expression does not appear to represent an artefact. The results of cytoplasmic expression were constant in almost all the antibodies tested in this study, showing an association with poor features more than nuclear expression. This is not the first study showing the cytoplasmic expression of these markers (BRCA1, Rad51, SMC6L1, and BARD1), although different antibody clones were used [450, 453, 454, 457, 458]. However, it would be better if blocking peptides was used to confirm antibody specificity and eliminating concerns about non-specific binding.

In the present study, low levels of nuclear HR proteins such as BRCA1 protein was correlated with poor survival, which is similar to a previously reported study investigating mRNA levels in lung cancer [459]. In addition, a low level of nuclear Rad51 was correlated with established poor prognostic factors, such as high histological grade, TN phenotype and poor predictor of patient survival: Graeser et al supports these findings [460]. Cancer cell lines with a HR defect, as an example of those that have BRCA1 or BRCA2 loss, tend to be unable to stimulate foci of Rad51 following DNA damage [55, 460]. The inability to make

Rad51 after DNA damage shows a functional readout of a defect in HR. In the present study, the HeLa BRCA1 cell line (deficient BRCA1), or *BRCA1* known mutation BC cases/ ER^- BC, showed lower levels of Rad51 (or any HR marker) than the control HeLa BRCA1 cell line (proficient BRCA1) or sporadic BC showing positive BRCA1 and ER. This finding proposes a defect in the HR pathway in *BRCA1* known mutation cases/ or ER negativity sporadic BC. In line with a previous study [461], the immunostaining of Rad51 in primary BC confirmed a direct relationship between high cytoplasmic Rad51 expression and TN status, but an inverse relationship was observed with nuclear expression. This observation suggests the possible role of steroid hormone receptors in the regulation of Rad51 [462]. Pedram et al investigated the effect of oestradiol (E2)/ER on the HR pathway, where E2 inhibition of ATR signalling produced a delay in the formation of Rad51 nuclear foci: this was observed after UV-irradiation [463]. It is thus possible to hypothesise that the absence of steroid hormone receptors may be a surrogate marker of E2/ER signalling, which may directly influence the DNA-DSB repair pathway [464, 465]. In the results presented in this chapter, a comparison of the Rad51 nuclei and cytoplasmic expression arising within mutated *BRCA1* carriers showed a high level of cytoplasmic Rad51, supporting the hypothesis that nuclear levels may be lower due to the mutation of *BRCA1,* which might inhibit the transfer of Rad51 into the nucleus of the cell. Mitra et al supported these findings in prostate cancer [466]. The level of DNA damage required to induce the cytoplasmic to nuclear transport of Rad51 has not yet been established. Gildemeister et al have investigated subcellular fractions of HeLa and HCT116 (colon cancer cell line) cells and have identified a considerable improvement in levels of nuclear Rad51, upon exposure to a moderate dose of IR. In addition, they have detected an increase in DNA damage induced (IR/using Western blot) in nuclear Rad51 in BRCA2-defective cell line Capan-1, providing further evidence that Rad51 nuclear transport is an essential aspect of the cellular response to DNA damage [457].

The HR pathway marker SMC6L1 [467] is correlated with the expression of BRCA1 [468]. In common with the other investigated HR markers, SMC6L1 was expressed in both the nucleus and cytoplasm of cancer cells. In gene-level analyses, *SMC6L1* was associated with overall and high-grade risk of BC [469]. Roy et al has proposed that SMC6(alternative term for SMC6L1) plays a role in the repair of DNA *in vivo* aimed towards the complex of SMC5–6 to ssDNA substrates developed throughout the HR processes and/or replication of DNA [467]. Interestingly, unlike the other HR markers, double positive nuclear and cytoplasmic expression of SMC6LI was associated with poor prognostic factors, such as TN, $CK5^+$, high histological grade, nuclear pleomorphism and mitosis. SMC6L1 showed no effect on patient survival, unless when co-expressed with another HR marker; for example, Rad51, where double positive SMC6L1.n and Rad51.n cancers were significantly associated with a longer BCSS. However, significance was lost in Multivariate analysis, as combination effect may be biased by other factors and not by the investigated marker, leading to loss the significant of the target markers. This is the first large study of clinico-pathological significance, in terms of the survival of SMC6L1 in BC. The findings in this study uncover the association of SMC6L1 in linking recognition of DNA damage repair pathways in BC.

DSBs that arise throughout replication, if allowed to remain unfixed by the end of the S phase, should be repaired prior to mitosis. For HR to occur during both the S and G2 phases, applying sister chromatids as a template, it is necessary that the sister chromatids are produced in closeness to each other. This is most likely initiated by cohesion, which offers an actual physical linkage of the sister chromatids from the S phase, until their eventual separation during the anaphase. Cohesion is required throughout the S phase. Cohesion is triggered by DSB, following completion of DNA replication, and this function is essential for the repair of sister-chromatid in cells in the G2 phase [470, 471]. Unsurprisingly, anything affecting the cohesion complex or its components through mutation or loading seriously affects DSB repair [472, 473]. In the present study, SMC6L1.n (HR marker and may promote sister chromatid homologous recombination by recruiting the SMC1-SMC3 cohesin complex to DSB [474]) showed a positive association with cytoplasmic CHK1, nuclear or cytoplasmic γH2AX, $CHK2^{+}$ and ATR. Interestingly, it showed no association with ATM. However, it is well known that the first activated kinase by DSB is ATM, and ATR was proven to work as downstream of ATM in DSB-induced checkpoint signalling in both phases; S/G2 [306]. SMC6L1 has been stated as an important target of ATM for the DSB-induced checkpoint of S phase [475]. Therefore, it is interesting that DSB damage-induced sister chromatid cohesion requires ATR more than ATM. The result here suggests that ATM may not be activated by SMC6L1 and the other DNA damage sensors proteins have roles in cohesion complex leading to a defect in DNA repair. However, it is unclear at this point, whether SMC6L1 is a direct target of ATR. Further studies should certainly provide interesting observation into the roles of ATR in DSB-responsive sister chromatid cohesion. However, real time PCR can be used in the future to detect the accumulation of ATR or any markers at the damage site by using fluorescent proteins against the target markers.

The major protein-binding partner to BRCA1 is BARD1. BARD1 was initially identified as being a nuclear protein and a tumour suppressor gene that has a role in DNA repair [476] by localising to nuclear dots of BRCA1 through the S phase and right after DNA damage [407, 408]. Rodriguez et al have showed that BARD1 is a nuclear–cytoplasmic shuttling protein. It has an N-terminal nuclear export sequence (NES), which assists the export to the nucleus through the chromosome region maintenance 1 (CRM1) transport receptor pathway. Analogous to the BRCA1 NES [477], the BARD1 NES can also be positioned directly inside the binding domain of BARD1-BRCA1; its export activity is obstructed upon interaction with BRCA1, leading to the nuclear anchorage of BARD1. Rodriguez et al described a unique case study of protein regulation, wherein BRCA1 and BARD1 control the subcellular localisation of each other through the reciprocal masking of their particular nuclear export signals [477]. In the present study, the co-expression of any BARD1/BRCA1 implies that there is a cytoplasmic shift in cellular BARD1. Considering that BARD1 and BRCA1 stabilise the expression of each other, [478] the expression of BARD1 and BRCA1 were compared in this study. BRCA1.c was co-expressed with BARD1.c in some but not all cases. Additionally, the protein BARD1 was localised to the cytoplasm (very few cases showed nuclear expression), while BRCA1 was more frequently expressed in the nucleus. The expression of BARD1.c was higher than that of BRCA1.n;

BRCA1 was localised to the cytoplasm and, in the majority of cases, to the nucleus. In addition, there was a high positive correlation between BRCA1.n and BARD1.c. These findings propose that the form of BARD1 expressed in breast tumours may not be able to directly interact with BRCA1. Wu et al using real time-PCR and cloned and sequenced BARD1 cDNAs, showed that the BARD1 overexpressed in ovarian cancer cells and localised to the cytoplasm is most probably an aberrant form of BARD1 [479]. Fabbro et al observed that BARD1 can increase the nuclear import of BRCA1, in addition to preventing the nuclear export of BRCA1 by masking its nuclear export signal [480]. This 'chaperone' process requires a direct interaction between the two proteins and BARD1 promotes BRCA1 recruitment to DNA repair-associated nuclear foci, upon damage of DNA [480, 481].

A recent study has further demonstrated that BARD1 shuttles between the nucleus and the cytoplasm: its location in the cell cytoplasm correlates with its apoptotic function, which is significantly decreased by BRCA1 [453, 454]. In contrast, Wu et al mirror the finding of this study by showing short DFI of cytoplasmic BARD1 with higher tumour grade in both ovarian and sporadic BCs [482]. BARD1 stimulates BRCA1-independent apoptosis through the P53 pathway after DNA damage [454]. In the present study, the expression of BARD1 and P53 was compared, although there was no correlation between their expressions. An excess of BARD1 over BRCA1 in known *BRCA1* germline mutations cases is expected to produce BARD1-induced P53-dependent apoptosis. It is assumed that it is likely that cancer cells would inactivate either BARD1 or P53 to avoid apoptosis; however, the tendency regarding mutations or epigenetic alterations may be tissue specific. It has been stated that BARD1 demonstrates pro-apoptotic activity and is associated with activated expression in mice cells immediately after genotoxic stress [483]. This activity is influenced by P53 and, to some extent, is suppressed by BRCA1 co-expression in mouse TAC-2 mammary epithelial cell lines and embryo stem cells. Interestingly, in this study, BARD1 showed no association with P53, but complex BRCA1.n^+/BARD1.c^+ showed a strong negative association with P53: this may show the effect of BRCA1 on BARD1 subcellular localisation and apoptosis by P53. Leading however, further studies showing the effect of P53 on complex BRCA1/BARD1 are warranted. To investigate the probability that BARD1, like its main binding partner BRCA1 has the ability to shuttle in and out of the nucleus, in cell lines for example MCF-7 or HeLa BRCA1 control (since both showed the highest expression of BRCA1 and BARD1 in this study), the CRM1 nuclear export receptor can be overexpressed and evaluated its influence on subcellular localisation of BARD1. In addition, to determine the subcellular distribution of endogenous BARD1, fractionated cell lysates from the cell lines can be prepared, and analysed for BARD1 using Western blotting. In addition, to determine whether the expression of BARD1 in cancers with mutations of BRCA1 is correlated with P53 mutation status, real time-PCR can be performed, and cDNA of P53 can be cloned and sequenced [453, 454].

It has been suggested that loss of the expression of PTEN may possibly result in a defect in HR. In terms of the phenotype of genomic instability in PTEN-deleted cells, Shen et al revealed that cells deficient in PTEN have defective DNA-DSB repair, quite possibly due

to loss or downregulation of Rad51, in addition to loss of PTEN at centromeres [484]. PTEN works on chromatin and controls the expression of Rad51, which usually decreases the incidence of spontaneous DSBs [484]. In the present study, all HR repair markers such as Rad51n^{-}.c^{+}, and SMC6L1.n^{+}.c^{+}, and only DNA-PK^{-} from NHEJ pathway were associated with loss of PTEN. In addition, RPPA showed that a low expression of PTEN was mainly seen in the HeLa BRCA1 cells (deficient BRCA1) (Appendix 2). These findings may explain the involvement of PTEN in HR repair. However, when BRCA1.c, but not BRCA1.n, was analysed as a continuous variable, correlations showed no significant with PTEN. It is more logic to consider the continuous variable as correlation is biological and certainly the cut-off points will reflect these correlations, but it can argue that from a clinical point of view, using categorical markers may be more helpful for patient management such as ER status in BC which is used as positive/ negative and not as continuous variables.

Cells that lack BRCA1 are known to lack the repair of DSBs through the conservative mechanism of HR; thus, they apply a substitute repair pathway, primarily NHEJ. Despite the fact that NHEJ has a significant role in the repair of DSBs [262], this pathway had no consideration for homology (sister chromatid) in repairing breaks and it is therefore considered an error-prone repair, ultimately causing elevated instability of the genome [399, 428, 485]. In this study, as the BRCA1 is extensively discussed, verifying the relationship between BRCA1 and NHEJ markers in identifying the risk of BC would probably offer a further perception of NHEJ, with regards to the role of HR in tumorigenesis of the breast [486]. This study reviews such an investigation, based on (a) using cases of known *BRCA1* germline mutations as a control group (b) a large and well-characterised series of clinically annotated, sporadic early-stage BC and, finally, (c) cell lines of BC with varying BRCA1 statuses. The findings here demonstrate that the expression of KU70/KU80 and DNA-PK was higher in tumours showing aggressive characteristics, such as high histologic grade, TN, basal-like phenotype and negative expression of BRCA1. Importantly, the expression of KU70/KU80 or DNA-PK was associated with the absence of the expression of protein in nuclear and positive cytoplasmic BRCA1. RPPA also confirmed this in the cell lines, demonstrating a high expression of KU70/KU80 in BRCA1 deficient cell lines (HeLa BRCA1), when compared with BRCA1 proficient cell lines (HeLa BRCA1 control). Ghezzi et al supported this observation in their research into colorectal cancer [487]. Unlike KU70/KU80, DNA-PK was higher in BRCA1 proficient cell lines than in the BRCA1 deficient cell line (HeLa), again this supports the finding by IHC.

The prognostic significance of the expression of KU70/KU80 and DNA-PK in the literature is inconsistent. In this study, KU70/KU80^{+} showed a trend for longer DFI in unselected tumours. This outcome is in line with the research of Pavon et al which highlighted a significant relationship between high tumour *KU70* mRNA and better overall survival in head and neck cancers [488]. However, in colorectal carcinomas, KU70/KU80 does not predict survival [489]. It may thus be suggested that the prognostic value of KU70/KU80 could possibly be organ specific. However, the present study showed that KU70/KU80^{-} and DNA-PK^{+} in TN cases had the worst BCSS. Thus, there is a possibility that

complexes of NHEJ markers could possibly differentiate between distinct tumour classes, such as ER+ and ER-. Herein, the variation between KU70/KU80 and DNA-PK may possibly be a chance result, since they are in the same pathway and thus are expected to show similar results; however, it is also possible that their particular features and functions may reveal this variation. DNA-PK is apparently limited to higher eukaryotes, whereas KU70/KU80 is expressed in the majority of organisms from yeast to man [490]. The function of Ligase IV is entirely reliant on KU70/KU80 but not on DNA-PK, highlighting that the components of the DNA-PK complex have several functions [491].

The tumour suppressor P53 is phosphorylated and activated by DNA-PK, which is dependent on the presence of DNA breaks for its activity [492]. It is widely acknowledged that P53 is mutated or deleted in several cancers, including BC [493], and is essential in the cellular response to IR by managing cell-cycle checkpoint control, inducing apoptosis, and, quite possibly, modulating DNA repair. In the present study, dichotomised, but not continuous; (lost significant), KU70/KU80 was positively associated with P53. It appears that, in addition to their function in the repair of DNA, DNA-PK and KU70/KU80 may play a role in apoptosis as a response to IR, at least in cells with functioning P53. Leading however, further studies showing the effect of P53 on NHEJ repair pathway are warranted. Determine whether the expression of NHEJ markers in BC (e.g in BRCA HeLa and MCF-7 cells, which showed high level of KU70/KU80 and DNA-PK respectively) is correlated with P53 mutation status, real time-PCR can be performed, and cDNA of P53 can be cloned and sequenced.

As outlined above, HR markers show a possible interaction with NHEJ, particularly in TN tumours, which could possibly have an impact on their ability to repair DSBs. In TN cases, as opposed to non-TN cases, the co-expression of KU70/KU80^{-} and BRCA1.n^{+} had an inverse effect on survival; thus, it appears acceptable to anticipate that HR markers may contribute to NHEJ activity and play a pivotal role in the outcome or development and progression of BC, particularly in TN tumours. Despite the fact that this demonstrates the co-expression of HR and NHEJ markers, several questions remain, in terms of which pathway is the driver and which marker actually regulates the repair of DSBs. Further functional studies are warranted, such as utilising the Comet assay to detect the main markers responds to DNA-DSB.

After receiving chemotherapy, cases showing high levels of Rad51.n or BRCA1.n protein demonstrated a shorter BCSS, in comparison to cases that received no chemotherapy. In contrast, the formation of Rad51 foci in response to DNA damage was recently demonstrated to be related to response to neoadjuvant anthracycline-based chemotherapy in BC [460]. As chemotherapy toxicity is associated with the ability of a compound to bind DNA and develop cross-links, in addition to triggering the death of cells through the induction of DSBs [494], it can suggest that a defect in the HR pathway may be responsible for failure to repair damage caused by these agents, leading to activate a backup error prone pathways such as NHEJ to induce the repair [399, 428, 485]. It is proposed that *BRCA1* helps

prevent familial BC; not only by stimulating the HR pathway, but also by possibly eliminating error-prone NHEJ and promoting error-free NHEJ methods [30]. In this study, *BRCA1* germline mutated BCs demonstrated a high level of an NHEJ protein (KU70/KU80) (but not DNA-PK), in addition to the negative expression of nuclear, not cytoplasmic, HR proteins (Rad51 & BRCA1): this may indicate that NHEJ (as an error-prone pathway) is the active pathway in the repair of DNA-DSBs in this class of BC. It is important to understand whether defects in this particular pathway may engage in the development of BC by studying the entire pathway and any overlap markers; thus, the potential effect that one selective marker from the distinct repair pathways of DNA-DSB has on patients' outcomes must be considered. Unfortunately, in this study, BCs containing a *BRCA1* mutation are relatively infrequent to comment on this group alone. Based on the present study, the cytoplasmic expression of HR markers showed a similar effect to NHEJ markers in BC patients, whereas the nuclear expression of HR had diverse effects. These findings confirm the diverse role of DNA-DSB repair proteins and emphasise the role of subcellular localisation, providing further evidence of the complexity of DNA damage response mechanisms.

The results of this study combine the power of IHC with the parallel analytic capability of protein microarray RPPA. RPPA was achieved using whole cell extracts and, interestingly, the nuclear (rather than the cytoplasmic) expressions of Rad51, BRCA1 and SMC6L1 were in-line with the RPPA findings, as was cytoplasmic BARD1 by IHC. This confirms the importance of subcellular localisation when investigating any protein (shows both localisations) and validates the assertion that nuclear or even cytoplasmic expression does not occur by chance. The subcellular localisation of HR markers suggests that the traffic of proteins between the nucleus and cytoplasm might play a role in the development of BC. Based on the present findings, it can hypothesis that tumours deficient in HR proteins, such as Rad51 and BRCA1, may activate a back-up HR pathway for the repair of DSBs, such as NHEJ (KU70/KU80 and DNA-PK), this was concluded based on the high level of NHEJ proteins.

Knowing the subcellular location of a protein is important for understanding its functions [395]. However, regardless co-expression of cellular localisation, BRCA1.c (regardless nuclear expression) showed no correlation with CK5, MTA1.n, γH2AX.n, CHK2, CHK1.n, Rad51.n, BARD1.c, but all these markers were statistically significant with nuclear BRCA1 (without considering the cytoplasmic expression). In addition, Pearson's correlation (continuous data analysis) confirm these finding, leading to hypothesis that the nuclear but not cytoplasmic BRCA1 may have the main role in association with these markers in BC. Additionally, the association between Rad51.n (regardless cytoplasmic expression) showed no correlation with MTA1.c, CHK1.c, and ID4.c, but all these markers were statistically significant with cytoplasmic Rad51 (regardless of nuclear expression). Again this may help to suggest that the cytoplasmic expression of Rad51 may have the main role in association with these markers in BC. Further studies are warranted to investigate the effect of cellular localisation of HR repair markers on their function. Improvement in understanding the molecular details in the cytoplasmic/nuclear actions of HR repair markers may help to find a

target for BC, especially the two cellular compartments showed different features of BC. In addition, to determine the subcellular distribution of any marker, fractionated cell lysates from cell lines can be prepared, and analysed for target marker using Western blotting.

Although HR and NHEJ proteins may be valuable markers in clinical studies of novel therapeutics that target the repair of DNA damage based on the hypothesis of synthetic lethality, such as PARP inhibitors, investigating the different proteins plays a role in the various repair pathways may offer valuable information, given the complexity of the DNA repair processes. Thus, studying further markers of HR and NHEJ repair would improve our understanding of the repair of DNA in BC.

Figure 4.21 summarises the key findings in this chapter and pathways involved in DNA-DSB in BC.

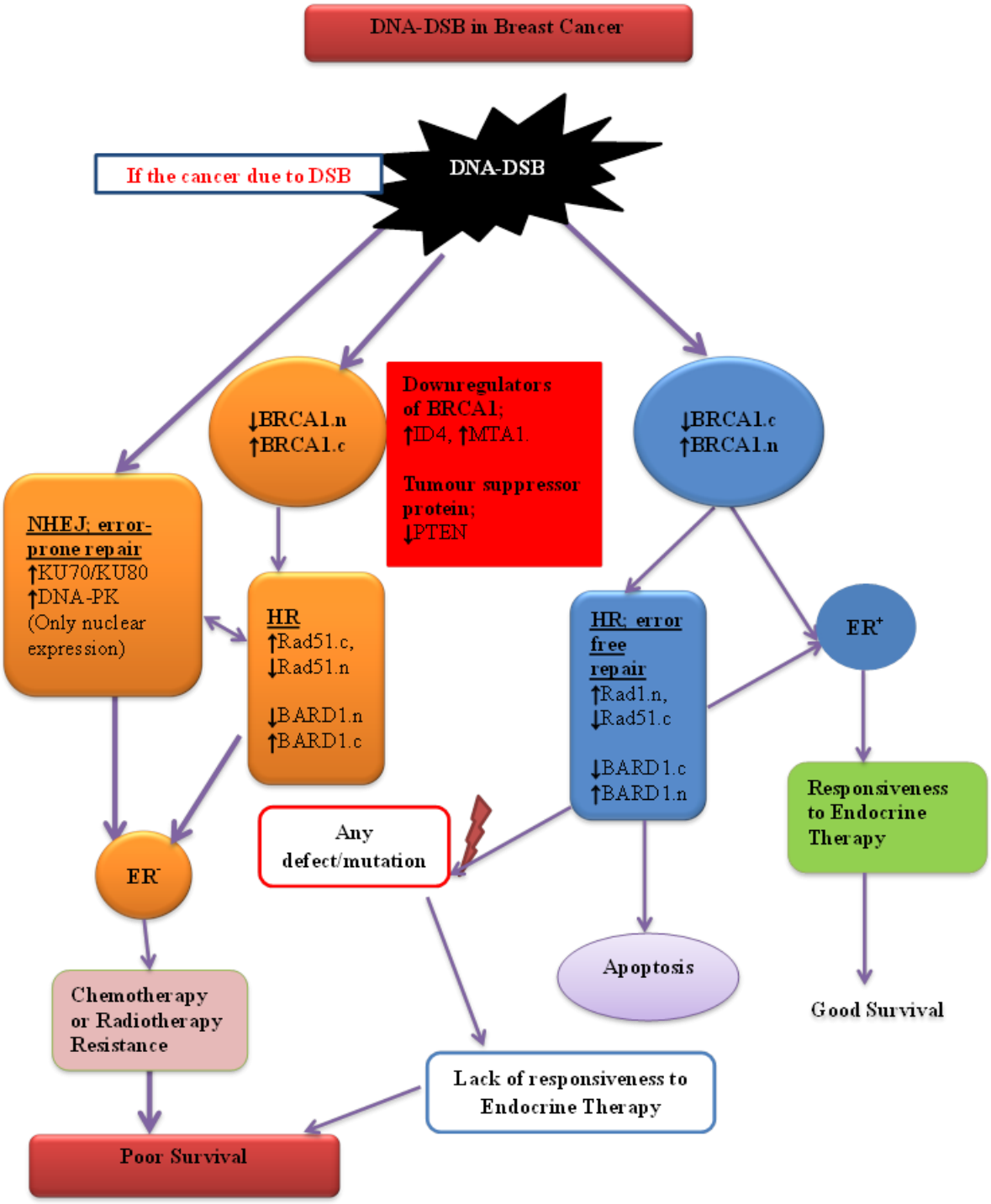

Figure 4.21 A summary of the key findings in this chapter and pathway involved in DNA-DSB repair in breast cancer. The cytoplasmic expression was mainly detected in HR markers and was associated with poor survival such as Rad51, whereas nuclear expression showed favourable outcome. However, NHEJ markers (KU70/KU80 and DNA-PK) were only expressed in the nucleus of cancer cells, and were highly associated with poor features in breast cancer.

Chapter 5

5.1 Introduction

5.1.1 SUMOylation and Association with DSB Repair in Breast Cancer

There are various essential standards in protecting the balance of the genetic information of any cell [495]; for example, the independence functions of genome maintenance and the immediate reaction to any kind of genomic injury, which in any other case would certainly result in disorder within important cellular functions by interfering with gene expression. There is also a reversible reaction to the physiological condition associated with the cell. In addition, the mechanism for the protection associated with the stability of the genome needs to clearly show a flexible reaction to distinct kinds of harmful agents, to which a cell may often be exposed [496]. Post-translational modifications, along with ubiquitin on its own and a small ubiquitin-related modifier (SUMO), have already been determined as major factors in the maintenance of the genome. SUMO is an ubiquitin-like protein that is covalently attached to a number of target proteins, in order to modify their function. In contrast to ubiquitination, SUMO does not target proteins for proteolytic breakdown; rather, it is involved in controlling a number of different protein functional properties, such as protein-protein interactions, as well as subcellular targeting [6].

In humans, there are three well-characterised SUMO isoforms: SUMO-2 and SUMO-3, which share a high level of similarity, and SUMO-1. SUMO follows a similar enzyme structural pattern as ubiquitin modification, requiring an E1-activating enzyme, an E2-conjugating enzyme (only one of these, Ubiquitin-Conjugating Enzyme 9 (UBC9), is recognised) and E3-ligating enzymes, including PIAS1-4 (protein inhibitor of activated STAT1-STAT4 (signal transducer and activator of transcription)) [497].

Apart from their DNA and protein binding ability, which can be mediated by the conserved region of (scaffold attachment factor-A/B/acinus/PIAS (SAP), PIAS proteins consist of a really interesting new gene (RING), a finger-like zinc-binding domain, in addition to a SUMO interaction motif: as a result, they function as SUMO-E3 ligases (E3) [498]. PIAS is thus able to interact with and even modulate a variety of protein activity, in addition to signalling cascades [499].

Similar to ubiquitin conjugation, SUMO requires an E1-activating enzyme and an E2 conjugase [500]. Despite the fact that this action can take place without an E3 ligase, it would not be efficient. The presence of mammalian E3 ligases was identified by a couple of independent studies employing yeast two-hybrid approaches, which indicated that family members of PIAS were SUMO ligases [501, 502]. These studies showed that both PIAS1 and PIASγ (PIAS4) *in vivo* and *in vitro* offered P53 and LEF1 (Lymphoid Enhancer-binding Factor 1) SUMO respectively [501-503]. In contrast to ubiquitin conjugation, SUMO appears

to modify the function of protein; not through degradation, but rather by changing function, localisation or even the extent of ubiquitylation. PIAS stimulated SUMO involves the motif of the RING finger of PIAS, largely because deletion or modification of this motif removes the SUMO of PIAS-binding partners [501, 502]. The functional effects of PIAS association and PIAS-induced SUMO appear to be different; for example, the removal of SUMO sites in LEF1 failed to change its targeting to nuclear bodies, while, in contrast, the deletion of the RING motif in PIAS4 blocked this targeting [502]. The loss of the RING finger domain from PIAS1 failed to block P53-mediated transcription [504], despite the fact that a study proposed that this impact could possibly be cell-type specific [501].

The modification of proteins by SUMO possesses several functions, including protein stability, apoptosis, response to stress, progression throughout the cell cycle, nuclear cystolic transport and transcriptional regulation [505, 506]. Thus, SUMO can easily, rapidly and reversibly adjust the properties of proteins and also negate the requirement for *de novo* protein synthesis. This makes such proteins suitable regulators for fine-tuning the repair of DNA, in addition to the pathways of damage response [507]. The contribution of ubiquitin, along with SUMO, in terms of the key pathways of genome maintenance, has been previously reviewed [7, 508]. It has been determined that the proteins of SUMO E3 increase the effectiveness of SUMO attachment simply by binding with both UBC9 (the E2 enzyme) and the substrate, consequently behaving as bridging factors. SUMO is simply triggered by the E1 enzyme and is directed to the E2 SUMO conjugate enzyme (UBC9) [509].

PIAS has been observed to have much wider functions in the transcriptional and cellular regulation of tissue development, in addition to carcinogenesis. It interacts with various other nuclear proteins; for example, nuclear hormone receptors, such as the androgen receptor (AR) [510] ER [511], P53 [512], MDM2 [513], BRCA1 [514] and MTA1 [515]. The exact roles of these proteins in BC are still unclear.

The declaration that SUMO of several replications and repair proteins enhance immediately after DNA damage delivers the attractive hypothesis that this response aids replication, in addition to facilitating repair. The recruitment of PIAS SUMO E3s (homologs of yeast Siz1 and Siz2) to DSB sites in human cells, as well as the impairment of HR, once SUMO is defective, helps support this hypothesis. It also offers powerful proof that SUMO has a functional role in the damage response to DSBs [7, 516-518].

The PIAS family plays a significant part in proficient DNA repair [7, 516]. Immediately upon damage to DNA, PIAS1 and PIAS4 are required, in order to finish the accretion of the necessary proteins for DNA repair, such as BRCA1, to the DNA damage site [7, 516]. The *in vitro* modification of SUMO of the BRCA1/BARD1-associated RING domain 1 heterodimer dramatically enhances its ligase activity, identifying it as a SUMO-regulated ubiquitin ligase [516]. PIAS1 has been recognised as a cofactor that prevents the transcriptional activation potential of STAT1, in addition to augmenting the transcriptional activity of nuclear hormone receptors, whereas PIAS4 represses STAT1 and AR without the

need to interfere with DNA binding. PIAS1 is required for complete BRCA1 recruitment towards a damaged DNA site, quite possibly throughout BRCA1 and RAP80 interaction, while PIAS4 is needed earlier in the DNA damage repair cascade, in order to recruit other DNA repair proteins [519]. Depletion of PIAS1 and PIAS4 induces sensitivity to irradiation and cisplatin in U2OS cells (human osteosarcoma cell line expressing wild type P53) [7, 497, 516].

PIAS1 highly promotes ER SUMO in the occurrence of E2 ligands as well as Tamoxifen. As a result, the SUMO of ER is ligand dependent: this indicates that hormone binding, in addition to its receptor configuration alteration, is essential for its interaction with the modification machinery of SUMO [511]. Despite the fact that PIAS1 provides E3 ligase activity, it is not necessary for the regulation of ER. PIAS1 has also been suggested as a factor in the regulation of innate immunity through epigenetic mechanisms [520]. It has been proposed that PIAS1 could possibly control oncogenic networks, through its ability to inhibit the tumour suppressor of P53, STAT proteins or BRCA1 [7, 501, 521]. However, the roles of PIAS1 in cancer cells seem to be specific. In prostate cancer, a high expression of PIAS1 increases proliferation by P21 inhibition [522], whereas a decrease in its expression stimulates the development of colon cancer [523].

In terms of the observation that SUMO usually targets various proteins appearing at the same stage of DSB repair, or perhaps many different subunits of a complex [517, 524], the influences of SUMO may be accomplished through the simultaneous modification of an array of functionally related proteins. These factors allow for difficulty in dissecting the functions of the SUMO of each substrate, as well as determining those substrates whose SUMO attributes the most to the survival of cells under specific circumstances. After the formation of a DSB, cells stimulate protein kinases of DDR, such as ATM, ATR and DNA-PK. Protein kinases then trigger phosphorylation of histone H2AX, in addition to the accumulation of proteins such as MDC1, BRCA1, CtIP and RNF8 into IR-induced foci (IRIF), which enhance the signalling of DSB and promote DSB repair [525, 526].

5.2 Hypothesis

The attachment of SUMO to target proteins in response to DNA-DSB regulates various cellular functions [509, 527]. Galanty et al [7] demonstrated that SUMO1, SUMO2 and SUMO3 accumulate at DSB sites in mammalian cells, together with the accrual of SUMO1 and SUMO2/3 in need of the E3 ligase enzymes PIAS4 and PIAS1. PIAS1 and PIAS4 are recruited to damage sites through mechanisms requiring their SAP domains; they are also essential for the effective association with BRCA1 in such regions. PIAS1 and PIAS4 promote DSB repair and confer the resistance of IR. These results thus present PIAS1 and PIAS4 as DDR factors and demonstrate how coordinated SUMO and ubiquitylation manipulate the recruitment of proteins to DSB sites [505, 506]. It is thus hypothesised that

SUMO contributes to the aggressive nature of BC particularly those associated with features similar to breast carcinoma arising in patients with *BRCA*1 germline mutations.

5.3 Aim

The aim of this chapter is to investigate the roles of SUMO markers; PIAS1, PIAS4 and UBC9 in BC, using IHC, TMA and RPPA to determine the association between the SUMO markers, pathological features, expression of tumour biomarkers and clinical outcomes.

5.4 Materials and Methods

A previously described in Chapter 2

5.4.1 Patient Samples

All data are as previously described in Chapter 2 Section 2.1.1. Three cohorts were used: A) 1904 unselected cases of female primary operable invasive tumours between 1986 and 1998, B) 386 cases selected from a consecutive series of primary operable ER negative tumours between 1998 and 2007 and C) 24 well-characterised series of breast tumours from patients with known *BRCA1* germline mutations. However, HRMA with employing PCR was used for *BRCA1* mutation detection in group C (this was performed by Dr Ahmed Benhasouna). All cases were obtained from the well-characterised Nottingham Tenovus primary breast carcinoma series.

5.4.2 Available Biomarkers' Data

Data on a wide range of biomarkers of known clinical and biological relevance to BC were accessible and saved on a web-based interface (Distiller; Slidepath Ltd, Dublin, Ireland). These include, ER, PgR, HER2, CK5, CK17, CK14, tumour suppressor proteins (P53 and PTEN), and cell proliferative marker (Ki-67) [270-272, 331].

5.4.3 Immunohistochemistry

As previously described in Chapter 2 Section 2.1.3.Three markers of SUMO (PIAS1, PIAS4 and UBC9) were investigated in this chapter.

5.4.3.1 Immunohistochemical Antibody Labelling using the Novolink Detection Method

As previously described in Chapter 2 Section 2.1.3.1. In this chapter all the markers were stained by the author. All these markers have already been previously successfully stained on TMA [528, 529].

5.4.3.2 Optimization of Antibodies used for IHC

As previously described in Chapter 2 Section 2.1.3.2. In addition to Western blotting, specificity of staining was confirmed by application of negative (with omission of the primary antibody) and positive controls. Positive controls were used according to the manufacturer's datasheet and/or from the human protein atlas available at http://www.proteinatlas.org/. This helped not only to test the specificity of staining but also to assess the pattern and intensity of protein expressions in the appropriate tissue. Details of the negative and positive controls used are summarised in Table 5.1 Moreover, some control TMA slides containing a variety of BC cases with some containing cores from different areas of the same cases in addition to normal parenchymal elements were used during optimisation to assess the degree of expression heterogeneity.

Table 5.1 Immunohistochemistry Positive and Negative Controls of Antibodies Used in this Chapter

Antibody	Positive control	Negative control	Reference
PIAS1	BC tissue	BC tissue	Abcam/ human protein atlas available at http://www.proteinatlas.org/
PIAS4	BC tissue	BC tissue	Novus Biologicals/ human protein atlas available at http://www.proteinatlas.org/
UBC9	Normal liver tissue or BC tissue	BC tissue	Novus Biologicals/ human protein atlas available at http://www.proteinatlas.org/
Stating of positive and negative controls was performed together in the same run. Negative staining was performed without adding the antibody and showed no staining. All were performed on TMA. All these markers have already been previously successfully stained on TMA [528, 529]			

5.4.3.3 Immunohistochemistry Scoring

As previously described in Chapter 2 Section 2.1.3.3. For evaluation of IHC of the TMA, a modified H-score was used [282]. For H-score, both the intensity of staining and the percentage of stained cells were considered within each tissue core. Staining intensity was scored as 0, 1, 2 or 3 for negative, weak, moderate and strong, respectively. The proportion (percentage) of positive cells for each intensity was subjectively estimated. Multiplication of the two indices (intensity and percentage positive cells) provided final scores that range from 0 to 300.

All the markers were scored by the author and re-scored each marker with at least 30% of a randomly chosen subset of cases. A statistical agreement test was performed (Kappa value) for each marker, where there was good agreement (≥0.5), and an average was taken. If there were discrepancies, the highest scoring was taken. Kappa values are summarised in Table 5.2.

PIAS4 were scored using light microscopy, whereas PIAS1 and UBC9 were scored visually using high-resolution digital images, using a web-based interface (Distiller, Slidepath Ltd., Dublin, Ireland).

Table 5.2 The Statistical Agreement between Different Scoring of Antibodies Used in this Chapter.

Markers	Percentage of re-scoring	Kappa value
PIAS1.n	30%	0.7
PIAS1.c	30%	0.9
PIAS4	100%	0.69
UBC9.n	30%	0.59
UBC9.c	30%	0.68
Kappa test was performed on IBM SPSS 21.0. An average was taken after re-scoring		

5.4.4 Specificity of the Antibodies by Western Blot

As previously described in Chapter 2 Section 2.1.4.4. Western blot was used on all markers except PIAS4. PIAS4 marker was previously stained by the author before starting cell culture work. A mixture of different cell lysates to detect only the specificity of an antibody has been applied in different studies and showed its reliability [283, 284]. In the present study, PIAS1 and UBC9 were detected in a mixture of different lysates (MCF-7, MDA-MB-231, HeLa BRCA1 and its control). All the antibodies tested in this chapter share the same positive controls which are MCF-7 and HeLa BRCA1 cell lines. The pre-stained marker 'full range rainbow marker' (Invitrogen Life Technologies) was used as a molecular weight standard. http://www.proteinatlas.org/ provides profile data for positive controls of all the markers used in this study. Table 5.3 summarises the details of WB for each marker.

Table 5.3 List of Antibodies Tested by Western Blot on a Mixture of Different Cell Lines.

Antibody	Cell lines	Specific positive cell lines*
PIAS1	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
UBC9	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
Cell lines and reagents were obtained from the group of Dr Madhusudan Srinivasan. Thawing and freezing procedures were done by Nada Albarakati. Passages, Bradford assay and gel electrophoresis were done by the author. Passages used in W.B were as follows; HeLa BRCA1; passages 29&30, HeLa BRCA1 control; passages 15&16, MCF-7; passages 25&26, MDA-MB-231; passages 15&16. * Data available at http://www.proteinatlas.org/.		

5.4.5 Reverse Phase Protein Microarray

RPPA was carried out as previously described in Chapter 2 Section 2.1.5. Cell lines preparation and protein extraction and interpretation of the results were carried out by the author. However, RPPA run and analysis was carried out by Dr Ola Nejm (Immunology, School of Life Sciences, University Hospital, Nottingham, UK) as a collaborative project. PIAS4, this marker was previously stained by the author before starting the collaboration with Dr Ola Nejm.

5.5 Statistical Analysis

All statistical analyses were done by the author using IBM SPSS statistic 21.0 software. For all statistical tests, a two-sided *P-value* of <0.01 was considered statistically significant.

5.5.1 The Determination of the Optimal Cut-offs

As described previously in Chapter 2 Section 2.1.6.1, the cut-off points of the biomarkers were dichotomised and obtained using different approaches: a) using the mean or median of the H-score of the staining according to distribution pattern whether normally or not normally distributed, or b) using x-tile software (version 3.6.1, 2003-2005, Yale University, USA). x-tile was used for PIAS1 and PIAS4 (if the mean/median was taken then more than three quarter of the cases will be considered negative). Median and mean were used as cut off for UBC9 nuclear and cytoplasmic expression respectively. Table 5.4 shows the details of the antibodies used in this chapter. Details of H-score histograms of all markers are presented in Appendix 3.

Table 5.4 Sources, Dilution, Cut-offs Point and Pre-Treatment Conditions of the Antibodies Used in this Chapter.

Antibody	Clone	Source	Dilution IHC	Dilution W.B RPPA	[+]Distribution	Cut-offs	IHC kit
PIAS1	Ab32219	Abcam	1:425 1h	1:1,000 1:1,000	Nuclear/ cytoplasmic	Nuclear ≥35H-score Cytoplasm ≥95H-score, x-tile.	Novolink
PIAS4	NBP1-31215	Novus Biologicals	1:250 1h	NT NT	Nuclear	≥160 H-score, x-tile	Novolink
UBC9	Ep2938Y	Novus Biologicals	1:225 1h	1:500 1:250	Nuclear/ cytoplasmic	Nuclear ≥160H-score, median Cytoplasm ≥200H-score, mean.	Novolink

IHC= immunohistochemistry. W.B= Western blotting. NT= not tested. All the antibodies were pre-treated in citrate antigen retrieval pH=6.0 in microwave for 20 minutes and stained on TMA. [+] Cellular localisation. 1h= 1 hour incubation with the primary antibody at room temperature.

5.5.2 Univariate Analysis with Clinico-Pathological al Parameters and Tumour Markers

The differences between all markers, with regards to clinico-pathological features, or with other tumour markers were analysed using the Pearson Chi-Squared test (x^2). Consequently, x^2 was also used in order to examine the inter-relations between markers themselves. In addition analysis of continuous variables was performed using the appropriate statistical test Pearson's correlation and ANOVA. One way ANOVA was used to find out which of different BC classes (by IHC or RPPA) were significantly different from each other (post hoc test; Tukey).

5.5.3 Univariate Analysis with Patients' Outcome

Patient's alive or those who died for any reason other than BC were not included. The Kaplan-Meier method was used to generate a univariate survival curve and the differences in survival among the biomarkers were evaluated using the log-rank test.

5.5.4 Multivariate Analysis with Patients' Outcome

If a marker in univariate analysis was statistically significant with patients' outcomes, then Cox regression was applied for multivariate analyses to test for confounders and prognostic or predictive independency of the investigated biomarker from standard prognostic/predictive factors such as tumour grade, tumour stage, and tumour size.

5.6 Results

5.6.1 Expression of SUMO Markers in Invasive Breast Cancer

Western blotting validated the specificity of antibodies in SUMO pathway, which was deemed validated by a single band at the correct protein size for both PIAS1 and UBC9 (see Figure 5.1).

PIAS4 was stained before applying W.B, however, positive and negative tissue controls were used to validate the specificity of the antibodies. Breast tumour tissue was used as a positive/negative control for PIAS4 and PIAS1 (Figure 5.2), and normal liver tissue as positive control and breast tumour tissue as negative control for UBC9 (Figure 5.3) (http://www.proteinatlas.org/).

Using IHC, PIAS4 showed nuclear staining in invasive breast tumours, whereas PIAS1 and UBC9 showed both nuclear and cytoplasmic staining. Figures 5.2 and 5.3 outline the staining pattern of these markers by IHC. Table 5.5 displays the frequencies of SUMO proteins in sporadic and known *BRCA1* germline mutations BCs, while Figure 5.4 represents the distribution of SUMO markers (mean) in different classes of BC by IHC. The four classes

included were classified based on BRCA1 and ER status (protein expression). Class 1; sporadic BRCA1 negative and ER negative, class 2; sporadic BRCA1 positive and ER positive, class 3; known *BRCA1* germline mutations BC (hereditary)/ER negative, and finally, class 4; known *BRCA1* germline mutation BC (hereditary)/ ER positive.

There was a strong expression of nuclear PIAS1, nuclear UBC9, (Figure 5.4) in sporadic $BRCA1^{+}/ER^{+}$ cancers, compared to sporadic $BRCA1^{-}/ER^{-}$ (for all $P<0.0001$). Additionally, UBC9.n showed a significant difference between sporadic $BRCA1^{+}/ER^{+}$ cancers and *BRCA1* hereditary BC that showing ER negativity ($P<0.0001$). In contrast, cytoplasmic expression of UBC9 in terms of *BRCA1* hereditary BC that showing ER positivity was the weakest amongst the other classes, and was highly significant between sporadic $BRCA1^{-}/ER^{-}$ cancers vs. *BRCA1* hereditary BC that showing ER^{+} ($P=0.002$), or both ER^{+} cases in sporadic or hereditary cases ($P=0.002$), and finally between the two groups of *BRCA1* hereditary BCs that showing ER^{-} vs. ER^{+} ($P=0.005$).

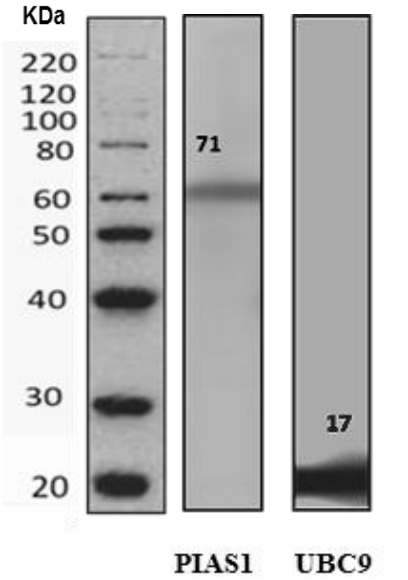

Figure 5.1 Detection of SUMO proteins level by Western blot in a mixture of cell lines, MDA-MB-231, MCF-7, HeLa BRCA1 and its control. The predicted size of each protein is labelled on the band. Passages used in W.B were as follows; HeLa BRCA1; passages29&30, HeLa BRCA1 control; passages 15&16, MCF-7; passages 25&26, and MDA-MB-231; passages 15&16.

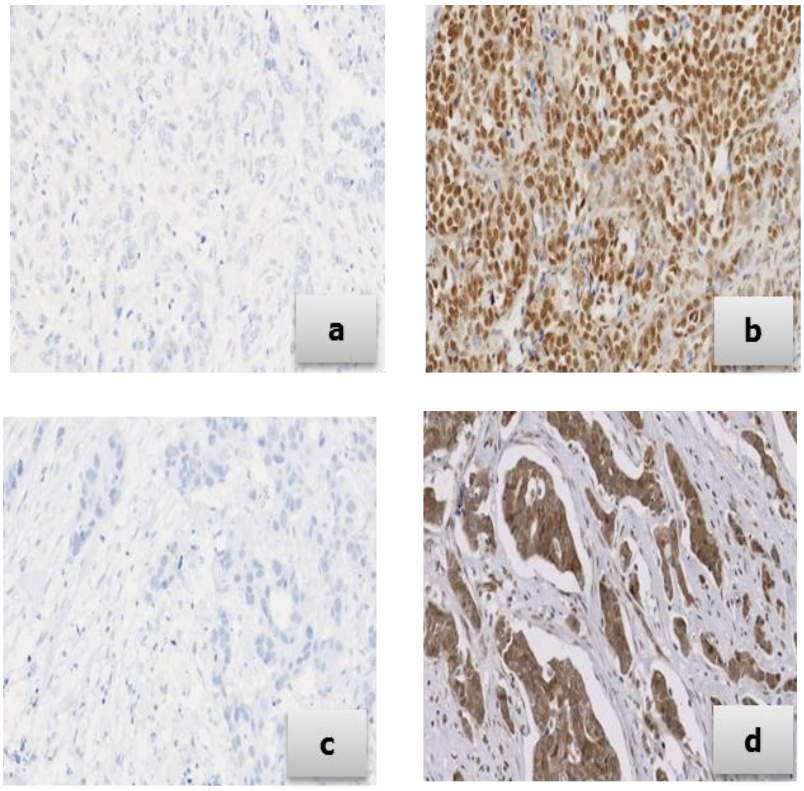

Figure 5.2 The immunostaining expression of PIAS4 and PIAS1 (SUMO proteins) detected by IHC on TMA. a; negative control of PIAS4 in invasive ductal carcinoma/ NST, grade 3 and stage 1. b; PIAS4 nuclear expression in invasive lobular carcinoma, grade 3 and stage 1, also was used as positive control. c; negative control of PIAS1 in invasive ductal carcinoma/ NST; stage 2 and grade 3. d; PIAS1 nuclear and cytoplasmic expression in invasive ductal carcinoma/NST; grade 2, and stage 1 which was also used as positive control. Magnification x20.

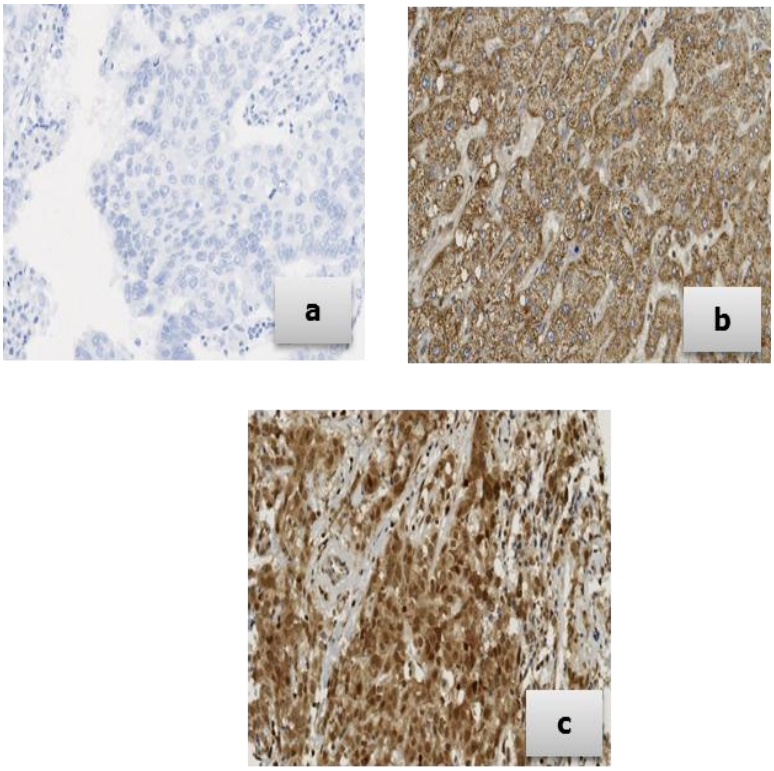

Figure 5.3 The immunostaining expression of UBC9 protein detected by IHC on TMA. a; negative control in invasive ductal carcinoma/NST; stage 2 and grade 3. b; normal liver tissue as positive control for UBC9. c; UBC9 nuclear and cytoplasmic expression in invasive ductal carcinoma/NST; grade 3 and stage 1. Magnification x20.

Table 5.5 Frequency of PIAS1, PIAS4, and UBC9 Expression in Breast Cancer.

SUMO marker	Sporadic BC		Known *BRCA1* germline mutation BC	
	(%)	Frequency	(%)	Frequency
PIAS1				
Nuclear	14.1%	180/1278	0%	0/23
Cytoplasmic	79.3%	1013/1278	91.3%	21/23
PIAS4	78.5%	1154/1470	91.7%	22/24
UBC9				
Nuclear	50.7%	751/1485	5.3%	1/19
Cytoplasmic	64.4%	957/1485	52.4%	6/19
Sporadic BC includes both unselected and ER-negative BC cases. The number of cases may be reduced due to loss cases during preparation of tissue for staining (TMA sectioning or IHC procedure).				

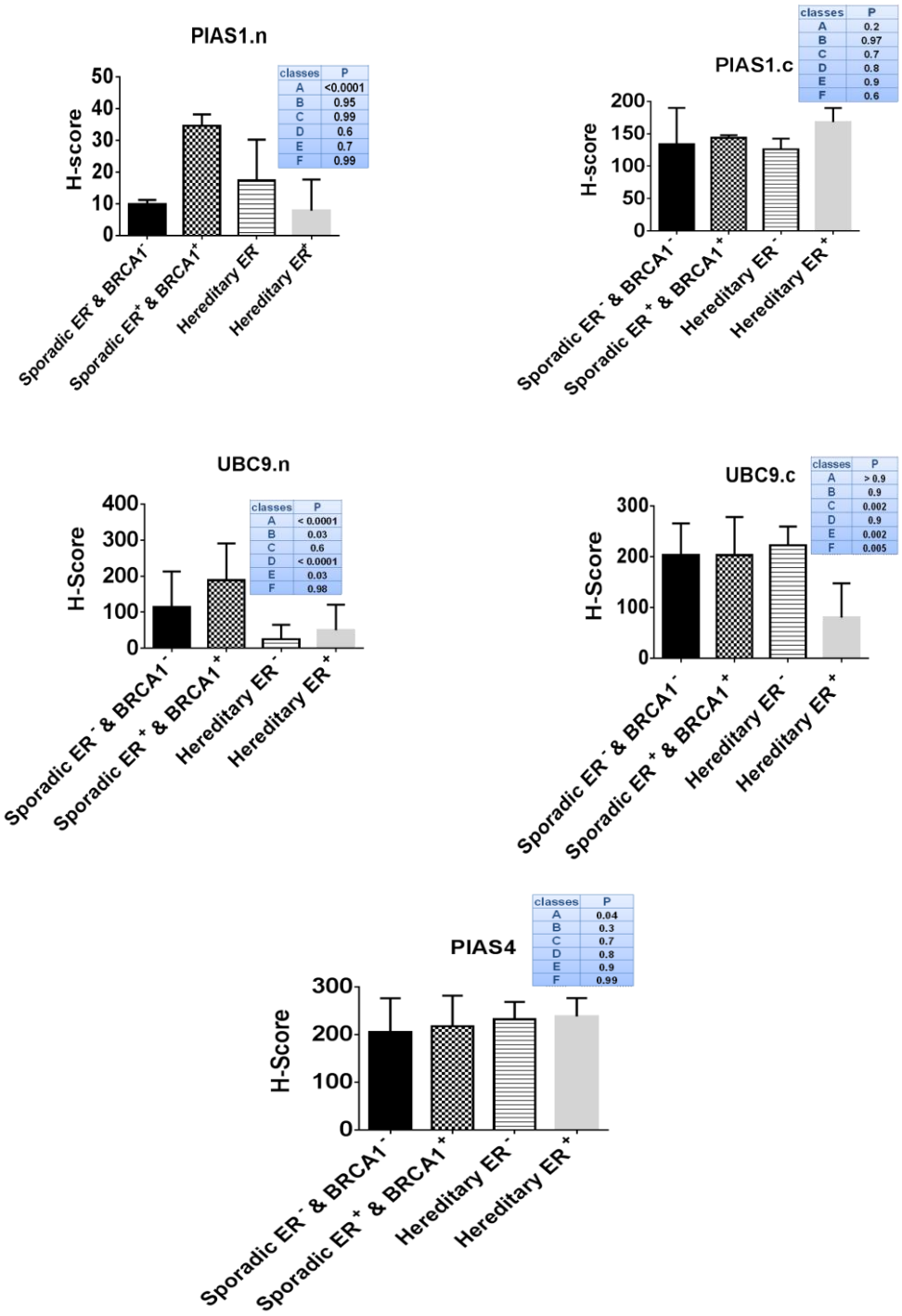

Figure 5.4 SUMO protein levels detected by IHC in breast cancer on TMA. Each bar represents different class based on hereditary or sporadic BRCA1 and ER status. n= nuclear and c= cytoplasmic expression. Error bars represent Mean (SD) and was created on H-score (ranges 0-300). A= sporadic cases [ER- & BRCA1-] vs. sporadic cases [ER+ & BRCA1+], B= sporadic cases [ER- & BRCA1-] vs. Hereditary cases [ER-], C= sporadic cases [ER- & BRCA1-] vs. Hereditary cases [ER+], D= sporadic cases [ER+ & BRCA1+] vs. Hereditary cases [ER-], E= sporadic cases [ER+ & BRCA1+] vs. Hereditary cases [ER+], and F= Hereditary cases [ER-] vs. Hereditary cases [ER+]. One way ANOVA test was used for each marker within the classes.

5.6.2 Correlation of SUMO Markers

Table 5.6 summaries the associations between the SUMO markers. There was a strong negative association between PIAS1.n, its cytoplasmic expression and positive association with UBC9.n (P<0.0001). The cytoplasmic expression of PIAS1^{+} was highly associated with UBC9.c^{+} (P<0.0001), but not nuclear expression (P=0.5). UBC9.n was positively associated with its cytoplasmic expression (P<0.0001). PIAS4 showed a positive association with both nuclear and cytoplasmic UBC9 and PIAS1.c (all P<0.0001).

Table 5.6 Correlation between SUMO Markers.

Markers		UBC9.n				UBC9.c			
		Negative N (%)	**Positive N (%)**	X^2	**P**	**Negative N (%)**	**Positive N (%)**	X^2	**P**
PIAS4	**Negative**	153(69.9)	395(48.1)	33	<0.0001	115(52.5)	266(32.3)	30	<0.0001
	Positive	66(30.1)	426(51.9)			104(47.5)	557(67.7)		
PIAS1.n	**Negative**	438(55.2)	35(30.4)	25	<0.0001	288(36.2)	45(39.1)	0.4	0.5
	Positive	356(44.8)	80(69.6)			507(63.8)	70(60.9)		
PIAS1.c	**Negative**	98(54.4)	377(51.5)	0.5	0.5	96(53.3)	237(32.3)	27.5	<0.0001
	Positive	82(45.6)	355(48.5)			84(46.7)	496(67.7)		
UBC9.c	**Negative**	386(52.9)	141(18.8)	188	<0.0001				
	Positive	344(47.1)	610(81.2)						
Markers		**PIAS1.n**				**PIAS1.c**			
		Negative N (%)	**Positive N (%)**	X^2	**P**	**Negative N (%)**	**Positive N (%)**	X^2	**P**
PIAS4	**Negative**	219(22.3)	36(24.4)	0.6	0.4	75(29.4)	143(16.5)	21	<0.0001
	Positive	762(77.7)	106(74.6)			180(70.6)	725(83.5)		
PIAS1.c	**Negative**	197(18)	65(36.7)	33	<0.0001				
	Positive	887(82)	112(63.3)						

N= number of cases. c. = cytoplasmic, n. = nuclear expression. The cut off points of positivity were as follows: ≥ 35 H-score for PIAS1.n, and ≥95 for PIAS1.c, ≥ 160 H-score for UBC9.n and ≥200 H-score for UBC9.c, ≥160 H-score for PIAS4.

5.6.3 Correlation of SUMO Markers with Other Tumour Markers

The correlation between categorical (X^2) SUMO proteins and other tumour biomarkers (regarding co-expression of cellular localisation of PIAS1 and UBC9) is summarised in Tables 5.7.

Table 5.8 shows the correlation on continuous data of SUMO proteins with other tumour markers (Pearson's correlation). Some correlations between categorical and continuous data were different therefore, only these categorical correlations were re-analysed, but regardless co-expression of cellular localisation, in order to compare each cellular localisation separately. Therefore, Table 5.9 shows the association between categorical (X^2) SUMO markers and other tumour biomarkers but regardless co-expression of subcellular localisation to make the comparison with continuous data (Pearson's correlation, Table 5.8) easier.

Regarding co-expression of cellular localisation (Table 5.7), there was a positive association between the expression of SUMO markers (PIAS1$c^+.n^-$ and UBC9 $c^+.n^+$) and BRCA1.n (both P<0.0001), ER (both P<0.0001), PgR (P= 0.001 and P<0.0001 respectively). Only UBC9 $c^+.n^+$ showed an association with BLBC (P<0.0001).

Regarding DNA-damage sensors and signal transducers, there was a high positive association between ATM and PIAS1$c^+.n^-$ or UBC9 $c^+.n^+$ (P<0.0001 and P= 0.007 respectively). In addition similar association was seen with CHK1.n^- or CHK1.c^+ and PIAS1$c^+.n^-$, PIAS4 and UBC9 $c^+.n^+$(for CHK1.n; P<0.0001, P=0.003 and P<0.0001 respectively, for CHK1.c; all P<0.0001), $CHK2^+$ with PIAS4 and UBC9 $c^+.n^+$ (all P<0.0001) (see Table 5.7).

In regards to DNA-DSB repair, there was a high positive association between HR markers such as SMC6L1.c, Rad51.n and BRCA1.c with all SUMO markers (all P<0.0001), and BRCA1.n with PIAS1.$n^-.c^+$ and UBC9 $c^+.n^+$ (all P<0.0001). However, for NHEJ, there was a positive association between KU70/KU80 or DNA-PK and all SUMO markers (all P<0.0001, except KU70/KU80 with UBC9; P=0.001).

Two down regulator markers for BRCA1 were investigated; MTA1 and ID4. MTA1.c^+ showed a high association with all SUMO markers (all P<0.0001, except with PIAS4; P=0.001) and MTA1.n with PIAS4 and UBC9 $c^+.n^+$ (P=0.003 and P<0.0001 respectively), however, ID4.c showed only a high association with PIAS1.$n^-.c^+$ (P<0.0001) (see Table 5.7).

Details of significant and non-significant associations are summarised in Appendix 3.

Table 5.8 shows the correlation on continuous data of SUMO proteins with other tumour markers. Regression analysis of continuous data confirmed the categorical data (Tables 5.7 and 5.9). However, when PIAS1.c was analysed as a continuous variable, some correlations showed different results. A significant correlation was observed with ER and Ki-67 (P=0.003, and P<0.0001 respectively; categorical data, Table 5.9), while the correlation lost significance in continuous data (P=0.7, P=0.05 respectively; Table 5.8). In addition, some other correlations lost their significance in categorical data such as PIAS1.n with MTA1.n (continuous; P<0.0001 and categorical ; P=0.02, Tables 5.8 and 5.9 respectively), PIAS1.c with SMC6L1.n (continuous; P=0.002 and categorical ; P=0.4, Tables 5.8 and 5.9 respectively), UBC9.c with Rad51.c (continuous; P<0.0001 and categorical ; P=0.3, Tables 5.8 and 5.9 respectively), and with CHK1.c (continuous; P<0.0001 and categorical ; P=0.5, Tables 5.8 and 5.9 respectively).

The high value of X^2 of all the results in this section can be referred to, i) a bias in the population of patients, ii) based on the data here, it does not seem to have any assumption issues, the main problem is normally when one of the expectation values is 5 or less, but in the findings presented here a large chi squared value is resulted when the expectations are not

less than 5. However, the data just seem to show that it is very likely that the association is not due to chance.

Table 5.7 Correlation between SUMO Markers with other Tumour Markers.

Parameters		PIAS4			
		Negative N(%)	Positive N (%)	X^2	P
PgR	Negative	175(59.7)	546(51.5)	6	0.012
	Positive	118(40.3)	515(48.5)		
TN	Negative	186(64.6)	783(72.6)	7	0.008
	Positive	102(35.4)	296(27.4)		
Rad51.c	Negative	35(16.4)	64(8.1)	13	<0.0001
	Positive	179(83.6)	731(91.9		
Rad51.n	Negative	159(74.3)	453(56.8)	21.5	<0.0001
	Positive	55 (25.7)	344(43.2)		
KU70/KU80	Negative	72(28.5)	69(7.9)	76	<0.0001
	Positive	181(71.5)	806(92.1)		
DNA-PK	Negative	67(35.6)	79(10.1)	77	<0.0001
	Positive	121(64.4)	702(89.9)		
SMC6L1.n	Negative	99(49)	236(29.9)	26	<0.0001
	Positive	103(51)	553(70.1)		
SMC6L1.c	Negative	86(42.6)	222(28.1)	15.6	<0.0001
	Positive	116(57.4)	567(71.9)		
CHK1.n	Negative	225(86.9)	695(78.4)	9.5	0.003
	Positive	34(13.1)	191(21.6)		
CHK1.c	Negative	149(57.5)	352(39.8)	14	<0.0001
	Positive	110(42.5)	533(60.2)		
CHK2	Negative	109(64.5)	268(47.6)	15	<0.0001
	Positive	60(35.5)	295(52.4)		
ATR	Negative	128(66.7)	330(50.5)	15.7	<0.0001
	Positive	64(33.3)	324(49.5)		
γH2AX.n	Negative	50(26.7)	85(11.3)	28.7	<0.0001
	Positive	137(73.3)	664(88.7)		
BARD1.c	Negative	65(29.7)	168(20.3)	9	0.003
	Positive	154(70.3)	661(79.7)		
BRCA1.c	Negative	170(71.1)	534(55.9)	18	<0.0001
	Positive	69 (28.9)	421(44.1)		
MTA1.n	Negative	108(51.2)	318(39.9)	9	0.003
	Positive	103(48.8)	478(60.1)		
MTA1.c	Negative	45(21.3)	99(12.4)	11	0.001
	Positive	166(78.7)	698(87.6)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. The cut off points of positivity were the same as previously published [273], and were as follows: ≥160 H-score for PIAS4, ≥1% for PgR, .≥50 for MTA1.n and ≥120 H-score for cytoplasmic, ≥20 H-score for CHK1.n and ≥80 H-score for CHK1.c, ≥105 H-score for CHK2, ≥ 40 H-score for γH2AX nuclear, ≥18 H-score for ATR. ≥ 40 H-score for BRCA1.c. ≥ 130 for BARD1.c, >240 H-score for SMC6L1.n and ≥230 H-score for SMC6L1.c. ≥150 H-score for DNA-PK, and ≥90 H-score for KU70/KU80. Triple negative (ER, PgR and HER-2).

Table 5.7 Correlation between SUMO Markers with other Tumour Markers Continued.

Parameters		PIAS1					
		$c^- n^-$ N (%)	$c^+ n^+$ N (%)	$c^- n^+$ N (%)	$c^+ n^-$ N (%)	X^2	P
ER	**Negative**	72(37.7)	28(26.7)	17(27)	408(46.9)	26	<0.0001
	Positive	119(62.3)	77(73.3)	46(73)	462(53.1)		
PgR	**Negative**	96(52.7)	41(38.3)	21(36.2)	463(55.4)	17.6	0.001
	Positive	86(47.3)	66(6.7)	37(63.8)	373(44.6)		
P53	**Negative**	130(71.4)	74(70.5)	39(72.2)	517(60.2)	13	0.005
	Positive	52(28.6)	31(29.5)	15(27.8)	342(39.8)		
Rad51.c	**Negative**	36(26.5)	13(15.9)	15(34.1)	44(5.9)	81.5	<0.0001
	Positive	100(73.5)	69(84.1)	29(65.9)	707(94.1)		
Rad51.n	**Negative**	106(77.4)	28(33.3)	18(40.9)	511(67.9)	60	<0.0001
	Positive	31(22.6)	56(66.7)	26(59.1)	242(32.1)		
KU70/KU80	**Negative**	38(22.4)	12(13.3)	15(27.3)	78(9.8)	30	<0.0001
	Positive	132(77.6)	78(86.7)	40(72.7)	717(90.2)		
DNA-PK	**Negative**	40(30.5)	7(10.6)	4(13.8)	79(12.9)	27	<0.0001
	Positive	91(69.5)	59(89.4)	25(86.2)	533(87.1)		
SMC6L1.c	**Negative**	55(42)	24(32.4)	18(52.9)	157(25.2)	25	<0.0001
	Positive	76(58)	50(67.6)	16(47.1)	467(74.8)		
ID4.c	**Negative**	103(52.8)	45(40.5)	39(60.9)	264(29.7)	58	<0.0001
	Positive	92(47.2)	66(59.5)	25(39.1)	625(70.3)		
CHK1.n	**Negative**	159(85)	21(20.2)	9(16.1)	699(82.6)	302	<0.0001
	Positive	28(15)	83(79.8)	47(83.9)	147(17.4)		
CHK1.c	**Negative**	54(28.9)	10(9.6)	16(28.6)	25(3)	157	<0.0001
	Positive	133(71.1)	94(90.4)	40(71.4)	820(97)		
Ki-67	**Negative**	82(47.7)	36(42.4)	29(53.7)	219(29)	34	<0.0001
	Positive	90(52.3)	49(57.6)	25(46.3)	535(71)		
ATM	**Negative**	84(64.1)	30(38.5)	13(38.2)	360(57.8)	18.4	<0.0001
	Positive	47(35.9)	48(61.5)	21(61.8)	263(42.2)		
ATR	**Negative**	68(60.7)	48(70.6)	20(58.8)	259(49.9)	13	0.004
	Positive	44(39.3)	20(29.4)	14(41.2)	260(50.1)		
γH2AX.n	**Negative**	18(14.6)	1(1.5)	1(3.4)	93(15.6)	12.6	0.006
	Positive	105(85.4)	65(98.5)	28(96.6)	503(84.4)		
γH2AX.c	**Negative**	24(19.5)	11(16.7)	11(37.9)	41(6.9)	44	<0.0001
	Positive	99(80.5)	55(83.3)	18(62.1)	556(93.1)		
BARD1.c	**Negative**	59(40.7)	14(19.4)	8(22.2)	119(18.2)	35	<0.0001
	Positive	86(59.3)	58(80.6)	28(77.8)	535(81.8)		
BRCA1.c	**Negative**	119(73.5)	44(51.2)	32(68.1)	414(55.6)	21	<0.0001
	Positive	43(26.5)	42(48.8)	15(31.9)	331(44.4)		
BRCA1.n	**Negative**	88(54.3)	30(34.9)	13(27.1)	453(60.6)	38	<0.0001
	Positive	74(45.7)	56(65.1)	35(72.9)	295(39.4)		
MTA1.n	**Negative**	72(52.9)	22(30.1)	14(37.8)	266(42.4)	11	0.01
	Positive	64(71.1)	51(69.9)	23(62.2)	362(57.6)		
MTA1.c	**Negative**	46(33.8)	11(15.1)	9(24.3)	57(9.1)	60	<0.0001
	Positive	90(66.2)	62(84.9)	28(75.7)	572(90.9)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. The cut off points of positivity were the same as previously published [273], and were as follows: ≥ 35 H-score for PIAS1.n, and ≥95 for PIAS1.c, ≥1% for PgR and ER, >34% for Ki-67, ≥50 for MTA1.n and ≥120 H-score for cytoplasmic, ≥20 H-score for CHK1.n and ≥80 H-score for CHK1.c, ≥ 40 H-score for γH2AX nuclear and ≥120 for cytoplasmic, ≥18 H-score for ATR, and ≥ 75% for ATM, ≥ 40 H-score for BRCA1.c and ≥93 for nuclear, ≥ 130 for BARD1.c, ≥230 H-score for SMC6L1.c. ≥150 H-score for DNA-PK, and ≥90 H-score for KU70/KU80, ≥5% for P53, ≥8 H-score nuclear and ≥80 H-score cytoplasmic for Rad51, and ≥ 100 H-score for ID4.c.

Table 5.7 Correlation between SUMO Markers with other Tumour Markers Continued.

Parameters		UBC9					
		$c^- n^-$ N (%)	$c^+ n^+$ N (%)	$c^- n^+$ N (%)	$c^+ n^-$ N (%)	X^2	P
ER	**Negative**	182(49.3)	217(37)	36(26.7)	183(54.8)	48	<0.0001
	Positive	187(50.7)	369(63)	99(73.3)	151(45.2)		
PgR	**Negative**	213(60.2)	268(48)	56(44.4)	205(62.7)	28	<0.0001
	Positive	141(39.8)	290(52)	70(55.6)	122(37.3)		
TN	**Negative**	231(65.3)	440(78)	114(87.7)	198(60.7)	54	<0.0001
	Positive	123(34.7)	124(22)	16(12.3)	128(39.3)		
BLBC	**Negative**	233(72.6)	415(80.7)	111(94.1)	196(68.8)	38	<0.0001
	Positive	88(27.4)	99(19.3)	7(5.9)	89(31.2)		
Rad51.n	**Negative**	184(76.7)	176(53.5)	44(59.5)	181(80.4)	58	<0.0001
	Positive	56(23.3)	153(46.5)	30(40.5)	44(19.6)		
KU70/KU80	**Negative**	50(18.9)	32(9)	12(16.4)	21(9.4)	17	0.001
	Positive	214(81.1)	325(91)	61(83.6)	202(90.6)		
DNA-PK	**Negative**	97(31.8)	22(4.3)	7(7.3)	54(17.8)	122	<0.0001
	Positive	208(68.2)	494(95.7)	89(92.7)	250(82.2)		
SMC6L1.n	**Negative**	140(42.6)	114(22.1)	26(24.8)	151(47.9)	74.5	<0.0001
	Positive	189(57.4)	402(77.9)	79(75.2)	164(52.1)		
SMC6L1.c	**Negative**	149(45.3)	126(24.4)	60(57.1)	66(21)	89	<0.0001
	Positive	180(54.7)	390(75.6)	45(42.9)	249(79)		
CHK1.n	**Negative**	205(79.5)	267(72.8)	45(55.6)	182(80.5)	24	<0.0001
	Positive	53(20.5)	100(27.2)	36(44.4)	44(19.5)		
CHK1.c	**Negative**	38(14.7)	23(6.3)	13(16)	12(5.3)	22	<0.0001
	Positive	222(85.3)	343(93.7)	68(84)	214(94.7)		
γH2AX.n	**Negative**	62(19.4)	15(2.9)	4(4.1)	77(26.4)	114	<0.0001
	Positive	257(80.6)	511(97.1)	93(95.9)	215(73.6)		
γH2AX.c	**Negative**	53(16.6)	39(7.4)	22(22.7)	8(2.7)	54	<0.0001
	Positive	267(83.4)	487(92.6)	75(77.3)	284(97.3)		
CHK2	**Negative**	147(70.3)	117(32.1)	27(37.5)	125(61.9)	97	<0.0001
	Positive	62(29.7)	248(67.9)	45(62.5)	77(38.1)		
Ki-67	**Negative**	122(38.7)	183(35.4)	53(45.7)	68(23.2)	26	<0.0001
	Positive	193(61.3)	334(64.6)	63(54.3)	228(76.8)		
ATM	**Negative**	151 (56.6)	201 (52.9)	37 (44)	150 (63.8)	12	0.007
	Positive	116 (43.4)	179 (47.1)	47 (56)	85 (36.2)		
ATR	**Negative**	181(69.3)	203(43.6)	56(58.3)	116(48.7)	47	<0.0001
	Positive	80(30.7)	263(56.4)	40(41.7)	122(51.3)		
BARD1.c	**Negative**	112(33.2)	77(14.3)	19(18.3)	60(18.9)	47	<0.0001
	Positive	225(66.8)	463(85.7)	85(81.7)	258(81.1)		
BRCA1.c	**Negative**	196(65.1)	246(51)	74(66.7)	160(55.6)	19.5	<0.0001
	Positive	105(34.9)	236(49)	37 (33.3)	128(44.4)		
BRCA1.n	**Negative**	202(67.1)	239(49.4)	37(33)	201(69.8)	70	<0.0001
	Positive	99(32.9)	245(50.6)	75(67)	87(30.2)		
MTA1.n	**Negative**	180(53.7)	131(24.6)	13(12)	190(59.7)	166	<0.0001
	Positive	155(46.3)	402(75.4)	95(88)	128(40.3)		
MTA1.c	**Negative**	115(34.2)	32(6)	24(22.2)	24(7.5)	149.2	<0.0001
	Positive	221(65.8)	502(94)	84(77.8)	294(92.5)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. The cut off points of positivity were the same as previously published [273], and were as follows: ≥ 160 H-score for UBC9.n and ≥200 H-score for UBC9.c, ≥1% for PgR and ER, >34% for Ki-67, ≥50 for MTA1.n and ≥120 H-score for cytoplasmic, ≥20 H-score for CHK1.n and ≥80 H-score for CHK1.c, ≥ 40 H-score for γH2AX nuclear and ≥120 for cytoplasmic, ≥18 H-score for ATR, and ≥ 75% for ATM, ≥ 40 H-score for BRCA1.c and ≥93 for nuclear, ≥ 130 for BARD1.c, ≥230 H-score for SMC6L1.c and >240 nuclear, ≥150 H-score for DNA-PK, and ≥90 H-score for KU70/KU80, ≥8 H-score nuclear and ≥80 H-score cytoplasmic for Rad51, and ≥ 105 H-score for CHK2. Triple negative (ER, PgR and HER-2).BLBC= TN + CK5 and CK14 and CK17.

Table 5.8 Pearson's Correlations between SUMO Proteins with other Tumour Markers.

Markers		PIAS1.n	PIAS1.c	PIAS4	UBC9.c	UBC9.n
PIAS1.n	**R**	*	*	*	*	0.182
	P					<0.0001
	N					912
PIAS1.c	**R**	-0.090				0.077
	P	0.001				0.020
	N	1278				912
PIAS4	**R**	0.047	0.207			0.246
	P	0.118	<0.0001			<0.0001
	N	1124	1124			1041
ER	**R**	0.128	0.012	0.112	-0.057	0.174
	P	<0.0001	0.703	<0.0001	0.059	<0.0001
	N	975	975	1099	1109	1109
PgR	**R**	0.085	0.098	0.164	0.059	0.154
	P	0.008	0.002	<0.0001	0.051	<0.0001
	N	966	966	1085	1098	1098
P53	**R**	-0.034	-0.010	0.007	0.101	-0.028
	P	0.288	0.746	0.812	0.001	0.347
	N	960	960	1084	1101	1101
Ki-67	**R**	-0.140	0.060	-0.063	0.131	-0.173
	P	<0.0001	0.051	0.028	<0.0001	<0.0001
	N	1072	1072	1233	1245	1245
BRCA1.c	**R**	0.004	0.157	0.113	0.110	0.046
	P	0.893	<0.0001	<0.0001	<0.0001	0.111
	N	1047	1047	1198	1184	1184
BRCA1.n	**R**	0.324	0.040	0.085	-0.013	0.287
	P	<0.0001	0.197	0.003	0.665	<0.0001
	N	1047	1047	1198	1184	1184
BARD1.c	**R**	0.070	0.256	0.104	0.269	0.266
	P	0.035	<0.0001	0.001	<0.0001	<0.0001
	N	912	912	1049	1298	1298
BARD1.n	**R**	-0.012	0.044	0.001	-0.032	0.061
	P	0.720	0.184	0.979	0.255	0.029
	N	912	912	1049	1298	1298
Rad51.c	**R**	-0.166	0.368	0.135	0.130	-0.066
	P	<0.0001	<0.0001	<0.0001	<0.0001	0.053
	N	1018	1018	1011	863	863
Rad51.n	**R**	0.422	0.138	0.195	0.028	0.248
	P	<0.0001	<0.0001	<0.0001	0.420	<0.0001
	N	1018	1018	1011	863	863
SMC6L1.c	**R**	-0.161	0.155	0.093	0.284	0.018
	P	<0.0001	<0.0001	0.003	<0.0001	0.518
	N	868	868	992	1265	1265
SMC6L1.n	**R**	-0.013	0.104	0.303	0.146	0.325
	P	0.711	0.002	<0.0001	<0.0001	<0.0001
	N	868	868	992	1265	1265
KU70/KU80	**R**	-0.021	0.151	0.464	0.127	0.135
	P	0.476	<0.0001	<0.0001	<0.0001	<0.0001
	N	1116	1116	1129	917	917
DNA-PK	**R**	0.065	0.151	0.370	0.317	0.432
	P	0.061	<0.0001	<0.0001	<0.0001	<0.0001
	N	844	844	970	1221	1221

* Repeated analysis or analysis between the marker itself. N= number of cases. R= Pearson's correlation, P=Probability value. This table shows the correlation on continuous data of SUMO proteins with other tumour markers. The cut-off points as described in table5.7.

Table 5.8 Pearson's correlations between SUMO Proteins with other Tumour Markers Continued.

Markers		PIAS1.n	PIAS1.c	PIAS4	UBC9.c	UBC9.n
CHK1.c	**R**	-0.100	0.426	0.228	0.207	0.095
	P	0.001	<0.0001	<0.0001	<0.0001	0.004
	N	1197	1197	1145	931	931
CHK1.n	**R**	0.794	0.008	0.136	-0.061	0.179
	P	<0.0001	0.786	<0.0001	0.063	<0.0001
	N	1197	1197	1145	931	931
CHK2	**R**	0.106	0.116	0.162	0.121	0.386
	P	0.007	0.004	<0.0001	<0.0001	<0.0001
	N	631	631	732	848	848
ATR	**R**	-0.077	0.066	0.078	0.173	0.101
	P	0.036	0.073	0.024	<0.0001	0.001
	N	739	739	848	1063	1063
ATM	**R**	0.108	0.045	0.073	-0.030	0.105
	P	0.001	0.181	0.022	0.356	0.001
	N	870	870	978	966	966
γH2AX.c	**R**	-0.081	0.158	0.085	0.376	0.125
	P	0.021	<0.0001	0.010	<0.0001	<0.0001
	N	818	818	937	1234	1234
γH2AX.n	**R**	0.171	0.039	0.259	0.085	0.530
	P	<0.0001	0.268	<0.0001	0.003	<0.0001
	N	818	818	937	1234	1234
ID4.c	**R**	-0.073	0.207	0.099	0.143	0.061
	P	0.014	<0.0001	<0.0001	<0.0001	0.030
	N	1153	1153	1302	1262	1262
ID4.n	**R**	0.090	-0.056	0.025	-0.069	-0.044
	P	0.002	0.055	0.358	0.013	0.114
	N	1165	1165	1325	1292	1292
MTA1.c	**R**	-0.056	0.244	0.198	0.511	0.281
	P	0.094	<0.0001	<0.0001	<0.0001	<0.0001
	N	880	880	1008	1295	1295
MTA1.n	**R**	0.133	0.069	0.205	0.090	0.480
	P	<0.0001	0.041	<0.0001	0.001	<0.0001
	N	880	880	1008	1295	1295
UBC9.c	**R**	-0.091	0.204	0.195		0.407
	P	0.006	<0.0001	<0.0001	*	<0.0001
	N	912	912	1041		1481

* Repeated analysis or analysis between the marker itself. N= number of cases. R= Pearson's correlation, P=Probability value. This table shows the correlation on continuous data SUMO proteins with other tumour markers. The cut-off points as described in table 5.7

Table 5.9 Correlation between PIAS1 and UBC9 with other Tumour Markers Regardless of Co-expression of Cellular Localisation.

Markers		PIAS1.c		P X^2	PIAS1.n		P X^2
		Negative N (%)	Positive N (%)		Negative N (%)	Positive N (%)	
ATM	**Negative**	99(59.3)	391(55.6)	0.4 0.4	445(58.9)	44(38.6)	<0.0001 16.5
	Positive	68(40.7)	312(44.4)		311(41.1)	70(61.4)	
BRCA1.c	**Negative**	152(72)	459(55)	<0.0001 20	534(58.7)	78(57.8)	0.08 0.45
	Positive	59(28)	375(45)		375(41.3)	57(42.2)	
BRCA1.n	**Negative**	102(48.1)	485(57.9)	0.01 7	542(59.4)	44(32.4)	<0.0001 35
	Positive	110(51.9)	352(42.1)		370(40.6)	92(67.6)	
ER	**Negative**	89(34.6)	439(44.8)	0.003 8.6	481(45.2)	46(26.9)	<0.0001 20
	Positive	168(65.4)	540(55.2)		582(54.8)	125(73.1)	
KU70/KU80	**Negative**	53(23.2)	91(10.2)	<0.0001 27	116(12)	27(18.5)	0.03 5
	Positive	175(76.8)	797(89.8)		851(88)	119(81.5)	
Ki-67	**Negative**	113(49.3)	256(30.4)	<0.0001 28.7	302(32.5)	68(47.9)	<0.0001 13
	Positive	116(50.7)	587(69.6)		626(67.5)	74(52.1)	
BARD1.c	**Negative**	67(36.6)	134(18.4)	<0.0001 28	179(22.3)	22(20.4)	0.6 0.2
	Positive	116(63.4)	595(81.6)		622(77.7)	86(79.6)	
CHK1.n	**Negative**	170(69.1)	722(75.8)	0.03 5	860(83.1)	30(18.6)	<0.0001 304
	Positive	76(30.9)	230(24.2)		175(16.9)	131(81.4)	
DNA-PK	**Negative**	45(27.8)	86(12.6)	<0.0001 23	119(16)	11(11.6)	0.3 1.2
	Positive	117(72.2)	596(87.4)		625(84)	84(88.4)	
MTA1.n	**Negative**	88(50)	290(41.2)	0.03 4.5	339(44.3)	36(32.7)	0.02 5
	Positive	88(50)	414(58.8)		427(55.7)	74(67.3)	
SMC6L1.n	**Negative**	61(36.3)	233(33.3)	0.4 0.55	262(34.6)	30(27.8)	0.2 2
	Positive	107(63.7)	467(66.7)		495(65.4)	78(72.2)	
Markers		**UBC9.n**			**UBC9.c**		
		Negative N (%)	**Positive N (%)**	**P X^2**	**Negative N (%)**	**Positive N (%)**	**P X^2**
BRCA1.c	**Negative**	356(60.4)	320(54)	0.02 5	270 (65.5)	407(52.8)	<0.0001 18
	Positive	233(39.6)	273(46)		142(34.5)	364(47.2)	
BRCA1.n	**Negative**	403(68.4)	276(46.3)	<0.0001 59	239(57.9)	441(57.1)	0.8 0.07
	Positive	186(31.6)	320(53.7)		174(42.1)	332(42.9)	
ER	**Negative**	365(51.9)	253(35.1)	<0.0001 41	219(43.4)	400(43.4)	0.99 0
	Positive	338(48.1)	468(64.9)		286(56.6)	522(56.6)	
PgR	**Negative**	418(61.4)	324(47.4)	<0.0001 27	270(56.1)	474(53.4)	0.3 0.9
	Positive	263(38.6)	360(52.6)		211(43.9)	414(46.6)	
Rad51.n	**Negative**	365(78.5)	220(54.6)	<0.0001 56	228(72.6)	358(64.4)	0.01 6
	Positive	100(21.5)	183(45.4)		86(27.4)	198(35.6)	
Rad51.c	**Negative**	39(8.4)	39(9.8)	0.5 0.45	32(10.3)	46(8.3)	0.3 0.9
	Positive	424(91.6)	361(90.2)		280(89.7)	507(91.7)	
CHK1.c	**Negative**	387(80)	312(69.6)	<0.0001 13	250(73.7)	449(75.6)	0.5 0.4
	Positive	97(20)	136(30.4)		89(26.3)	145(24.4)	
SMC6L1.c	**Negative**	215(33.4)	186(30)	0.2 1.7	210(48.3)	193(23.1)	<0.0001 83
	Positive	429(66.6)	435(70)		225(51.7)	641 (76.9)	

N= number of cases. c. = cytoplasmic, n. = nuclear expression. This table was created to make the comparison between the continuous and categorical data much easier, since this table does not consider co-expression of subcellular localisation, leading to compare each cellular localisation separately. Cut-off points as Table 5.7.

5.6.4 Correlation of SUMO Markers with Clinico-Pathological Features

Table 5.10 summarises the association between the SUMO markers (PIAS1, PIAS4, and UBC9) and clinico-pathological features (Pearson *X2*). The majority of tumours showed PIAS1.c^+.n^-, PIAS4$^+$ or UBC9.n^+.c^+ were associated with poor features. Thus, PIAS1.c^+.n^- or UBC9.n^+.c^+ were associated with higher tumour grade (grade III), high mitotic frequency, higher nuclear pleomorphism and moderate NPI (all P<0.0001), in addition to a tumour size in excess of 1.5cm (P=0.006, and P=0.003 respectively). The expression of PIAS4$^+$ (P=0.002) and UBC9.n^+.c^+ (P=0.005) showed a significant association with tumour stage 1. All markers were highly associated with invasive ductal no special type BC (P<0.0001 for UBC9 and PIAS1 and P=0.006 for PIAS4). Details of significant and non-significant associations are summarised in Appendix 3.

As discussed in the previous chapters, the high value of X^2 can be referred to, i) a bias in the population of patients, ii) based on the data here, it does not seem to have any assumption issues, the main problem is normally when one of the expectation values is 5 or less, but in the results presented here a large chi squared value is resulted when the expectations are not less than 5. However, the data just seem to show that it is very likely that the association is not due to chance.

Table 5.10 Correlation between SUMO Markers with Clinico-Pathological Parameters.

Parameters		PIAS1					
		c^-n^- N (%)	c^+n^+ N (%)	c^-n^+ N (%)	c^+n^- N (%)	X^2	P
Size	**≤ 1.5cm**	72(36.9)	47(42.3)	24(37.5)	256(29)	12	0.006
	>1.5cm	123(63.1)	64(57.7)	40(62.5)	628(71)		
Grade	**1**	38(19.5)	33(29.5)	11(17.2)	74(8.3)	90	<0.0001
	2	64(32.8)	33(29.5)	33(51.6)	228(25.5)		
	3	93(47.7)	46(41.1)	20(31.3)	591(66.2)		
Tubules	**1**	12(6.3)	8(7.6)	1(1.7)	25(2.9)	24	0.001
	2	49(25.9)	45(42.9)	22(37.9)	250(28.7)		
	3	128(67.7)	52(49.5)	35(60.3)	595(68.4)		
Pleomorphism	**1**	3(1.6)	2(1.9)	3(5.2)	5(0.6)	84	<0.0001
	2	84(44.4)	52(49.5)	29(50)	201(23.2)		
	3	102(54)	51(48.6)	26(44.8)	661(76.2)		
Mitosis	**1**	79(41.8)	46(43.8)	32(5.2)	178(20.5)	85	<0.0001
	2	24(12.7)	20(19)	13(22.4)	168(19.3)		
	3	86(45.5)	39(37.1)	13(22.4)	524(60.2)		
Tumour Type	**Invasive Ductal/NST**	106(55.2)	58(52.3)	25(39.7)	649(73.5)	92	<0.0001
	lobular	23(12)	5(4.5)	9(14.3)	27(3.1)		
	Atypical Medullary	4(2.1)	1(0.9)	1(1.6)	27(3.1)		
	***Mixed**	48(25)	40(36)	24(38.1)	158(17.9)		
	****other**	11(5.7)	7(6.3)	4(6.3)	22(2.5)		
NPI	**Excellent**	28(14.4)	24(22)	9(14.1)	40(4.5)	80	<0.0001
	Good	34(17.5)	25(22.9)	13(20.3)	126(14.2)		
	Moderate 1	49(25.3)	30(27.5)	21(32.8)	304(34.3)		
	Moderate 2	51(26.3)	19(17.4)	18(28.1)	252(28.4)		
	Poor	24(12.4)	10(9.2)	2(3.1)	116(13.1)		
	Very poor	8(4.1)	1(0.9)	1(1.6)	48(5.4)		

n. = nuclear and c. = cytoplasmic expression. N= number of cases. *Lobular or tubular mixed BCs. **Mucinous, Alveolar Lobular, Miscellaneous including Metaplastic, Adenoid Cystic, Spindle, and Tubulolobular. NPI= Nottingham Prognostic Index. Excellent NPI (2.08–2.4), good NPI (2.42 to ⩽3.4), a moderate prognostic I NPI (3.42 to ⩽4.4), moderate prognostic II NPI (4.42 to ⩽5.4), poor NPI (5.42 to ⩽6.4), and a very poor NPI (6.5–6.8). The cut-off points were as follows; ≥ 35 H-score for PIAS1.n, and ≥95 for PIAS1.c.

Table 5.10 Correlation between SUMO markers with Clinico-Pathological Parameters Continued.

Parameters		UBC9					
		$c^- n^-$ N (%)	$c^+ n^+$ N (%)	$c^- n^+$ N (%)	$c^+ n^-$ N (%)	X^2	P
Size	**≤ 1.5cm**	108(28.6)	190(31.4)	56(40)	81(23.8)	14	0.003
	>1.5cm	270(71.4)	416(68.6)	84(60)	259(76.2)		
Stage	**1**	261(68)	355(58.4)	84(59.6)	197(58.1)	18	0.005
	2	88(22.9)	188(30.9)	37(26.2)	116(34.2)		
	3	35(9.1)	65(10.7)	20(14.2)	26(7.75)		
Grade	**1**	47(12.2)	84(13.8)	20(14.2)	22(6.4)	45	<0.0001
	2	101(26.3)	164(27)	61(43.3)	72(21.1)		
	3	236(61.5)	360(59.66)	60(42.6)	248(72.5)		
Pleomorphism	**1**	4(1.1)	7(1.2)	6(4.4)	3(0.9)	47	<0.0001
	2	114(30.9)	183(30.6)	60(43.8)	62(18.3)		
	3	251(68)	409(68.3)	71(51.8)	273(80.8)		
Mitosis	**1**	95(25.7)	169(28.3)	60(43.8)	58(17.1)	55	<0.0001
	2	67(18.1)	115(19.2)	30(21.9)	48(14.2)		
	3	208(56.2)	316(52.7)	47(34.3)	233(68.7)		
Tumour Type	**Invasive Ductal/NST**	253(67.3)	418(69.6)	63(45)	253(74.9)	97.6	<0.0001
	lobular	17(4.5)	18(3)	26(18.6)	4(1.2)		
	Atypical Medullary	13(3.5)	11(1.8)	3(2.1)	11(3.3)		
	***Mixed**	79(21)	132(22)	42(30)	61(18)		
	****other**	14(3.7)	22(3.7)	6(4.3)	9(2.7)		
NPI	**Excellent**	28(7.3)	48(8)	16(11.6)	16(4.7)	43	<0.0001
	Good	62(16.2)	99(16.6)	35(25.4)	32(9.4)		
	Moderate 1	139(36.4)	186(31.1)	38(27.5)	118(34.7)		
	Moderate 2	98(25.7)	156(26.1)	25(18.1)	101(29.7)		
	Poor	35(9.2)	87(14.5)	19(13.8)	52(15.3)		
	Very poor	20(5.2)	22(3.7)	5(3.6)	21(6.2)		

PIAS4					
Parameters		**Negative N (%)**	**Positive N (%)**	X^2	**P**
Tumour stage	**1**	214(68.6)	665(57.9)	13	0.002
	2	69(22.1)	367(31.9)		
	3	29(9.3)	117(10.2)		
Tumour Type	**Invasive Ductal/NST**	198(64.7)	774(68)	15	0.006
	lobular	12(3.9)	63(5.5)		
	Atypical Medullary	18(5.9)	23(2)		
	***Mixed**	65(21.2)	239(21)		
	****other**	13(4.2)	40(3.5)		

n. = nuclear and c. = cytoplasmic expression. N= number of cases. *Lobular or tubular mixed BCs. **Mucinous, Alveolar Lobular, Miscellaneous including Metaplastic, Adenoid Cystic, Spindle, and Tubulolobular. NPI= Nottingham Prognostic Index. Excellent NPI (2.08–2.4), good NPI (2.42 to ⩽3.4), a moderate prognostic I NPI (3.42 to ⩽4.4), moderate prognostic II NPI (4.42 to ⩽5.4), poor NPI (5.42 to ⩽6.4), and a very poor NPI (6.5–6.8). The cut off points of positivity were as follows: ≥ 160 H-score for UBC9.n and ≥200 H-score for UBC9.c and ≥160 H-score for PIAS4.

5.6.5 Relationship between SUMO Markers and Patients' Outcomes by Univariate Analysis

SUMO markers showed no effect on patient survival (Figures 5.5-5.9). Cases showing PIAS1.n^- and received chemotherapy had better BCSS than those with PIAS1.n^+ (P=0.006, Figure 5.7a). Interestingly, negative or positive expressions of UBC9.n demonstrated no difference in BCSS after receiving chemotherapy (P=0.003, Figure 5.9a), whereas, cytoplasmic expression has worse BCSS than negative one after receiving chemotherapy (P=0.004, Figure 5.9b).

Nuclear expression UBC9^{+}, but not cytoplasmic, conferred a better BCSS after receiving endocrine treatment (amongst ER^{+} tumours) than UBC9.n^{-} (nuclear; P=0.001, Figure 5.9c, cytoplasmic; P=0.006, Figure 5.9d).

Multivariate analysis for combinations (such as PIAS1 with chemotherapy or PIAS1 with endocrine therapy) does not usually perform, because multivariate analysis applies for the main marker results (+ vs -) as combination effect may be biased by the other factor and not by the target marker.

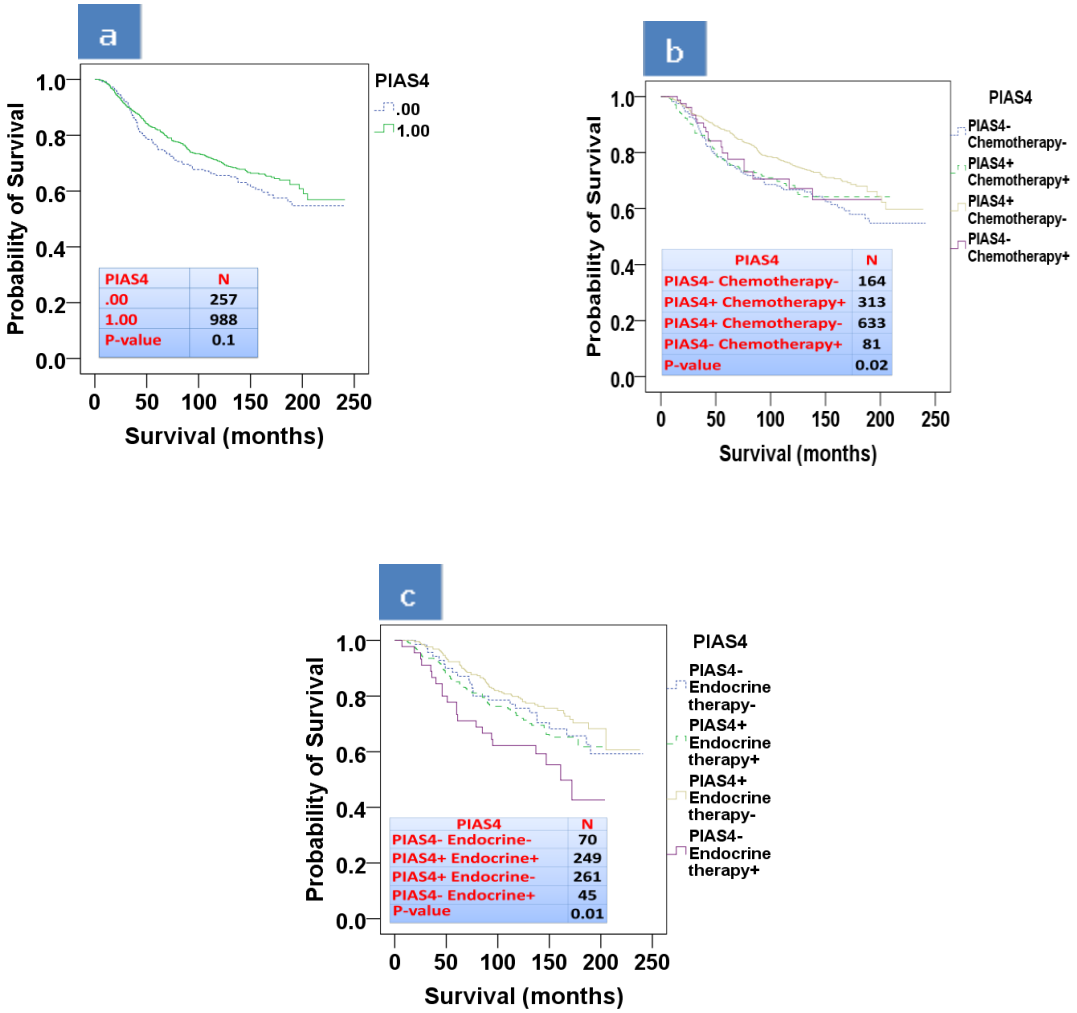

Figure 5.5 The associations between PIAS4 with BCSS and the effect of treatment on patient's outcome. N; number of cases. Only patients who died from breast cancer were considered. a; shows association between PIAS4 and BCSS , where 0= negative and 1= positive expression. b; expression of PIAS4 and BCSS based on chemotherapy in unselected cases. c; expression of PIAS4 and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. P<0.01 was considered significant. Some of ER-positive tumours did not receive endocrine therapy, because the treatment plan for those cases was as patients with a score of NPI≤ 3.4 received no adjuvant therapy.

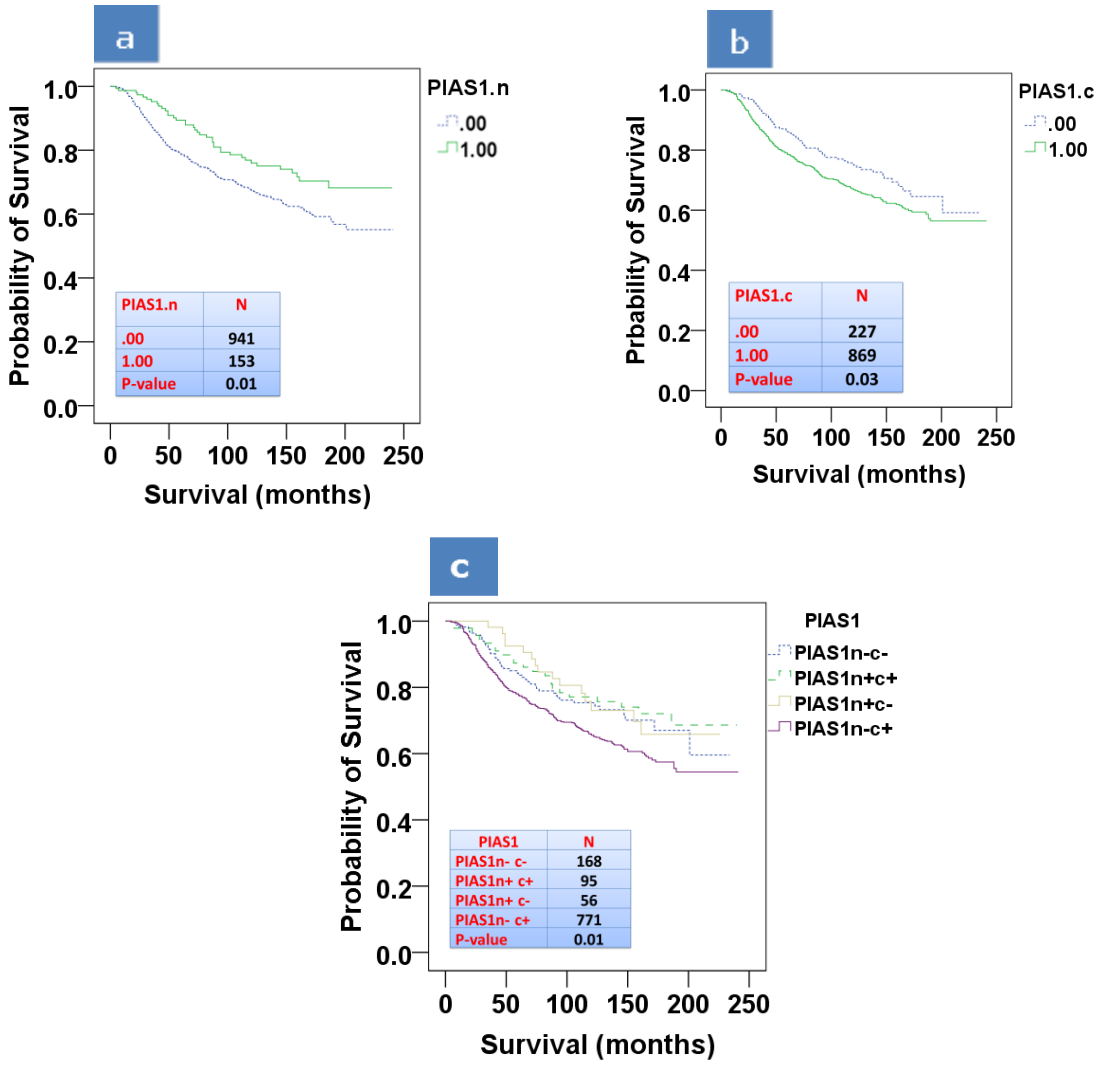

Figure 5.6 The associations between PIAS1 with BCSS. 0= negative and 1= positive expression of PIAS1. Where n= nuclear expression and c= cytoplasmic expression of PIAS1 and N; number of cases. Only patients who died from breast cancer were considered. a; shows association between nuclear expression of PIAS1 and BCSS, whereas b; shows cytoplasmic expression. c; co-expression of nuclear and cytoplasmic PIAS1 and its association with BCSS. P<0.01 was considered significant.

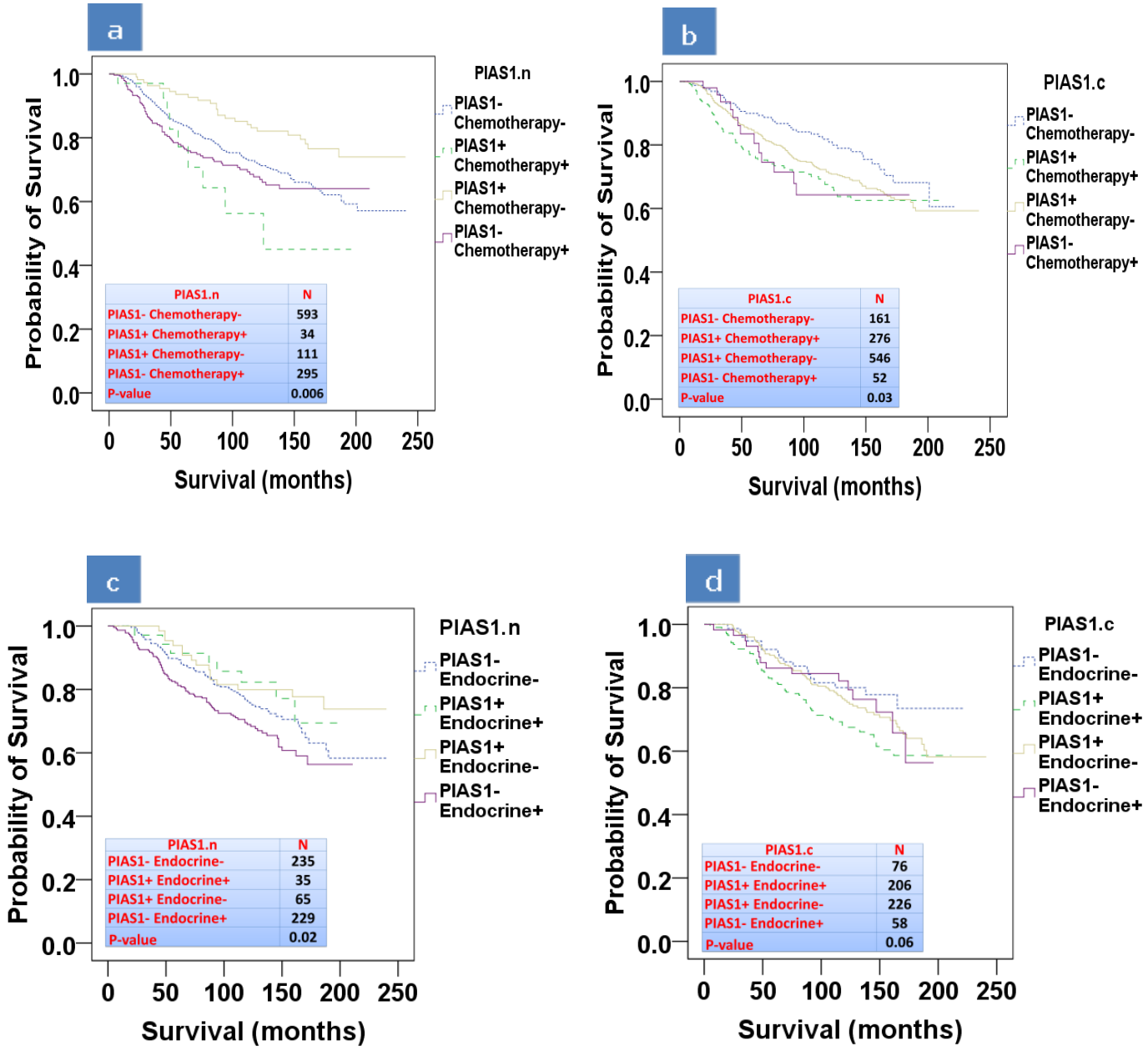

Figure 5.7 The associations between PIAS1 with BCSS and the effect of treatment on patient's outcome. N; number of cases. Only patients who died from breast cancer were considered. a; shows association between nuclear expression of PIAS1 and BCSS based on chemotherapy in unselected cases and b; cytoplasmic expression of PIAS1 and BCSS based on chemotherapy in unselected cases. c; nuclear expression of PIAS1 and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. d; cytoplasmic expression of PIAS1 and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. P<0.01 was considered significant. Some of ER-positive tumours did not receive endocrine therapy, because the treatment plan for those cases was as patients with a score of NPI≤ 3.4 received no adjuvant therapy.

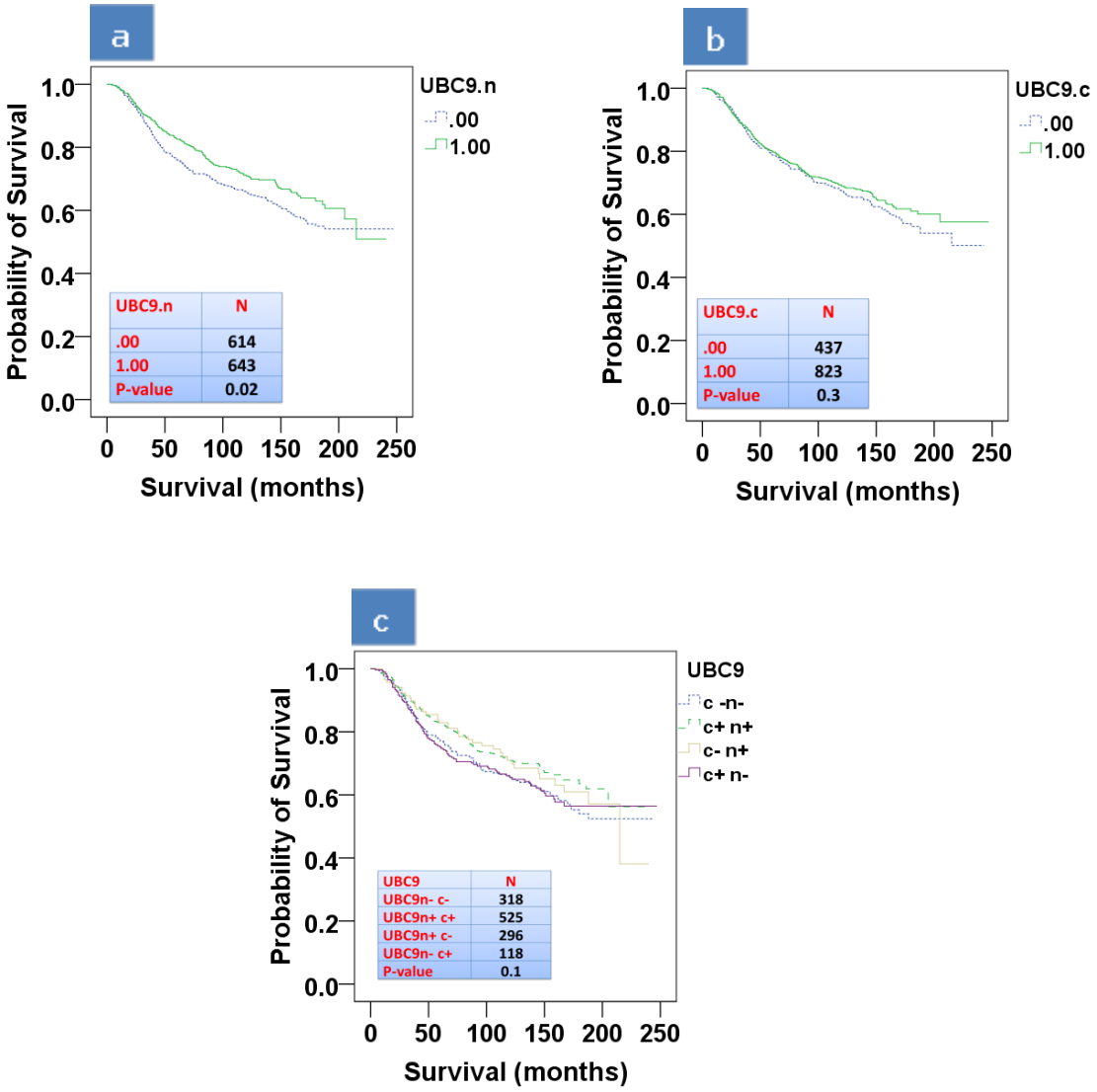

Figure 5.8 The associations between UBC9 with BCSS. 0= negative and 1= positive expression of UBC9. Where n= nuclear expression and c= cytoplasmic expression of UBC9 and N; number of cases. Only patients who died from breast cancer were considered. a; shows association between nuclear expression of UBC9 and BCSS, whereas b; shows cytoplasmic expression. c; co-expression of nuclear and cytoplasmic expression of UBC9 and its association with BCSS. P<0.01 was considered significant.

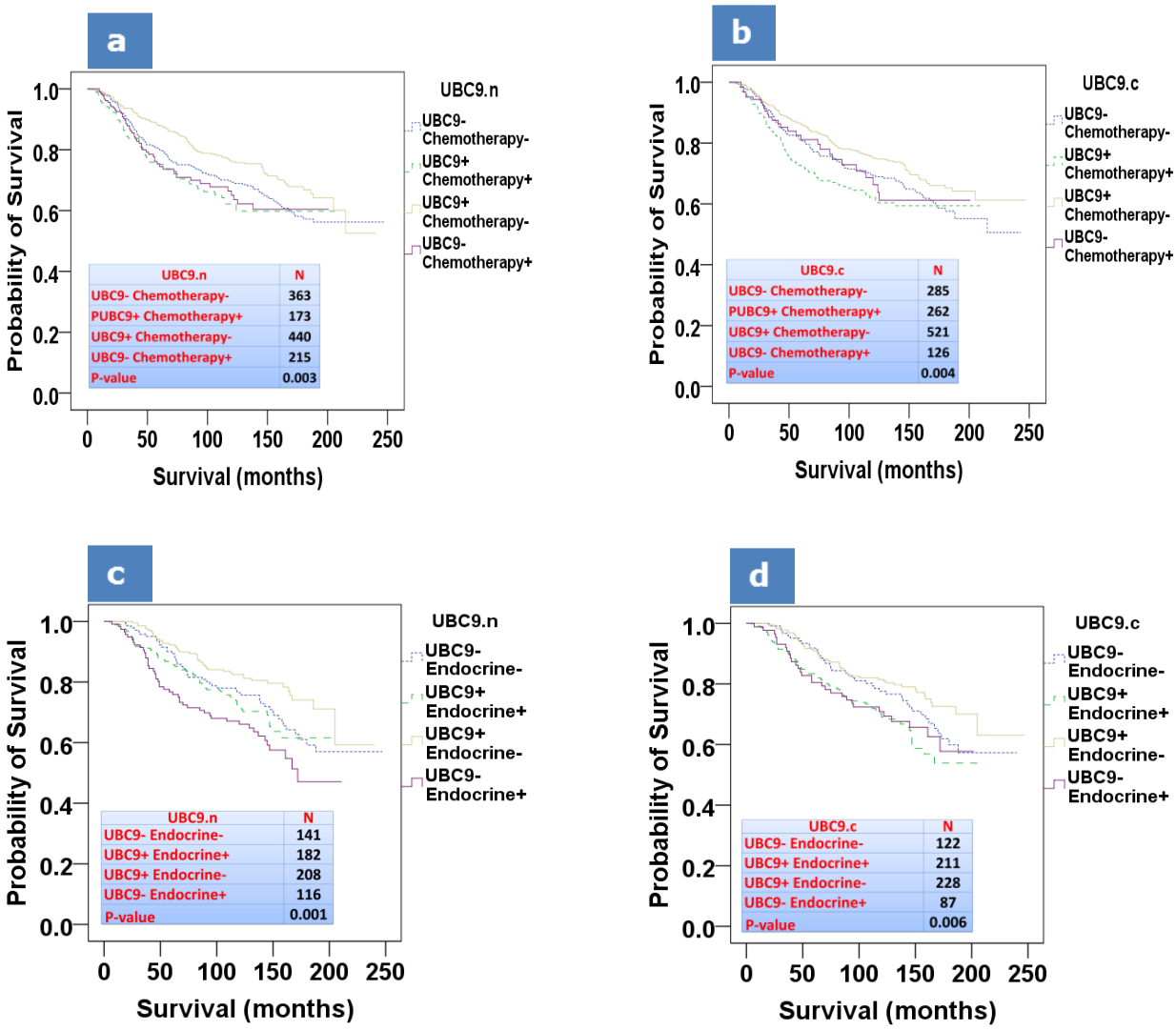

Figure 5.9 The associations between UBC9 with BCSS and the effect of treatment on patient's outcome. N; number of cases. Only patients who died from breast cancer were considered. a; shows association between nuclear expression of UBC9 and BCSS based on chemotherapy in unselected cases and b; cytoplasmic expression of UBC9 and BCSS based on chemotherapy in unselected cases. c; nuclear expression of UBC9 and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. d; cytoplasmic expression of UBC9 and BCSS based on receiving/or not endocrine therapy in only ER-positive cases. P<0.01 was considered significant. Some of ER-positive tumours did not receive endocrine therapy, because the treatment plan for those cases was as patients with a score of NPI≤ 3.4 received no adjuvant therapy.

5.6.6 Expression of SUMO Markers in Cell Lines by Reverse Phase Protein Microarray

RPPA was used to evaluate the expression level of SUMO markers in the four cell lines (BRCA1 deficient HeLaSilenciX® cells and its control cells (proficient BRCA1), MCF-7 and MDA-MB-436 cells). RPPA confirmed the IHC results, particularly with regards to nuclear expression. The RPPA findings demonstrated higher levels of expression of UBC9 in HeLa BRCA1 control cell lines (BRCA1.C) and MCF-7, when compared to other cell lines. In contrast, PIAS1 was higher in BRCA1 or MDA-MB-436 than BRCA1.C or MCF7. Only

expression of UBC9 in BRCA1 deficient HeLa vs. MCF-7 was of statistical significant (P=0.005, Figure 5.10).

However, a variation of GAPDH expression was noted and was mostly likely due to that there is variation in the fluorescence intensity between different microarray experiments. Therefore the signals of each marker were normalised to the signals of GAPDH of the same experiment performed on the same day.

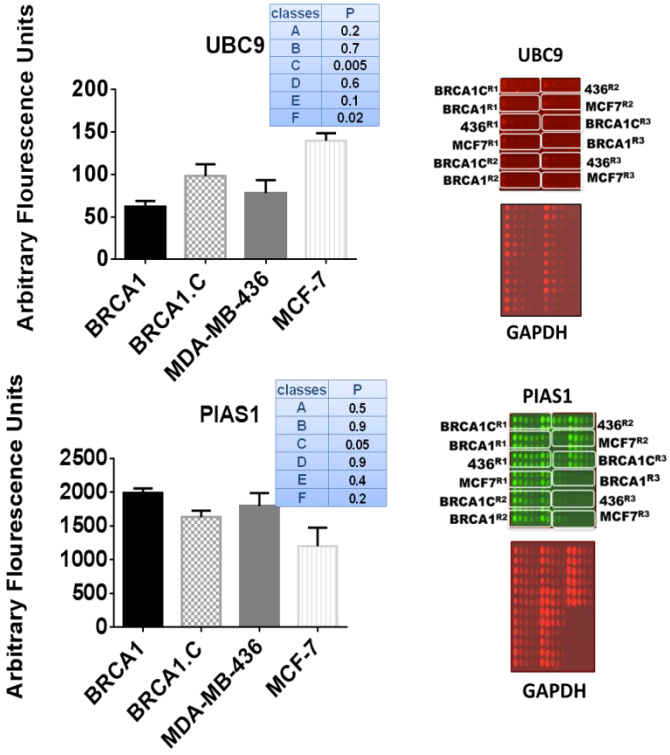

Figure 5.10 The SUMO protein levels detected by reverse phase protein microarray in different cell lines (BRCA1 deficient HeLaSilenciX® cells and its control [BRCA1 and BRCA1.C respectively], MCF-7 and MDA-MB-436 cells). For image of nitrocellulose slide spotted with different cell lysates; the red square represents the 700 channel for detection of mouse antibody while green square the 800 channel for rabbit antibody. Images of scanned nitrocellulose slides printed with extracted protein from cell lines and probed with the antibodies against the target proteins. Five 2-fold dilutions of each sample were printed in duplicate. Background was subtracted and the intensity of each spot was normalised to its corresponding GAPDH level. Each (^{R}) represents different passage of each sample, therefore, three different passages of each sample were used. Error bars represent Mean (SD). HeLa BRCA1; between passage 21 and 30, HeLa BRCA1 control; between passage 15 and 20, MCF-7; between passage 25 and 32, and MDA-MB-436; between passage 12 and 20. A= BRCA1 vs. BRCA1.C, B= BRCA1 vs. MDA-MB-436, C= BRCA1 vs. MCF-7, D= BRCA1.C vs. MDA-MB-436, E= BRCA1.C vs. MCF-7, and F= MDA-MB-436 vs. MCF-7. One way ANOVA test was used.

5.7 Discussion

SUMO is the process whereby protein function is modified, either by changing the extent of localisation or ubiquitylation, rather than just degradation attributable to ubiquitin

[502, 511]. Mammalian cells express SUMO1 in addition to the highly related protein SUMO2 and also SUMO3 (SUMO2/3). These, to some degree, are functionally unnecessary proteins [530] that are structurally associated with ubiquitin and therefore covalently attach onto target proteins by way of a SUMO-conjugation system consisting of an E1 activating enzyme (SAE1/SAE2), an E2 ligase (UBC9) and various E3 ligases (PIAS) with different types of target-protein specificities [531]. Engagement of the SUMO pathway in areas of DDR was previously described [527].

Investigating the characteristics of the SUMO proteins PIAS1, PIAS4 and UBC9 in a series of ER- and ER+ BCs, in addition to those harbouring *BRCA1* germline mutations, and investigate correlation with other tumour markers and DNA-DSB repair markers has allowed further understanding of the involvement of SUMO in repair pathways in the disease. This is the first characterisation of SUMO E2 and E3 ligases in a large cohort of BCs. Expression of PIAS1, PIAS4 and UBC9 were correlated with clinical outcome, pathological responses and the expression of other biomarkers, in order to demonstrate their role in different classes of BC.

The level of SUMO expression in BC is ambiguous, despite the fact that preliminary findings in Clevenger's et al laboratory study [532] propose that the dysregulation of PIAS expression occurs in human BCs. The expression of SUMO markers studied in this chapter, using IHC, was detected in the nucleus of the cancer cells, as anticipated [523, 533], although cytoplasmic expression was also detected using the antibodies targeted to UBC9 and PIAS1. Regarding the cellular localisation (in nucleus), the frequencies of PIAS4 and UBC9 in BC were higher than PIAS1. This is in agreement with Coppola et al who documented that a significant reduction in the protein expression of PIAS1 occurs in colon cancer [534]. Chen et al observed a high level of UBC9 in human BCs [535], while Wei et al demonstrated a high expression of PIAS4 in gastric tumours [536]. The data from this study suggests that UBC9 positivity, nuclear^{-}/cytoplasmic^{+} PIAS1 and high expression of nuclear PIAS4 are associated with more aggressive phenotypes of BCs, such as those that are ER negative or BRCA1 negative, and BRCA1 down regulator proteins (ID4, MTA1): this finding has been previously documented in other studies [535, 536]. SUMO markers also occur significantly more frequently amongst TN tumours, as opposed to non-TN tumours. TN tumours are usually highly proliferative and poorly differentiated, in addition to showing comprehensive genetic instability [204]. TN cancers share these and various additional features with *BRCA1*-associated breast tumours, which includes high incidence of P53 mutations and poor prognosis. The present study shows that an increased frequency of SUMO markers may possibly represent an additional feature shared by tumours that occur in carriers of the *BRCA1* mutation, in addition to TN cancers.

It is well known that the trafficking of proteins in and out of the nucleus regulates signal transduction, gene expression, the progression of the cell-cycle and apoptosis. Controlled nuclear transport can also be crucial for progression and is necessary in the proper response to stress. Thus, in terms of SUMO markers as nuclear localised proteins, unexpected

cytoplasmic expression may play an important role in tumorigenesis, especially as they demonstrated roles other than nuclear expression. It can be hypothesised that, once SUMO markers are excluded from the nucleus, they are unable to work properly. In the cytoplasm, SUMO markers may be entirely degraded or retained: this hypothesis needs further in vitro studies to be confirmed.

The relationship between PIAS and ERα has been previously described [511]. Mutations that halt SUMO modification damage induced transcription of ERα without the need to influence the cellular localisation of ERα. Aside from highlighting PIAS1 as an E3 ligase for ERα, Sentis et al [511] also determined that PIAS1 and UBC9 modulated ERα-dependent transcription separately from their conjugation activity with SUMO-1. In the present study, the SUMO markers showed a significant positive association with ER, which suggests that SUMO markers may play a role in hormone receptors in BC. In addition this may explain the high level of UBC9 in MCF-7 cell lines or in breast tissue expressing ER^+ and $BRCA1^+$. The expression of SUMO biomarkers in human BC amongst *BRCA1* mutation carriers and sporadic cases were investigated, which indicated that expression of SUMO proteins is aberrantly decreased more frequently amongst familial *BRCA1* tumours than in sporadic BRCA1 tumours. The sporadic BRCA1 tumours with reduced SUMO biomarkers were often ER negative. The low expression of nuclear PIAS1 (where the main function is) in this study is further verification that the majority of SUMO markers may possibly show an additional feature shared by *BRCA1* familial cancers, in demonstrating lack of ER (since the expression of PIAS1 is dependent upon the presence of ER). Thus, these results suggest that SUMO may modulate the ERα-dependent cellular response, in addition to offering a connection between SUMO and the pathways of oestrogen. However, *in vitro* studies to investigate the role of ER in SUMO are warranted. ERα deletion mutants can be constructed by PCR however, *in vitro* SUMO conjugates can be separated on SDS-PAGE and analysed by Western blotting.

The noticeable difference between IHC and RPPA used for the study of PIAS1 expression could be due to the difference in the samples used, for example using cervical cell lines (HeLa BRCA1 cell lines), and it is known that cell lines express few tissue-specific gene/protein expression [537, 538]. In addition, the composition of the cell culture medium may be precisely why patterns of protein expression differentiate cancer cell lines from breast tumour tissue. In addition, there are common variations between the conditions of cells growing *in vitro* and that of a tissue sample. Cell lines are rapidly dividing, and general variations in expression of genes comprise of an up-regulation of genes related to proliferation [537, 538]. Compounds may have a significant and diverse effect on the altered pathways between cell lines and tumour tissue. Although the study focused on the protein expression, rather than function, determination of the cell cycle phase of the cell lines may help to know if this has an effect on protein expression.

The role of UBC9 and PIAS4 has already been outlined as down-regulating the expression of BRCA1 [516, 539], in the present findings, a large number of tumours that

expressed UBC9 or PIAS4 showed a positive association with BRCA1 down-regulator proteins such as ID4 and MTA1[540], thereby provide a clue that may explain the low expression of BRCA1 protein in these tumours. However, these finding need further investigation to assess the effect of SUMO markers on BRCA1 expression.

The results presented in this chapter are in-line with the hypothesis that DSBs are repaired by one or more alternative pathways, as well as being dependent of each other [541]. In the present study, tumours that expressed SUMO biomarkers showed not only a strong expression of KU70/KU80 (NHEJ), but also a lack of expression of the HR-associated markers such as Rad51 and BRCA1. This may suggest that NHEJ, the error-prone mechanism [542], is the effective pathway in the repair of DNA-DSBs in BC. However, real time PCR could be used to confirm the detection of the accumulation of KU70/KU80 or any markers at the damage site by using fluorescent proteins against the target markers.

Markers of the cell cycle, DNA damage sensors and signal transducers such as Ki-67, P53, CHK1 and ATM, have been demonstrated to be good predictors of BRCA1 dysfunction in BC [270]. The expression of Ki-67 is related to the proliferation of abnormal cells, with poor outcomes [331]. It is possible that UBC9 and PIAS4 may play a role in the regulation of the cell cycle and however, P53 SUMO has been documented previously [503]. Park et al described the involvement of UBC9 in the regulation of the cell cycle, where UBC9 negatively regulates BRCA1 via different promoters, such as P21, P27 and Gadd45 [543]. The association of UBC9 and PIAS4 with abnormal P53 expression in BCs suggests that these tumours may experience disorganised cell-cycle control and thus prompt rapid abnormal cell division: a hallmark of tumour aggressiveness. PIAS1 may possibly act as a tumour suppressor in breast tumours, as deficiency of this gene is associated with the proliferation of abnormal cells. Further study is required on PIAS1, in order to establish its potential role as a tumour suppressor. The detection of mutations and polymorphisms in PIAS1 as tumour suppressor genes can be achieved by single-strand conformation polymorphism analysis.

Increasing evidence indicates that SUMO plays a role in protecting the integrity of the genome, in addition to modulating DNA repair [544-546]. Modification of SUMO has also been involved in the maintenance of checkpoints and the modulation of cell-cycle transitions [547]. In addition, SUMO has an effect on the repair of DNA through the cell-cycle-specific modification of DNA-repair factors, or simply by aiming towards some proteins that have roles in cell-cycle-regulated DNA-repair pathways. It has been reported that levels of CHK1 and CHK2 are regulated by ubiquitin-mediated proteolysis through two distinct E3 ligase complexes [548, 549]. In the present study, the findings show an association between at least one of the E3 proteins (PIAS1 and PIAS4) with DNA-damage sensors and signal transducers markers, indicating that these markers may be modified by SUMO, leading to a defect in the DNA damage sensors proteins and participation in the aggressive nature of BC.

ATM functions upstream as an example of BRCA1 in the same pathway, given that BRCA1 is directly phosphorylated by ATM kinase on serine residues S1423 and S1524, thus modulating BRCA1 function [550]. In the present study, SUMO markers were highly associated with at least one markers of the following; CHK1, ATM, Ki-67 and P53. Taking into account the proposition that DNA damage response is an anti-cancer barrier, the results in this study are consistent with a scenario where the primary cancer-predisposing defect (e.g., BRCA1) would probably weaken the control of genome integrity, in addition to the aberrantly enhanced results of unrepaired DSBs, resulting in the activation of ATM. This could trigger the ATM-regulated cell-cycle checkpoints and cell death pathways, which may lead to inactivation of ATM, if these kinds of lesions were to develop in the direction of malignancy. Thus, the SUMO of the BRCA1 defect, at some point, results in the improved inactivation frequency of ATM. It remains unknown whether the patients suffer from a defect in repair or that the cancer was mainly due to DNA-DSBs thus further studies are warranted to investigate and confirm this perhaps by using the Comet assay which is a standard technique for evaluation of damage or repair of DNA, biomonitoring and genotoxicity testing [551, 552]. Figure 5.11 summarises the main findings of this chapter. In response to DNA-damage the level of ATM should be increased leading to increase level of BRCA1 expression. Here the low level of ATM may explain a defect in the response to the damage or the cancer itself has other causes than a defect in DNA-DSB repair.

Knowing the subcellular location of a protein is important for understanding its functions [395]. However, in continuous data, PIAS1.c showed no correlation with BRCA1.n, but was statistically significant with nuclear PIAS1, similarly was seen with other nuclear markers such as ER, ATM, MTA1.n and CHK1.n. Therefore, it can be hypothesised that nuclear, but not cytoplasmic, PIAS1 may have the main role in association with BRCA1.n or any marker expressed in the nucleus (such as ATM and ER), in BC. Further studies are warranted to investigate the effect of cellular localisation of PIAS1 and UBC9 on their function.

When SUMO markers were analysed as continuous variables, some correlations lost/improved the significance in comparison to categorical data. It is more logic to consider the continuous variable as correlation is biological and certainly the cut-off points will reflect these correlations, but it can argue that from a clinical point of view, using markers as a dichotomised data may be more helpful for patient management such as HER-2 status in BC which is used as positive/ negative and not as continuous variables.

To conclude, the findings of this chapter confirm the previous results, in addition to revealing possible new features or roles for the high level expression of SUMO markers in relation to the negative/low expression of BRCA1 in BC, especially in terms of DNA-DSB repair pathways and DNA damage sensors and signal transducers pathways. In addition, these markers may be accustomed to screening for BRCA1, TN and BLBC.

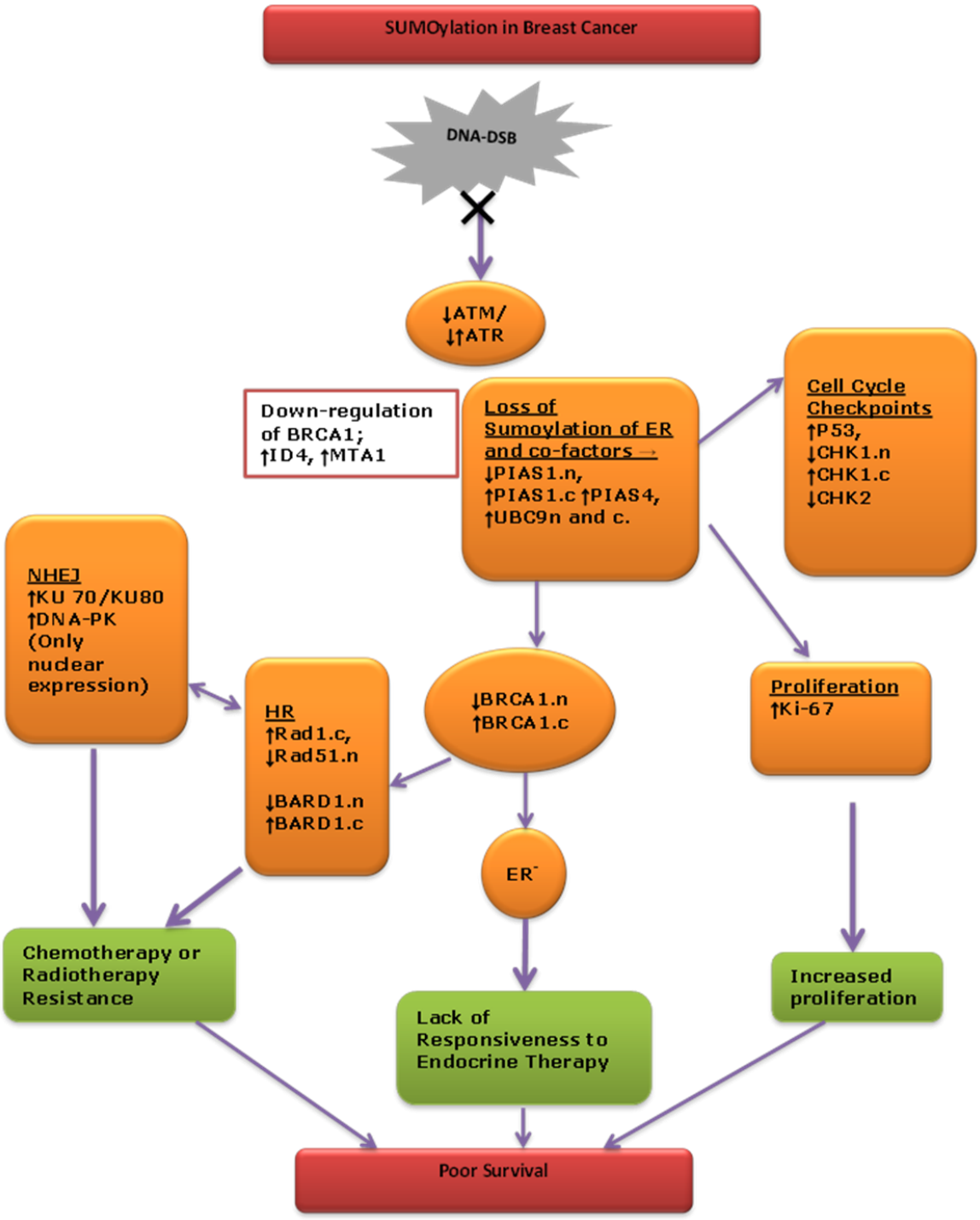

Figure 5.11 A summary of the key findings of SUMO markers and their involvement in DNA damage sensors and signal transducers, and DNA-DSB repair pathways in BC. **X** represents a defect in the response to DNA damage by showing low level expression of ATM. High expression of SUMO biomarkers showed not only a strong expression of KU70/KU80 (NHEJ), but also a lack of expression of the HR-associated markers. Increased association with MTA1 and ID4 in SUMO markers may participate in the low expression of BRCA1.

Chapter 6

6.1 Introduction

6.1.1 Nucleocytoplasmic Transport in B.C

The mechanisms of nucleocytoplasmic transport have already been described as being generally associated with several cellular processes, such as gene expression, progression of the cell-cycle, apoptosis and transduction of signals [553]. It is accepted that modulation of the nuclear import of macromolecules is essential for changing cellular phenotypes throughout progression and malignant cell transformation [554]. The transport of nucleocytoplasmic proteins, such as Nucleophosmin (NPM), is important in cellular homeostasis, as the proper and regular response to endogenous and environmental stimuli depends on the communication between the nucleus and the cytoplasm [555, 556]. This particularly applies to kinases and phosphatases, several of which transfer to and outside of the nucleus in response to oxidants or various other stressors. The separation of the nucleus and cytoplasm arises in part signalling and various other events. However, this compartmentalisation may obstruct the communication of intracellular signalling in the event that components of the nuclear transport apparatus are influenced by ROS. This result is due to the fact that the factors of nuclear transport are important cellular targets for oxidants [557]. Nucleocytoplasmic transport arises through cylindrical structures spanning the nuclear envelope, often known as nuclear pore complexes (NPCs) [553]. Despite the fact that ions, small molecules and small proteins (less than 20 kDa) are able to easily pass through NPCs by diffusion, NPCs control and restrict the passage of macromolecules (more than 40 kDa) to those with the appropriate signals [555, 556]. The direction of transport through NPCs is dependent on a transmission identified as the nuclear localisation signal (NLS) [558]; in addition nucleocytoplasmic transport is controlled by soluble receptors that identify NLS within their particular cargoes. The majority of these transport receptors are members of a large family of homologous proteins referred to as karyopherins or importins. In human cells, a minimum of 22 β importin and six α importin proteins have already been identified [558, 559]. Proteins transferred inside the nucleus carry NLS, which can be identified simply by importin α/importin β heterodimers. NLS is recognised and bound by Importin α, whereas importin β permits the complex passage through NPC. In addition, Importin α can function on its own by binding to nuclear cargoes, with no need for the assistance of importin β [560].

Karyopherin α-2 (KPNA2) is a member of the family of proteins associated with the active transport of cargo proteins that contain an NLS from the cytoplasm to the nucleus. Despite the fact that the KPNA2 function has not been fully investigated, it mediates the transport of the nucleus of some tumour suppressors [561, 562]. Studies of co-transfection with green fluorescent protein (GFP)-CHK2 and wild type or even mutant KPNA2 highlighted a role for KPNA2 in the nuclear import of CHK2 [561]. In BC, the nuclear expression of the protein KPNA2 is related to higher tumour grade and stage, positive lymph node status, negative ER and PgR receptors and increased proliferation [563, 564].

Furthermore, the expression of KPNA2 is significantly related to poor survival and was shown to be an independent prognostic factor in BC [565]. These findings suggest that KPNA2 plays an important role in the proliferation of BC.

NPM was initially identified as a highly conserved nucleolar phosphoprotein that is largely expressed in the granular component of the nucleolus [566]. More recently, however, it was asserted that NPM has a role in the regulation of cell growth; thus, proliferation, apoptosis, transformation, cancer pathogenesis and nuclear shuttling is expected [567]. The exact function of NPM in oncogenesis is controversial [568], as NPM is mutated or rearranged in many haematological disorders [569], including AML. The expression of NPM is rapidly increased in response to mitogenic stimuli, with higher protein amounts observed in highly proliferating and malignant cells [570]: this is due to the fact that the expression of NPM rises rapidly in the early G1 phase, during mitosis [571]. It has been documented that the NPM protein is overexpressed in different tumours and it is proposed as a marker for different cancers, such as gastric [567] colon [572] and breast [573] cancers.

In the nucleolus, the ubiquitin ligase F-box protein (FBW7γ) is bound and stabilised by NPM, which has been suggested as a factor in the ubiquitination and degradation of the oncogenic transcription factor Myc [574]. Without functional NPM, FBW7γ is mislocalised to the cytoplasm and degraded, ultimately causing enhanced Myc levels and the proliferation of cells. Similarly, in AML, many mutations of NPM expose a *de novo* nuclear export signal, resulting in the cytoplasmic localisation of the protein [575].

6.2 Hypothesis

It is recognised that modulation of the nuclear import of macromolecules is essential for changing cellular phenotypes throughout progression and for malignant cell transformation as a result, it is hypothesised that the nucleocytoplasmic transport markers contributes to the development and progression of BC and may have some roles in the subcellular localisation expression of many markers.

6.3 Aim

The aim of this chapter is to investigate the roles of KPNA2 and NPM in BC in order to determine the association between the subcellular localised markers and nucleocytoplasmic transport markers. In addition, it aims to investigate the pathological features of the expression of KPNA2 and NPM and their association with clinical outcomes.

6.4 Materials and Methods

As previously described in Chapter 2

6.4.1 Patient Samples

All data are as previously described in Chapter 2 Section 2.1.1. Three cohorts were used: A) 1904 unselected cases of female primary operable invasive tumours between 1986 and 1998, B) 386 cases selected from a consecutive series of primary operable ER negative tumours between 1998 and 2007 and C) 24 well-characterised series of breast tumours from patients with known *BRCA1* germline mutations. However, HRMA with employing PCR was used for *BRCA1* mutation detection in group C (this was performed by Dr Ahmed Benhasouna). All cases were obtained from the well-characterised Nottingham Tenovus primary breast carcinoma series.

6.4.2 Available Biomarkers' Data

Data on a wide range of biomarkers of known clinical and biological relevance to BC were accessible and saved on a web-based interface (Distiller; Slidepath Ltd, Dublin, Ireland). These include ER, PgR, and HER-2 [270-272, 331].

6.4.3 Immunohistochemistry

As previously described in Chapter 2 Section 2.1.3. Two markers of nucleocytoplasmic transport (NPM and KPNA2) were investigated in this chapter.

6.4.3.1 Immunohistochemical Antibody Labelling Using the Novolink Detection Method

As previously described in Chapter 2 Section 2.1.3.1. In this chapter both markers were stained by the author. Both markers have been previously successfully stained on TMA [576-579].

6.4.3.2 Optimisation of Antibodies used for IHC

As previously described in Chapter 2 Section 2.1.3.2. In addition to Western blotting, specificity of staining was confirmed by application of negative (with omission of the primary antibody) and positive controls. Positive controls were used according to the manufacturer's datasheet and/or from the human protein atlas available at http://www.proteinatlas.org/. This helped not only to test the specificity of staining but also to

assess the pattern and intensity of protein expressions in the appropriate tissue. Details of the negative and positive controls used are summarised in Table 6.1. Moreover, some control TMA slides containing a variety of BC cases with some containing cores from different areas of the same cases in addition to normal parenchymal elements were used during optimisation to assess the degree of expression heterogeneity.

Table 6.1 Immunohistochemistry Positive and Negative Controls of Antibodies Used in this Chapter.

Antibody	Positive control	Negative control	Reference
KPNA2	BC tissue	BC tissue	Abcam/ human protein atlas available at http://www.proteinatlas.org/
NPM	Normal liver tissue or BC tissue	BC tissue	Abcam/ human protein atlas available at http://www.proteinatlas.org/
Stating of positive and negative controls was performed together in the same run. Negative staining was performed without adding the antibody and showed no staining. All were done on TMA. Both markers have been previously investigated on TMA [576-579].			

6.4.3.3 Immunohistochemistry Scoring

As previously described in Chapter 2 Section 2.1.3.3. For evaluation of IHC of the TMA, a modified H-score was used [282]. For H-score, both the intensity of staining and the percentage of stained cells were considered within each tissue core. Staining intensity was scored as 0, 1, 2 or 3 for negative, weak, moderate and strong, respectively. The proportion (percentage) of positive cells for each intensity was subjectively estimated. Multiplication of the two indices (intensity and percentage positive cells) provided final scores that range from 0 to 300.

All cases were scored without prior knowledge of the patients' pathological or outcome data. The author re-scored each marker with at least 30% of a randomly chosen subset of cases. A statistical agreement test was performed (Kappa value) for each marker, where there was good agreement ($\geq$0.5), and an average was taken. If there were discrepancies, the highest scoring was taken. Kappa values are summarised in Table 6.2. Both markers were scored and re-scored by the author using high resolution digital images using a web-based interface (Distiller; Slidepath Ltd, Dublin, Ireland).

Table 6.2 The Statistical Agreement between Different Scoring of Antibodies used in this Chapter.

Markers	Percentage of re-scoring	Kappa value
KPNA2	100%	0.630
NPM	30%	0.845
Kappa test was performed on IBM SPSS 21.0. An average was taken after re-scoring		

6.4.4 Specificity of the Antibodies by Western Blot

As previously described in Chapter 2 Section 2.1.4.4. Western blot was used on both markers. A mixture of different cell lysates to detect only the specificity of an antibody has been applied in different studies and showed its reliability [283, 284]. In the present study, NPM and KPNA2 were detected in a mixture of different lysates (MCF-7, MDA-MB-231, HeLa BRCA1 and its control). NPM and KPNA2 share the same positive controls which are MCF-7 and HeLa BRCA1 cell lines. The pre-stained marker 'full range rainbow marker' (Invitrogen Life Technologies) was used as a molecular weight standard. http://www.proteinatlas.org/ provides profile data for positive controls of NPM and KPNA2. Table 6.3 summarises the details of W.B for each marker.

Table 6.3 List of Antibodies Tested by Western Blot on a Mixture of Different Cell Lines.

Antibody	Cell lines	Specific positive cell lines*
NPM	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
KPNA2	A mixture of MCF-7, MDA-MB-231, HeLa BRCA1 and its control	MCF-7 or HeLa BRCA1 cell lines
Cell lines and reagents were obtained from the group of Dr Madhusudan Srinivasan. Thawing and freezing procedures were done by Nada Albarakati. Passages, Bradford assay and gel electrophoresis were done by the author. Passages used in W.B were as follows; HeLa BRCA1; passages 29&30, HeLa BRCA1 control; passages 15&16, MCF-7; passages 25&26, MDA-MB-231; passages 15&16. * Data available at http://www.proteinatlas.org/		

6.4.5 Reverse Phase Protein Microarray

RPPA was carried out as previously described in Chapter 2 Section 2.1.5. Cell lines preparation and protein extraction and interpretation of the results were carried out by the author. However, RPPA run and analysis was carried out by Dr Ola Nejm (Immunology, School of Life Sciences, University Hospital, Nottingham, UK) as a collaborative project.

6.5 Statistical Analysis

All statistical analyses were done by the author using IBM SPSS statistic 21.0 software. For all statistical tests, a two-sided *P-value* of <0.01 was considered statistically significant.

6.5.1 The Determination of the Optimal Cut-offs

As described previously in Chapter 2 Section 2.1.6.1. The cut-off points of the KPNA2 and NPM were dichotomised and obtained using different approaches: a) using the mean or median of the H-score of the staining according to distribution pattern whether normally or not normally distributed, or b) using x-tile software (http://x-tile.software.informer.com/version 3.6.1, 2003-2005, Yale University, USA) (Table 6.4). If the cut-off by mean or median was very high (e.g H-score >200) then x-tile was considered,

however, x-tile was used for NPM because the mean of H-score was very high (mean= 226) and median of H-score was used for KPNA2. Table 6.4 shows the details of the antibodies used in this chapter. Details of H-score histograms of both markers are presented in Appendix 4.

Table 6.4 Sources, Dilution, Cut-offs Point and Pre-Treatment Conditions of the Antibodies used in this Chapter.

Antibody	Clone	Source	Dilution IHC	Dilution W.B RPPA	[+]Distribution	Cut-offs	IHC kit
NPM	Ab55708	Abcam	1:400 1h	1:5,000 1:500	Nuclear	Nuclear ≥180 H-score, x-tile.	Novolink
KPNA2	Ab84440	Abcam	1:400 1h	1:1,000 1:500	Nuclear	Nuclear ≥30 H-score, median.	Novolink

IHC= immunohistochemistry. W.B= Western blotting. All the antibodies were pre-treated in citrate antigen retrieval pH=6.0 in microwave for 20 minutes and stained on TMA. [+] Cellular localisation. 1h= 1 hour incubation with the primary antibody at room temperature.

6.5.2 Univariate Analysis with Clinico-Pathological Parameters and Tumour Markers

The differences between all markers, with regards to clinico-pathological features, or with other tumour markers were analysed using the Pearson Chi-Squared test (x^2). Consequently, x^2 was also used in order to examine the inter-relations between markers themselves. In addition analysis of continuous variables was performed using Pearson's correlation and ANOVA. One way ANOVA was used to find out which of different BC classes (by IHC or RPPA) were significantly different from each other (post hoc test; Tukey).

6.5.3 Univariate Analysis with Patients' Outcome

Patients who were alive or those who died for any reason other than BC were not included. The Kaplan-Meier method was used to generate a univariate survival curve and the differences in survival among the biomarkers were evaluated using the log-rank test.

6.5.4 Multivariate Analysis with Patients' Outcome

If a marker in univariate analysis was statistically significant with patient's outcome, then Cox regression was applied for multivariate analyses to test for confounders and prognostic or predictive independency of the investigated biomarker from standard prognostic/predictive factors such as tumour grade, tumour stage, and tumour size.

6.6 Results

6.6.1 Expression of Nucleocytoplasmic Transport Markers in Invasive Breast Cancer

Western blotting validated the specificity of the KPNA2 and NPM antibodies showing a single band at the correct protein size (see Figure 6.1). In invasive tumours, KPNA2 and NPM demonstrated nuclear staining (see Figure 6.2). Although the majority of cases showed the positive nuclear expression of NPM, only (N=20/1339) cases demonstrated positive cytoplasmic expressions. As NPM generally functions in the nucleus, and very few cases showed cytoplasmic expression only nuclear staining was considered.

Table 6.5 outlines the frequencies of nucleocytoplasmic transport proteins in sporadic and known *BRCA1* germline mutations BC (hereditary), while Figure 6.3 represents the distribution (mean) of NPM and KPNA2 in different classes of BC by IHC. The four classes included were classified based on BRCA1 and ER status. Class 1; sporadic BRCA1 negative and ER negative, class 2; sporadic BRCA1 positive and ER positive, class 3; known *BRCA1* germline mutations BC that showing ER negativity, and finally class 4; known *BRCA1* germline mutation BC and showing ER positivity. KPNA2 showed the weakest expression in ER^+ BC, irrespective of BRCA1 status. However, there was a high significant difference between sporadic $ER^+/BRCA1^+$ and known *BRCA1* germline mutations BC that showing ER negativity ($P<0.0001$). In addition a highly significant difference between sporadic $ER^+/BRCA1^+$ and sporadic $ER^-/BRCA1^-$ BC ($P<0.0001$). In contrast, NPM expression was not significantly different between the classes.

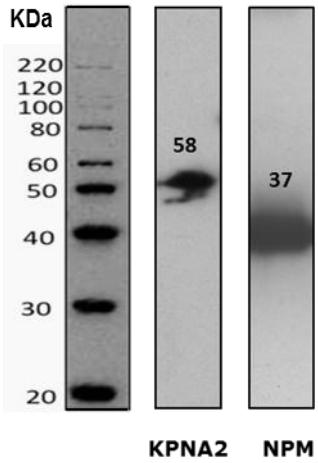

Figure 6.1 Detection of nucleocytoplasmic transport proteins level by Western blot in a mixture of cell lines, MDA-MB-231, MCF-7, HeLa BRCA1 and its control. The predicted size of each protein is labelled on the band. Passages used in W.B were as follows; HeLa BRCA1; passages29&30, HeLa BRCA1 control; passages 15&16, MCF-7; passages 25&26, and MDA-MB-231; passages 15&16.

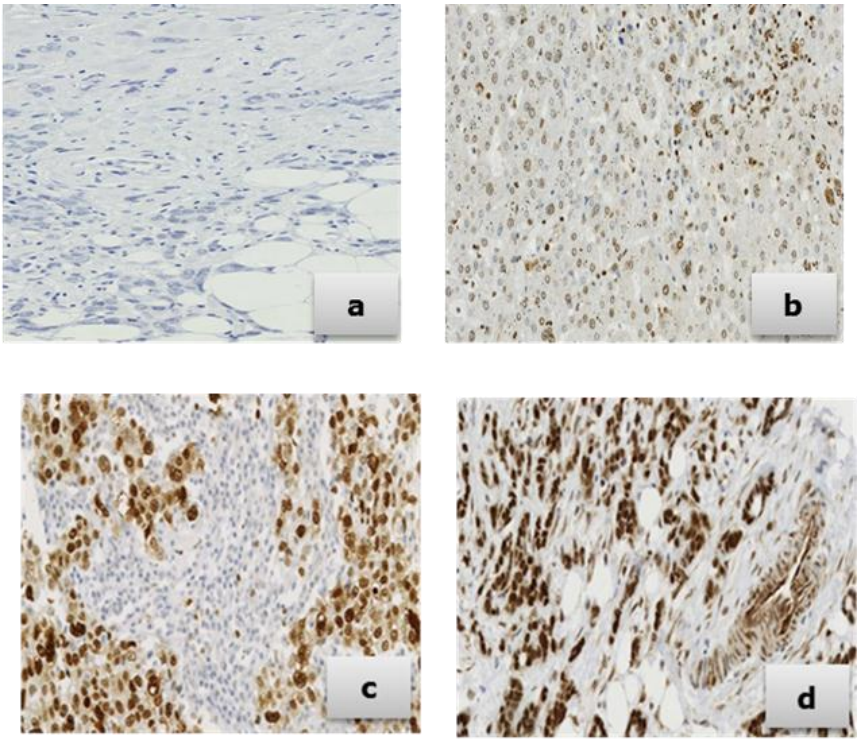

Figure 6.2 Immunostaining expression of nucleocytoplasmic transport proteins in breast cancer. Where a; negative control for both KPNA2 and NPM, in classical lobular breast cancer; stage 1 and grade 2. b; positive control for NPM in normal liver tissue. c; nuclear expression of NPM in tubular mixed breast cancer; stage 1 and grade 2. d; nuclear expression of KPNA2 in invasive ductal carcinoma/NST; stage 1 and grade 3 which is also a positive control. Magnification x20.

Table 6.5 Frequency of KPNA2 and NPM expressions in Breast Cancer.

Marker	Sporadic BC		known *BRCA1* germline mutations BC	
	(%)	Frequency	(%)	Frequency
KPNA2	51.3	715/1393	89.5	17/19
NPM	81.1	1086/1339	94.7	18/19
Sporadic BC includes both unselected and ER-negative BC cases. The number of cases may be reduced due to loss cases during preparation of tissue for staining (TMA sectioning or IHC procedure).				

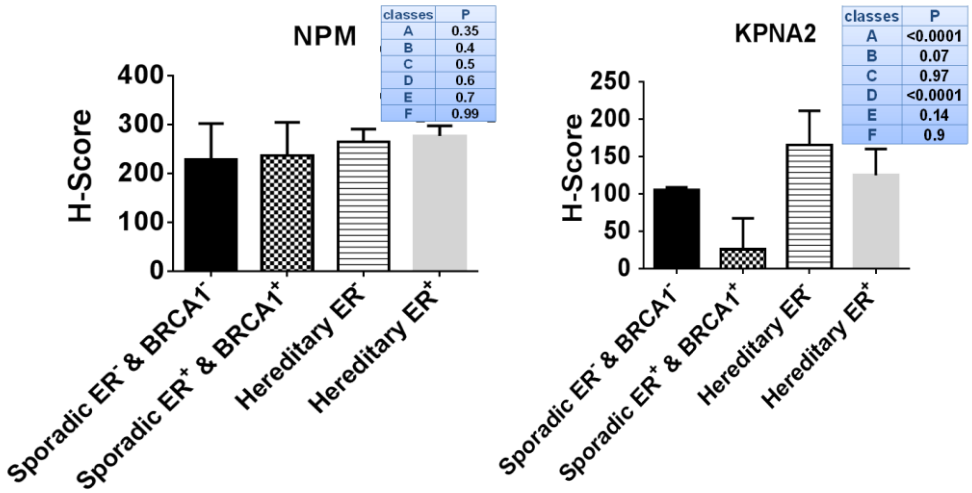

Figure 6.3 NPM and KPNA2 protein levels detected by IHC in breast cancer on TMA. Each bar represents different class based on hereditary or sporadic BRCA1 and ER status. Error bars represent Mean (SD) and was created on H-score (ranges 0-300). A= sporadic cases [ER^- & $BRCA1^-$] vs. sporadic cases [ER^+ & $BRCA1^+$], B= sporadic cases [ER^- & $BRCA1^-$] vs. Hereditary cases [ER^-], C= sporadic cases [ER^- & $BRCA1^-$]vs. Hereditary cases [ER^+], D= sporadic cases [ER^+ & $BRCA1^+$]vs. Hereditary cases [ER^-], E= sporadic cases [ER^+ & $BRCA1^+$] vs. Hereditary cases [ER^+], and F= Hereditary cases [ER^-] vs. Hereditary cases [ER^+]. ANOVA test was used for each marker within the classes.

6.6.2 Correlation of Nucleocytoplasmic Transport Markers with Other Tumour Markers

The association between the KPNA2 and NPM markers and other tumour biomarkers are summarised in Table 6.6 (Pearson x^2). There was a highly significant association between KPNA2 and ER^-, PgR^- , and TN tumours (but not between NPM and these biomarkers) (all P<0.0001). At least one of nucleocytoplasmic transport markers was significantly associated with the subcellular localisation of the HR repair markers, including $SMC6L1n^+.c^+$ (NPM and KPNA2; P<0.0001), $BRCA1n^-.c^+$ (NPM and KPNA2; P=0.009 and P<0.0001 respectively), $BARD1n^-.c^+$ (NPM; P<0.0001), and $Rad51n^-.c^+$ (KPNA2; P<0.0001), with DNA signal transducers and repair markers; $\gamma H2AXn^+.c^+$ (NPM and KPNA2; P<0.0001); $CHK1n^-.c^+$ (NPM and KPNA2; P=0.001 and P<0.0001 respectively), and with SUMO markers; $UBC9n^+.c^+$ (NPM and KPNA2; P<0.0001); $PIAS1n^-.c^+$; (KPNA2; P<0.0001). Details of significant and non-significant associations are summarised in Appendix 4.

Table 6.7 shows the Pearson's correlation using continuous data of NPM, KPNA2 with other tumour markers that showed both nuclear and cytoplasmic expression. Continuous data confirmed the categorical except that continuous PIAS1.c was not associated with KPNA2 (continuous; P=0.26; Table 6.7, categorical P<0.0001; Table 6.6). In addition, the significant was improved between ER and NPM in continuous analysis (continuous; P<0.0001; Table 6.7, categorical P=0.5; Appendix 4).

The high value of X^2 of all the results in this section can be referred to, i) a bias in the population of patients, ii) based on the data here, it does not seem to have any assumption

issues, the main problem is normally when one of the expectation values is 5 or less, but in the results presented here a large chi squared value is resulted when the expectations are not less than 5. However, the data just seem to show that it is very likely that the association is not due to chance.

Table 6.6 Correlation between Nucleocytoplasmic Transport Markers with other Tumour Markers.

Markers		NPM			
		Negative N (%)	positive N (%)	X^2	P
γH2AXn	Negative	62(31.3)	83(9.4)	67	<0.0001
	Positive	136(68.7)	800(90.6)		
γH2AX	n-c-	7(3.5)	9(1)	67	<0.0001
	n+c+	120(60.6)	720(81.5)		
	n-c+	55(27.8)	74(8.4)		
	n+c-	16(8.1)	80(9.1)		
BARD1.c	Negative	68(29.3)	180(18.1)	14.5	<0.0001
	Positive	164(70.7)	813(81.9)		
BARD1	n-c-	69(29.6)	179(18.1)	19	<0.0001
	n+c+	13(5.6)	105(10.6)		
	n-c+	151(64.8)	706(71.2)		
	n+c-	0	1(0.1)		
BRCA1	n-c-	74 (38.3)	236(26.5)	11.5	0.009
	n+c+	26 (13.5)	137(15.4)		
	n-c+	44 (22.8)	267 (30)		
	n+c-	49 (25.4)	251(28.2)		
SMC6L1.n	Negative	120(54.8)	278(29.5)	50.5	<0.0001
	Positive	99(45.2)	665(70.5)		
SMC6L1.c	Negative	101(46.1)	254(26.9)	31	<0.0001
	Positive	118(53.9)	689(73.1)		
SMC6L1	n-c-	68(31.1)	134(14.2)	63	<0.0001
	n+c+	66(30.1)	545(57.8)		
	n-c+	52(23.7)	144(15.3)		
	n+c-	33(15.1)	120(12.7)		
CHK1	n-c-	20(12.7)	33(4.7)	15.5	0.001
	n+c+	36(22.8)	145(20.6)		
	n-c+	97(61.4)	498(70.7)		
	n+c-	5(3.2)	28(4)		
CHK1.c	Negative	25(15.8)	61(8.7)	7	0.007
	Positive	133(84.2)	643(91.3)		
UBC9	n-c-	116(49.4)	195(19.9)	112	<0.0001
	n+c+	40(17)	545(46.4)		
	n-c+	70(29.8)	243(24.8)		
	n+c-	9(3.8)	86(8.8)		
UBC9.n	Negative	186(79.1)	438(44.8)	89.5	<0.0001
	Positive	49(20.9)	540(55.2)		
UBC9.c	Negative	125(53)	282(28.8)	50	<0.0001
	Positive	111(47)	698(71.2)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. The cut off points of positivity were as follows: ≥ 40 H-score for BRCA1.c, and ≥93 for BRCA1.n, >240 H-score for SMC6L1.n, and ≥ 230 H-score for SMC6L1.c. γH2AX ≥40 H-score nuclear & ≥120 H-score cytoplasmic, UBC9; nuclear ≥160H-score &cytoplasm; ≥200H-score, CHK1; ≥20 H-score nuclear & ≥80 H-score cytoplasmic, BARD1; ≥130 H-score cytoplasmic & >0 H-score nuclear.

Table 6.6 Correlation between Nucleocytoplasmic Transport Markers with other Tumour Markers Continued.

Markers		KPNA2			
		Negative N (%)	positive N (%)	X^2	P
ER	Negative	153(23.9)	447(63.8)	215	<0.0001
	Positive	487(76.1)	254(36.2)		
PgR	Negative	234(37.7)	473(71.5)	148	<0.0001
	Positive	387(62.3)	189(28.5)		
Triple Negative	Negative	533(85.3)	373(55.8)	134	<0.0001
	Positive	92(14.7)	296(44.2)		
HER-2	Negative	585(90.4)	521(76.6)	45.5	<0.0001
	Positive	62(9.6)	159(23.4)		
Ki-67	Negative	306(54.2)	105(17)	180	<0.0001
	Positive	259(45.8)	513(83)		
Rad51.c	Negative	55(15.2)	25(5.7)	20	<0.0001
	Positive	308(84.8)	416(94.3)		
Rad51.n	Negative	222(60.7)	328(73.9)	16	<0.0001
	Positive	144(39.3)	116(26.1)		
Rad51	n-c-	35(9.6)	14(3.2)	38	<0.0001
	n+c+	121(33.3)	103(23.4)		
	n-c+	187(51.5)	313(71)		
	n+c-	20(5.5)	11(2.5)		
γH2AX.c	Negative	82(16)	40(6.2)	29	<0.0001
	Positive	429(84)	601(93.8)		
γH2AXn	Negative	47(9.2)	111(17.3)	16	<0.0001
	Positive	463(90.8)	530(82.7)		
γH2AX	n-c-	9(1.8)	11(1.7)	46.5	<0.0001
	n+c+	390(76.5)	501(78.2)		
	n-c+	38(7.5)	100(15.6)		
	n+c-	73(14.3)	29(4.5)		
BRCA1.n	Negative	226(43.4)	429(72.5)	97	<0.0001
	Positive	295(56.6)	163(27.5)		
BRCA1	n-c-	119 (22.9)	218(36.9)	96	<0.0001
	n+c+	105 (20.2)	64 (10.8)		
	n-c+	107 (20.6)	209(35.4)		
	n+c-	189 (36.3)	99 (16.8)		
*BARD1/BRCA1	Negative	30(4.9)	60(9)	8	0.005
	Positive	577(95.1)	605(91)		
SMC6L1.c	Negative	225(38.8)	153(23.3)	35	<0.0001
	Positive	355(61.2)	503(76.7)		
SMC6L1	n-c-	135(23.3)	84(12.8)	39	<0.0001
	n+c+	277(47.8)	365(55.6)		
	n-c+	78(13.4)	138(21)		
	n+c-	90(15.5)	69(10.5)		
CHK1	n-c-	39(9.2)	17(3.7)	27	<0.0001
	n+c+	107(25.2)	81(17.7)		
	n-c+	259(60.9)	348(76.1)		
	n+c-	20(4.7)	11(2.4)		
CHK1.n	Negative	298(70.1)	365(79.7)	11	0.001
	Positive	127(29.9)	93(20.3)		
CHK1.c	Negative	59(13.9)	28(6.1)	15	<0.0001
	Positive	366(86.1)	429(93.9)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. TN= negativity of ER& PgR & HER-2. The cut off points of positivity were as follows: ER and PgR; ≥1, HER-2; ≥10%, ≥ 40 H-score for BRCA1.c, and ≥93 for BRCA1.n, >240 H-score for SMC6L1.n, and ≥ 230 H-score for SMC6L1.c. γH2AX ≥40 H-score nuclear & ≥120 H-score cytoplasmic CHK1; ≥20 H-score nuclear & ≥80 H-score cytoplasmic, BARD1; ≥130 H-score cytoplasmic & >0 H-score nuclear, Ki-67; >34%, ≥8 H-score for Rad51.n and ≥80H-score for Rad51.c. During mitosis, NPM co-localises with BRCA1 and BARD1. *There is no consideration for cellular localisation, any expression was considered positive either in cytoplasm or nucleus for both proteins.

Table 6.6 Correlation between Nucleocytoplasmic Transport Markers with other Tumour Markers Continued.

Markers		KPNA2			
		Negative N (%)	**positive N (%)**	X^2	**P**
UBC9	**n-c-**	175(30.1)	157(23.2)	57	<0.0001
	n+c+	238(40.9)	279(41.3)		
	n-c+	104(17.9)	217(32.1)		
	n+c-	65(11.2)	23(3.4)		
UBC9.n	**Negative**	279(47.9)	374(55.3)	7	0.009
	Positive	303(52.1)	302(44.7)		
UBC9.c	**Negative**	240(41.1)	181(26.7)	29	<0.0001
	Positive	344(58.9)	496(73.3)		
PIAS1	**n-c-**	88(22.4)	50(10.9)	44.5	<0.0001
	n+c+	46(11.7)	28(6.1)		
	n-c+	239(60.8)	373(81.3)		
	n+c-	20(5.1)	8(1.7)		
PIAS1.c	**Negative**	111(28)	58(12.6)	32	<0.0001
	Positive	286(72)	403(87.4)		
PIAS1.n	**Negative**	327(83.2)	425(92.2)	16.3	<0.0001
	Positive	66(16.8)	36(7.8)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. The cut off points of positivity were as follows: PIAS1; nuclear ≥35H-score & cytoplasm ≥95H-score, UBC9; nuclear ≥160H-score &cytoplasm ≥200H-score.

Table 6.7 Pearson's Correlations of Nucleocytoplasmic Transport Markers with other Tumour Markers.

Markers		KPNA2	NPM
HR Repair Markers			
BRCA1.c	**R**	0.060	0.067
	P	0.044	0.027
	N	1113	1086
BRCA1.n	**R**	-0.309	0.038
	P	<0.0001	0.205
	N	1113	1086
BARD1.n	**R**	-0.025	0.062
	P	0.377	0.030
	N	1250	1225
BARD1.c	**R**	-0.017	0.229
	P	0.542	<0.0001
	N	1250	1225
Rad51.c	**R**	0.194	-0.036
	P	<0.0001	0.312
	N	804	781
Rad51.n	**R**	-0.202	0.029
	P	<0.0001	0.415
	N	804	781
SMC6L1.c	**R**	0.186	0.197
	P	<0.0001	<0.0001
	N	1237	1162
SMC6L1.n	**R**	0.075	0.415
	P	0.008	<0.0001
	N	1237	1162
DNA Damage Signal Transducers and Repair Markers			
CHK1.c	**R**	0.144	0.067
	P	<0.0001	0.049
	N	882	862
CHK1.n	**R**	-0.148	0.013
	P	<0.0001	0.706
	N	882	862
YH2AX.c	**R**	0.143	0.055
	P	<0.0001	0.072
	N	1152	1081
YH2AX.n	**R**	-0.206	0.330
	P	<0.0001	<0.0001
	N	1152	1081
SUMO Markers			
PIAS1.n	**R**	-0.150	-0.050
	P	<0.0001	0.148
	N	858	843
PIAS1.c	**R**	0.038	-0.0003
	P	0.262	0.921
	N	858	843
UBC9.n	**R**	-0.160	0.363
	P	<0.0001	<0.0001
	N	1259	1213
UBC9.c	**R**	0.155	0.203
	P	<0.0001	<0.0001
	N	1259	1213
Proliferation			
Ki-67	**R**	0.46	-0.010
	P	<0.0001	0.736
	N	1184	1136
Hormone Receptors			
PgR	**R**	-0.313	0.058
	P	<0.0001	0.069
	N	1024	984
ER	**R**	-0.449	0.125
	P	<0.0001	<0.0001
	N	1027	991

The table represents continuous data for all the markers. N= number of cases. R= Pearson's correlation, P=Probability value. c. = cytoplasmic, n. = nuclear expression. Cut-off points were as described in table 6.6.

6.6.3 Correlation of Nucleocytoplasmic Transport Markers with Clinico-Pathological Features

Table 6.8 summarises the association between KPNA2, NPM and the various clinico-pathological features of BC (Pearson x^2). The majority of tumours with poor prognostic features, such as larger tumour size, higher tumour grade (grade III), higher nuclear pleomorphism, higher tubular formation and moderate NPI (all P<0.0001), were associated with KPNA2$^+$. Both markers were investigated in conjunction with stage of tumour and the results were as follows: NPM (P=0.006) and KPNA2 (P=0.007). Both markers were mainly invasive ductal no special type BC (both P<0.0001). Details of significant and non-significant associations are summarised in Appendix 4.

The high value of X^2 of the findings in this section can be referred to, i) a bias in the population of patients, ii) based on the data here, it does not seem to have any assumption issues, the main problem is normally when one of the expectation values is five or less, but in the results presented here a large X^2 is noticed when the expectations are not less than 5. However, the data just seem to show that it is very likely that the association is not due to chance.

Table 6.8 Relationship between Nucleocytoplasmic Transport markers with Clinico-Pathological Parameters.

Parameters		KPNA2			
		Negative N (%)	Positive N (%)	X^2	P
Age	**<50**	205(30.5)	299(41.9)	20	<0.0001
	>50	468(69.5)	414(58.1)		
Size	**≤ 1.5cm**	239(35.6)	152(21.6)	33	<0.0001
	>1.5cm	432(64.4)	551(78.4)		
Stage	**1**	434(64.4)	404(56.9)	10	0.007
	2	183(27.2)	218(30.7)		
	3	57(8.5)	88(12.4)		
Grade	**1**	145(21.5)	18(2.5)	326	<0.0001
	2	263(39)	84(11.8)		
	3	266(39.5)	611(85.7)		
Tubules	**1**	40(6.2)	7(1)	104	<0.0001
	2	251(38.7)	133(18.9)		
	3	357(55.1)	565(80.1)		
Pleomorphism	**1**	15(2.3)	3(0.4)	220	<0.0001
	2	295(45.7)	77(10.9)		
	3	335(51.9)	624(88.6)		
Mitosis	**1**	287(44.3)	57(8.1)	316	<0.0001
	2	141(21.8)	85(12.1)		
	3	220(34)	563(79.9)		
NPI$^+$	**Excellent**	88(13.1)	10(1.4)	214	<0.0001
	Good	164(24.5)	36(5.1)		
	Moderate 1	209(31.2)	248(35.4)		
	Moderate 2	132(19.7)	225(32.1)		
	Poor	61(9.1)	136(19.4)		
	Very poor	16(2.4)	46(6.6)		
Tumour Type	**Invasive Ductal/NST**	363 (55.3)	586 (82.9)	169	<0.0001
	lobular	47 (7.2)	11 (1.6)		
	Atypical Medullary	7 (1.1)	31 (4.4)		
	***Mixed**	206 (31.4)	68 (9.6)		
	****other**	34 (5.2)	11 (1.6)		

Cut-off for nuclear KPNA2 was ≥30H-score. There was no cytoplasmic expression detected. $^+$ and * and ** are presented in the next table.

Table 6.8 Relationship between Nucleocytoplasmic Transport markers with Clinico-Pathological Parameters Continued.

NPM					
Parameters		**Negative N (%)**	**Positive N (%)**	X^2	**P**
Tumour Stage	**1**	170(67.7)	622(57.5)	10	0.006
	2	65(25.9)	338(31.3)		
	3	16(6.4)	121(11.2)		
Tumour Type	**Invasive Ductal/NST**	164(67.2)	738 (68.8)	20	<0.0001
	lobular	7 (2.9)	60 (5.6)		
	Atypical Medullary	15 (6.1)	17 (1.6)		
	***Mixed**	52 (21.3)	223 (20.8)		
	****other**	6 (2.5)	35 (3.3)		

N= number of cases. Cut-off used for NPM was ≥180H-score. * Lobular or tubular mixed BCs. ** Mucinous, Alveolar Lobular, Miscellaneous including Metaplastic, Adenoid Cystic, Spindle, and Tubulolobular. + NPI= Nottingham Prognostic Index. Excellent NPI (2.08–2.4), good NPI (2.42 to ⩽3.4), a moderate prognostic I NPI (3.42 to ⩽4.4), moderate prognostic II NPI (4.42 to ⩽5.4), poor NPI (5.42 to ⩽6.4), and a very poor NPI (6.5–6.8). NST= no special type.

6.6.4 Relationship between Nucleocytoplasmic Transport Markers and Patients' Outcomes by Univariate analysis

The positive expression of KPNA2 demonstrated a significant association with shorter BCSS and a shorter time to the induction of recurrence (P<0.0001 and P=0.003, Figures 6.4a-b respectively), while NPM had no association with patients' outcomes (Figures 6.4c-d). The association between NPM&KPNA2 markers and the effect of treatment on patient's outcome is presented in Appendix 4.

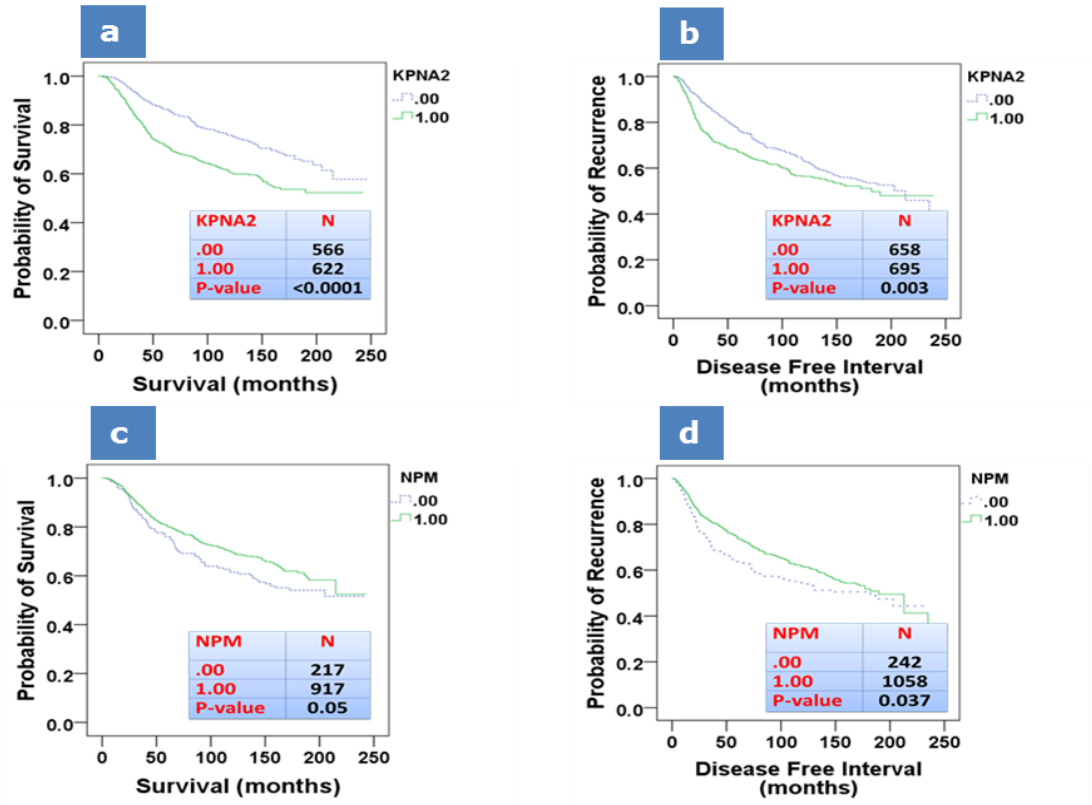

Figure 6.4 The associations between KPNA2 & NPM and BCSS or DFI. N; number of cases. Only patients who died from breast cancer were considered. a; shows association between KPNA2 and BCSS , and b; with DFI. c; shows association between NPM and BCSS , and d; with DFI. Where 0= negative and 1= positive expression. P<0.01 was considered significant.

6.6.5 Expression of Nucleocytoplasmic Transport Proteins and Patients' Outcomes by Multivariate Analysis

Table 6.9 and Figure 6.5 show Cox-regression analyses for predictors of BCSS and DFI for KPNA2. KPNA2 was not an independent prognostic marker for BC. Although NPM showed a trend for longer DFI in univariate analysis, it was a highly independent prognostic marker in multivariate analysis.

Table 6.9 Cox-regression Analyses for Predictors of BCSS and DFI

Parameters	P-value	95.0% CI		Parameters	P-value	95.0% CI	
		Lower	Upper			Lower	Upper
BCSS				**DFI**			
Tumour Stage	<0.0001	1.3	2.1	**Tumour Stage**	<0.0001	1.3	1.9
Tumour Grade	0.001	1.4	2.5	**Tumour Grade**	0.14	0.94	1.5
Tumour Size	0.066	0.98	1.82	**Tumour Size**	0.06	0.99	1.6
NPI	0.23	0.87	1.8	**NPI**	0.663	0.842	1.312
BLBC	0.4	0.66	1.117	**BLBC**	0.5	0.6	1.0
KPNA2	0.4	0.87	1.4	**KPNA2**	0.4	1.219	1.955

Parameters	P-value	95.0% CI	
		Lower	Upper
DFI			
Tumour Stage	<0.0001	1.452	2.234
Tumour Grade	0.006	1.096	1.706
Tumour Size	0.001	1.172	1.942
NPI	0.839	0.710	1.321
BLBC	0.004	0.495	0.872
NPM	<0.0001	0.50	0.81

NPI=Nottingham prognostic index. BLBC= as defined by Triple negative +positive expression of CK5 and CK14 and CK17. Only markers in univariate analysis that were statistically significant with patients' outcomes were applied for Cox regression (IBM SPSS 21.0) for multivariate analyses. However, positive expression of NPM showed a trend for longer DFI in univariate analysis.

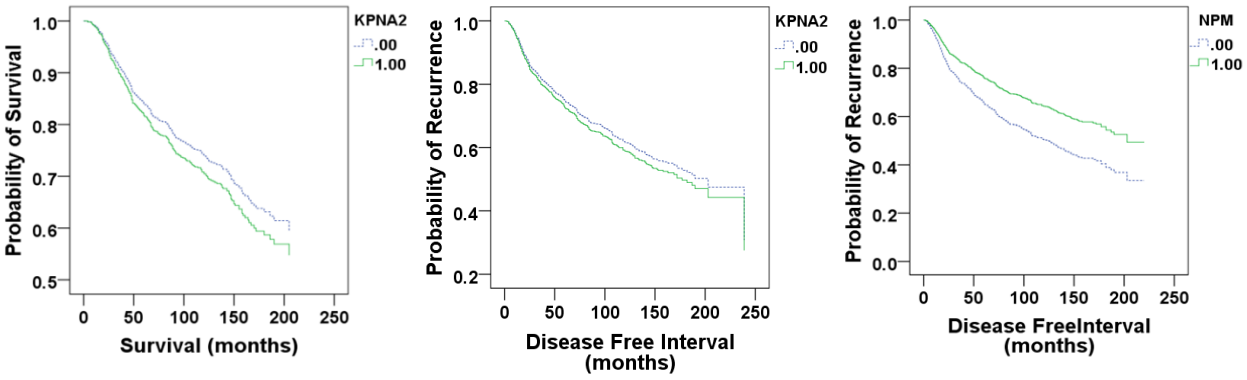

Figure 6.5 Multivariate Cox-regression analyses for BCSS and DFI for nuclear KPNA2 and nuclear NPM. Tumour grade, stage and size, NPI, and BLBC, were included. 0= negative expression, and 1=positive expression.

6.6.6 Expression of Nucleocytoplasmic Transport Markers in Cell Lines by Reverse Phase Protein Microarray

RPPA was used to evaluate the expression levels of nucleocytoplasmic transport markers in different cell lines, such as BRCA1 deficient HeLaSilenciX® cell and its control (proficient BRCA1), MCF-7 and MDA-MB-436 cells. RPPA does not confirm the IHC results of KPNA2 and demonstrates higher levels of the expression of KPNA2 in the BRCA1 control cell line (BRCA1.C) than the BRCA1 deficient HeLa (P=0.0004). Additionally, KPNA2 expression in BRCA1.C was significantly higher than MDA-MB-436 (P<0.0001), and finally BRCA1.C vs. MCF-7 showed a significant difference (P<0.0001). In line with IHC, the RRPA analysis of NPM highlighted similar expression amongst all the cell lines, and showed no statistical significant (Figure 6.6).

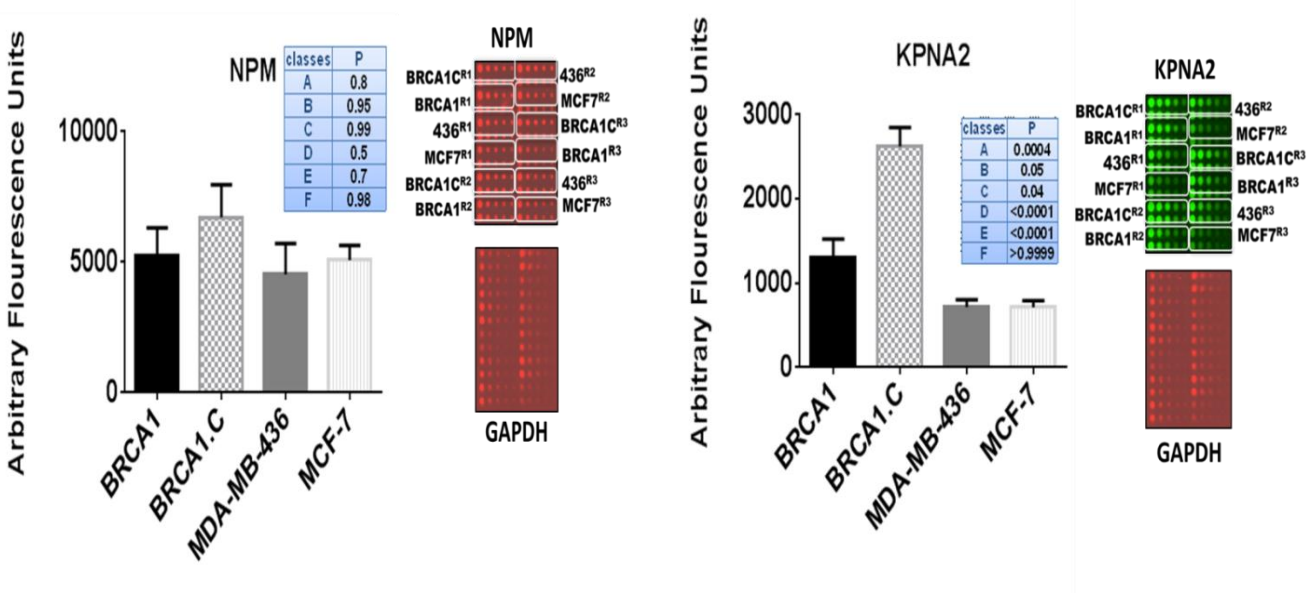

Figure 6.6 The nucleocytoplasmic transport protein levels detected by reverse phase protein microarray in different cell lines (BRCA1 deficient HeLaSilenciX® cells and its control [BRCA1 and BRCA1.C respectively], MCF-7 and MDA-MB-436 cells). For image of nitrocellulose slide spotted with different cell lysates; the red square represents the 700 channel for detection of mouse antibody while green square the 800 channel for rabbit antibody. Images of scanned nitrocellulose slides printed with extracted protein from cell lines and probed with the antibodies against the target proteins. Five 2-fold dilutions of each sample were printed in duplicate. Background was subtracted and the intensity of each spot was normalised to its corresponding GAPDH level. Each (^{R}) represents different passage of each sample; therefore, three different passages of each sample were used. Error bars represent Mean (SD). HeLa BRCA1; between passage21 and 30, HeLa BRCA1 control; between passage 15 and 20, MCF-7; between passage 25 and 32, and MDA-MB-436; between passage 12 and 20. A= BRCA1 vs. BRCA1.C, B= BRCA1 vs. MDA-MB-436, C= BRCA1 vs. MCF-7, D= BRCA1.C vs. MDA-MB-436, E= BRCA1.C vs. MCF-7, and F= MDA-MB-436 vs. MCF-7. One way ANOVA test was used.

6.7 Discussion

Profiling the localisation of proteins in several subcellular compartments is as important as profiling the expression of proteins and post-translational modification patterns. Understanding the localisation of proteins offers valuable insight into understanding state of activation, interaction networks and the biological functions of such proteins [580]. Such understanding may also aid the identification of potential targets in the process of drug discovery. For example, secreted proteins and plasma membrane proteins are conveniently accessible to targeted drugs, due to their particular localisation in the extracellular space or even on the surface of the cell [581].

DNA-DSB repair markers are usually located in the nucleus of a cell. In this study, these markers, detected by IHC, showed additional cytoplasmic expression. Interestingly, the effects of nuclear expression on patient survival switched from being a good predictor of BCSS to a poor predictor of this, when expressed in the cytoplasm. This is consistent with the clinical observation that cytoplasmic expression in most of the markers investigated is associated with poor prognostic features, such as TN, negativity of ER, PgR, absence of BRCA1 and higher histological grade. Thus, the identification of specific action in some proteins that signals between nucleus and cytoplasm in BC may help to detect a novel therapeutic target. In the present study, two proteins known to play a role in subcellular localisation (KPNA2 and NPM) were investigated and correlated with the expression of any marker showed nuclear and cytoplasmic expression, in order to investigate the expression of NPM and KPNA2 with these markers.

NPM is highly associated with the uncontrolled growth of cancer cells. The physiological function of NPM in tumorigenesis is unclear, but it possesses both tumour-suppressive and oncogenic activity [582]. NPM may play a role in oncogenesis by activating the oncogenic potential of a fused protein partner; Anaplastic lymphoma kinase (ALK), or myeloid leukemia factor 1(MLF1) [583]. It has been documented that the protein NPM is overexpressed in different tumours, such as gastric [567], colon [572] and breast tumours [573]. It is anticipated that tumour cell growth requires a sustained supply of nutrients and is in need of continued biogenesis of ribosome. NPM is therefore a crucial factor in this process and it naturally follows that there should be an approach towards the use of NPM in the treatment of various cancers. Furthermore, considering that NPM has oncogenic potential, when over-expressed, the protein could possibly translate or even amplify multiple oncogenic signalling mechanisms throughout carcinogenesis [582]. Herein, the DFI rate was better in patients demonstrating a high expression of NPM and multivariate analysis indicated that expression of NPM is an independent prognostic factor in BC patients. In contrast, a study on oral squamous cell carcinoma showed that the DFI rate was significantly better in patients with a low expression of NPM [584]. However, the discrepancy in these findings could be referred to the tissue used; breast and oral squamous cancers. The mechanism by which the overexpression of NPM is involved in the progression of cancer has not yet been determined and thus requires further investigation.

During mitosis, NPM co-localises with BRCA1 and BARD1 [585]; however, it has been demonstrated that the expression of BRCA1 is cell-cycle dependent, with the most significant expression presenting in the G2 and M phases. Due to the fact that BRCA1 and BARD1 stabilise each other [586], it is quite possible that the activity of ubiquitin ligase of BRCA1-BARD1 is rapidly increased in these stages. The complex of BRCA1-BARD1 in cells leads to the stabilisation, rather than degradation, of NPM [585]. In the present study, the complex of BARD1-BRCA1 showed a trend for association with NPM (P=0.016; Appendix 4). Given the variety of overlapping roles between NPM and BRCA1, it may suggest that NPM is an effective candidate, in terms of being a substrate of the ubiquitin ligase of BRCA1-BARD1, and may very well be important in the role of BRCA1 as a tumour suppressor, participating in its subcellular localisation. The mechanism with which the activity plays a role in the biology of BRCA1 needs to be established. To validate the *in vivo* co-localisation of NPM with BRCA1-BARD1, the subcellular localisation of these proteins may be investigated. Cell lines such as proliferating Swiss 3T3 cells can be stained with the indicated antibodies, followed by fluorescein isothiocyanate (green) conjugated secondary antibodies. This will stain the nucleus, which indicates the co-localisation of NPM with BRCA1-BARD1 in mitosis [585]. In addition, immunoprecipitation and immunoblotting methods; detection of *in vivo* ubiquitinated substrates, as well as *in vitro* Ub ligation assay can be carried out in further studies.

KPNA2 is a member of the family of various proteins associated with the active transport of cargo proteins, which contains an NLS from the cytoplasm to the nucleus. It has been reported as an important marker of tumorigenesis and progression of BC [587, 588]. Gluz et al support the findings in this study, highlighting the overexpression of nuclear KPNA2 in BCs, which was significantly associated with aggressive tumour features such as higher grade, negative hormone receptor status, shorter overall survival and DFI [564], although in the present study the effect of KPNA2 on patients' outcomes was not independent prognostic marker. Consistent with BC studies, similar associations of the expression of KPNA2 with features of poor prognosis were observed in other types of cancer, such as melanoma and ovarian cancers [589, 590]. This is the first study to investigate the nucleocytoplasmic transport markers of various pathways, in terms of a large cohort and specific subtypes.

The overexpression of cell-cycle genes such as *BUB1*, *CDC6*, *CDC25A*, *E2F1*, *KNSL5* and *UBCH10* is a sign of cell proliferation, which is known to be related to poor clinical outcome in BC [591]. Dai et al examined the molecular functions and biological processes of fifty prognostic genes, including *KPNA2*, and highlighted how the majority of the highly expressed genes in tumours with a poor outcome are cell-cycle associated genes [591]. In the present study, a strong correlation between KPNA2 protein expression and Ki-67, it can be hypothesised that, the finding may highlight the essential role of KPNA2 in the proliferation signalling of BC. This finding is supported by Saki et al but in regards to Oesophageal Squamous Cell Carcinoma [592]. Thus, the expression of KPNA2 could

possibly be related to the induction of proliferation and the progressive nature of BC. However, further studies are warranted to confirm this hypothesis.

KPNA2 mediates the transport of the nucleus of some tumour suppressors [561, 562]. Sandrock et al demonstrated that a low expression of KPNA2 inhibits the nuclear import of the small GTPase Ras-related C3 botulinum toxin substrate 1 (Rac1) [593]. However, the function of nuclear Rac1 regulates the signalling pathways that control gene expression and progression through the cell cycle [593, 594]. Tseng et al highlighted how *KPNA2* controls both the nuclear localisation of the MRN complexes and the formation of radiation-induced focus [595] by demonstrating how the suppression of *KPNA2* leads to the inactivation of double strand breaks (induced by IR) and the suppression of *NSB*-mediated DNA repair.

It has been proposed that KPNA2 participates in the nuclear translocation of BRCA1. The role of BRCA1 in carcinogenesis is pinpointed towards functions in the repair of DNA, in addition to control of the cell cycle checkpoint [596]. The importance of NLS in the translocation of BRCA1 has been identified, together with the direct interaction between KPNA2 and BRCA1 [597]. The observation that a *BRCA1* mutant deficiency of both NLSs can be observed in the nucleus [481, 598] has resulted in the identification of an alternative process in the importing of BRCA1 [480]. The value of the two alternative pathways continues to be identified.

At least one of the nucleocytoplasmic transport markers studied in this chapter (KPNA2 and NPM) was highly associated with the negative nuclear and positive cytoplasmic expression of different markers such as Rad51, BRCA1 and PIAS1, although the mechanism inducing cytoplasmic localisation remains to be determined. In the present study, it is likely that nuclear expression of HR repair, DNA damage signal transducer and SUMO markers bind to or interact with another marker leading to change their role when expressed in the cytoplasm. The aberrant high expression of KPNA2 and NPM in cancer tissue has been related to unfavourable patient features, prompting the possibility that these proteins play a role in carcinogenesis. Additionally, both markers were highly associated with the early stages of tumours. These combined results suggest that NPM and/or KPNA2 may be useful as a diagnostic biomarker in differentiating between the various stages of the progression of BC. In line with a previous study on bladder cancer, the expression of NPM has been linked with the stage of tumour progression [599]. In general, KPNA2 or NPM showed a significant positive association with cytoplasmic expression of most of the markers in this study such as Rad51 and PIAS1, but a negative one with its nuclear expression. Although both markers are known to have role in nuclear import, each one has its own role on the direct of transport of protein. However, this is just a hypothesis drawn as result of the finding, but further studies are warranted. Therefore, *in vitro* system, digitonin-permeabilised vertebrate cells can be developed in order to investigate biochemical events in the macromolecules transport across the envelope of the nucleus. Figure 6.7 shows a summary of the key findings in this chapter.

Despite the fact that ions, small molecules and small proteins (less than 20 kDa) are able to easily pass through NPCs by diffusion, NPCs control and restrict the passage of macromolecules (more than 40 kDa) to those with the appropriate signals [555, 556]. In the present study, it is important to note that the exclusion of fusion proteins from the nucleus to the cytoplasm is not size-dependent, because some markers are more than 40 kDa such as BRCA1 (~220 kDa), PIAS1 (71kDa) and CHK1 (54 kDa). Therefore, it can hypothesise that there is a defect in NPCs leading to uncontrolled travel of the macromolecule from and to the nucleus. However, a biochemical assay for formation of annulate lamellae (several pairs of parallel, smooth membranes, each pair containing regularly spaced pores similar to those of the nuclear envelope) can be developed and used to investigate the mechanism of annulate lamellae assembly in general and individual nucleoporins assembly into pore complexes in particular.

Again as described in the previous chapter the noticeable difference between IHC and RPPA used for the study of KPNA2 expression could be due to the difference in the samples used, for example using cervical cell lines (HeLa BRCA1 cell lines). Additionally, variations in environmental selection pressures may also clarify the differential patterns of the expression of protein in tumour tissue and the cell lines.

In theory, if the nuclear import/export of any proteins investigated in this study (Rad51, BRCA1, BARD1, SMC6L1, CHK1, γH2AX, PIAS1, and UBC9) is dependent on interaction with KPNA2 or NPM, then an increase in the expression of KPNA2 or NPM should occur and should have an effect on nuclear export to the cytoplasm. Thus, high levels of nuclear import proteins (KPNA2 or NPM) with other markers (HR repair, CHK1, and SUMO markers) in the cytoplasm caused a disappearance or the low expression of proteins (DNA-DSB or SUMO markers) in the nucleus. The expression of these markers (such as Rad51) in the cytoplasm of the cell may play a part in a poor prognosis of BC. From the results of this study, it can conclude that the poor prognosis of patients is largely associated with the negative nuclear and positive cytoplasmic expression of any marker tested here, such as Rad51. This finding may demonstrate the role of KPNA2 or NPM as nuclear export markers (by binding their cargo in the cytoplasm, after which they can interact with the nuclear pore complex and pass through its channel) [553]. The results of this study suggest that the expression of DNA-DSB repair marker (Rad51) and its nuclear vs. cytoplasmic compartmentalisation in breast tumour cells act as a prognostic marker, which may be utilised to facilitate the clinical management of patients. To conclude, further studies on the roles of the biology of subcellular localisation in breast oncogenesis, using cellular and animal models, are warranted, as are further clinical studies, in order to clarify whether subcellular localisation of proteins such as HR and SUMO markers can improve prognostication in BC.

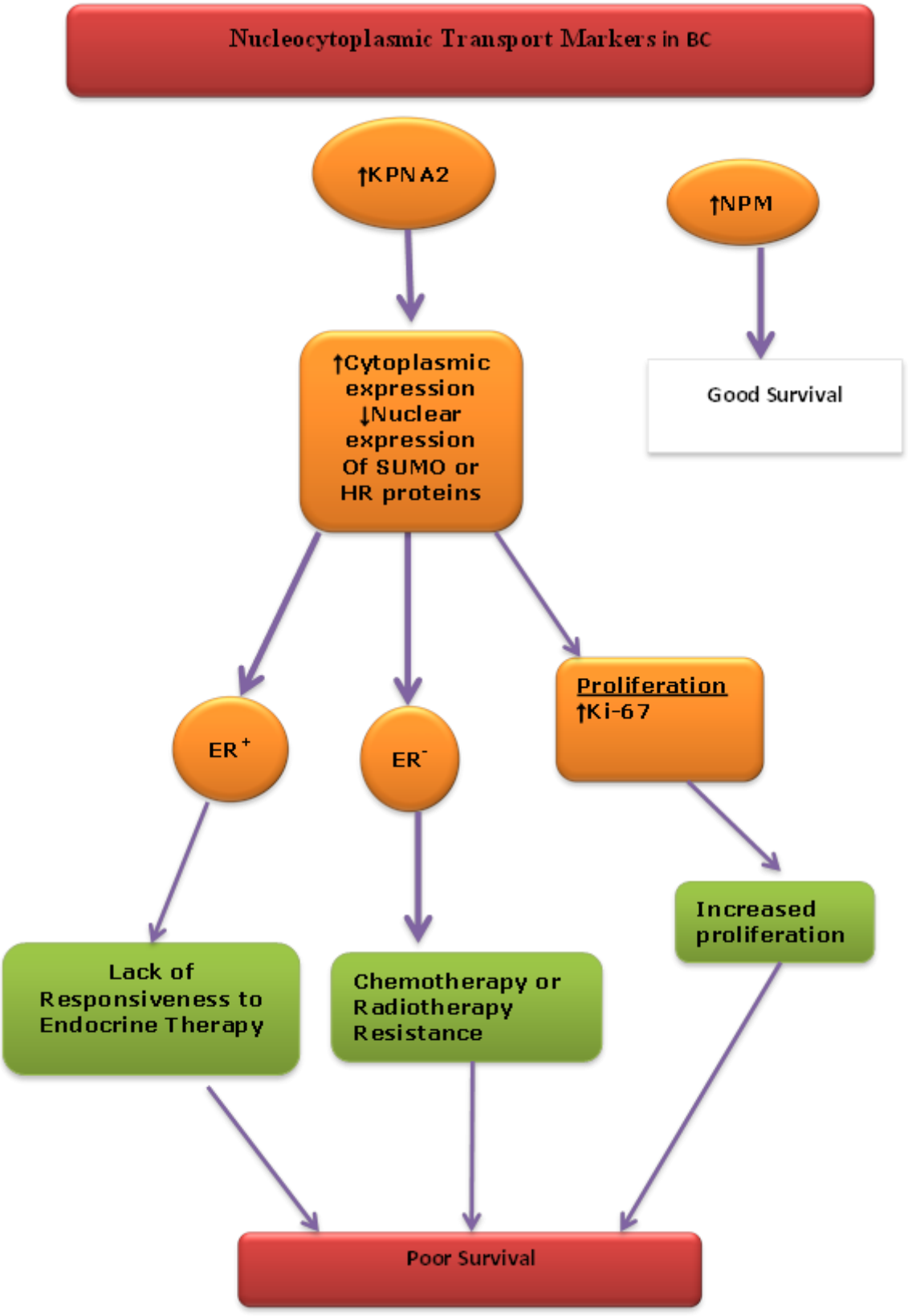

Figure 6.7 A summary of the key findings of nucleocytoplasmic transport markers in BC. Where, KPNA2 was mainly associated with ER negative BC and positive expression of Ki-67, whereas, positive expression of NPM was an independent good prognostic marker in BC.

Chapter 7

7.1 General Results

7.1.1 Correlation of Biomarkers as Continuous Variables

Table 7.1 shows the correlation of various markers analysed as continuous variables using Pearson's correlation. Generally, the markers of this study are likely to show a significant positive association with any other markers but similar in cellular localisation, such as BRCA1.n with nuclear expression of HR repair; Rad51, SUMO markers; PIAS1, PIAS4 and UBC9, DNA-damage sensors and signal transducer markers; ATM, CHK1, and CHK2 except ATR, and only DNA-PK from NHEJ. Similarly was seen between other markers (e.g. Rad51.n with CHK1.n or CHK2 with PIAS4). In contrast, markers that were expressed in the cytoplasm had a negative significant association with those expressed in the nucleus such as the association between SMC6L1.c and CHK1.n/ BRCA1.n/PIAS1.n as well as the association between Rad51.c and BRCA1.n/ CHK1.n/ PIAS1.n.

However, BARD1.c, but not nuclear expression, showed a positive association with most of the markers that were expressed in the nucleus such as CHK2, BRCA1.n, Rad51.n, and NPM. KU70/KU80 was positively associated with both cytoplasmic and nuclear expression of most of the markers such as Rad51, SMC6L1, and UBC9.

Regarding ATM, it only showed a positive association with nuclear expression markers such as Rad51, PIAS1, UBC9, γH2AX, CHK2, and CHK1, but however, both nuclear and cytoplasmic BRCA1 had a significant positive association with ATM, and finally ATM showed a negative association only with KPNA2.

Finally, KPNA2 had a negative association with those markers that had a nuclear expression such as BRCA1, Rad51, CHK1, CHK2, γH2AX, PIAS1, and UBC9 except SMC6L1 and PIAS4. However, a positive association were noticed between the cytoplasmic expressions of the same markers with KPNA2.

Figure 7.1 summarises the findings of this study, which demonstrates different pathways (HR and NHEJ repair, SUMO, DNA-damage sensors and signal transducers) that are known to have direct or indirect roles in the repair of DNA-DSB in BC. In response to DNA- damage the level of ATM should be increased leading to increase levels of BRCA1 expression. Here the low level of ATM may explain a defect in the response to the damage or the cancer itself has other causes than a defect in DNA-DSB repair. Generally, the figure illustrates that, there are two hypotheses: first; there is no DSB which may be explained by the low level of ATM as result of no damage to be censored leading to low level of BRCA1 expression, secondly; there is a DNA-DSB, due to the high expression level of γH2AX which is a general hallmark of DNA-DSB[600], but however, an error in the repair may occur. However, high level of NHEJ repair markers(error-prone repair pathway) [399, 428], and low expression level of HR repair (error- free repair pathway) [414, 601, 602], may have some role in defect of the repair.

Table 7.1 Pearson's Correlation between all the Markers in this Study.

Markers		BRCA1.c	BRCA1.n	BARD1.n	BARD1.c	Rad51.c	Rad51.n	SMC6L1.c	SMC6L1.n
BRCA1.c	**R**	*	-0.129	-0.009	0.009	0.174	-0.064	0.090	0.093
	P		<0.0001	0.764	0.748	<0.0001	0.047	0.003	0.002
	N		1756	1178	1178	955	955	1106	1106
BRCA1.n	**R**	-0.129	*	0.044	0.218	-0.088	0.449	-0.122	0.002
	P	<0.0001		0.129	<0.0001	0.007	<0.0001	<0.0001	0.953
	N	1756		1178	1178	955	955	1106	1106
BARD1.n	**R**	-0.009	0.044	*	0.169	0.052	-0.024	0.037	0.051
	P	0.764	0.129		<0.0001	0.132	0.493	0.195	0.072
	N	1178	1178		1477	851	851	1237	1237
BARD1.c	**R**	0.009	0.218	0.169	*	0.085	0.131	0.212	0.201
	P	0.748	<0.0001	<0.0001		0.014	<0.0001	<0.0001	<0.0001
	N	1178	1178	1477		851	851	1237	1237
Rad51.c	**R**	0.174	-0.088	0.052	0.085	*	0.031	0.119	-0.027
	P	<0.0001	0.007	0.132	0.014		0.283	0.001	0.436
	N	955	955	851	851		1178	813	813
Rad51.n	**R**	-0.064	0.449	-0.024	0.131	0.031	*	-0.084	0.031
	P	0.047	<0.0001	0.493	<0.0001	0.283		0.016	0.382
	N	955	955	851	851	1178		813	813
SMC6L1.c	**R**	0.090	-0.122	0.037	0.212	0.119	-0.084	*	0.416
	P	0.003	<0.0001	0.195	<0.0001	0.001	0.016		<0.0001
	N	1106	1106	1237	1237	813	813		1387
SMC6L1.n	**R**	0.093	0.002	0.051	0.201	-0.027	0.031	0.416	*
	P	0.002	0.953	0.072	<0.0001	0.436	0.382	<0.0001	
	N	1106	1106	1237	1237	813	813	1387	
KU70/KU80	**R**	0.119	-0.108	0.011	0.063	0.164	0.159	0.143	0.280
	P	<0.0001	<0.0001	0.734	0.058	<0.0001	<0.0001	<0.0001	<0.0001
	N	1055	1055	918	918	1030	1030	869	869
CHK1.c	**R**	0.240	-0.052	0.019	0.202	0.300	0.011	0.125	0.194
	P	<0.0001	0.090	0.558	<0.0001	<0.0001	0.722	<0.0001	<0.0001
	N	1081	1081	939	939	1037	1037	890	890
CHK1.n	**R**	0.010	0.352	-0.007	0.071	-0.150	0.457	-0.135	-0.004
	P	0.747	<0.0001	0.841	0.030	<0.0001	<0.0001	<0.0001	0.900
	N	1081	1081	939	939	1037	1037	890	890
CHK2	**R**	0.013	0.317	0.116	0.124	-0.081	0.208	0.129	0.256
	P	0.701	<0.0001	0.001	<0.0001	0.051	<0.0001	<0.0001	<0.0001
	N	858	858	808	808	580	580	790	790
ATR	**R**	0.091	-0.085	-0.032	0.055	-0.011	-0.042	0.235	0.098
	P	0.004	0.007	0.304	0.078	0.783	0.272	<0.0001	0.002
	N	1000	1000	1011	1011	673	673	991	991
ATM	**R**	0.119	0.234	0.016	0.038	0.022	0.157	-0.028	0.044
	P	<0.0001	<0.0001	0.616	0.250	0.535	<0.0001	0.394	0.187
	N	1158	1158	940	940	780	780	906	906
γH2AX.c	**R**	0.103	-0.033	-0.020	0.250	0.224	0.027	0.391	0.202
	P	0.001	0.281	0.500	<0.0001	<0.0001	0.459	<0.0001	<0.0001
	N	1052	1052	1146	1146	773	773	1167	1167
γH2AX.n	**R**	0.018	0.293	0.068	0.284	-0.038	0.263	0.042	0.439
	P	0.561	<0.0001	0.021	<0.0001	0.298	<0.0001	0.151	<0.0001
	N	1052	1052	1146	1146	773	773	1167	1167
PIAS4	**R**	0.113	0.085	0.001	0.104	0.135	0.195	0.093	0.303
	P	<0.0001	0.003	0.979	0.001	<0.0001	<0.0001	0.003	<0.0001
	N	1198	1198	1049	1049	1011	1011	992	992
PIAS1.n	**R**	0.004	0.324	-0.012	0.070	-0.166	0.422	-0.161	-0.013
	P	0.893	<0.0001	0.720	0.035	<0.0001	<0.0001	<0.0001	0.711
	N	1047	1047	912	912	1018	1018	868	868
PIAS1.c	**R**	0.157	0.040	0.044	0.256	0.368	0.138	00.155	0.104
	P	<0.0001	0.197	0.184	<0.0001	<0.0001	<0.0001	<0.0001	0.002
	N	1047	1047	912	912	1018	1018	868	868
UBC9.n	**R**	0.046	0.287	0.061	0.266	-0.066	0.248	0.018	0.325
	P	0.111	<0.0001	0.029	<0.0001	0.053	<0.0001	0.518	<0.0001
	N	1184	1184	1298	1298	863	863	1265	1265
UBC9.c	**R**	0.110	-0.013	-0.032	0.269	0.130	0.028	0.284	0.146
	P	<0.0001	0.665	0.255	<0.0001	<0.0001	0.420	<0.0001	<0.0001
	N	1184	1184	1298	1298	863	863	1265	1265
KPNA2	**R**	0.060	-0.309	-0.025	-0.017	0.194	-0.202	0.186	0.075
	P	0.044	<0.0001	0.377	0.542	<0.0001	<0.0001	<0.0001	0.008
	N	1113	1113	1250	1250	804	804	1237	1237
NPM	**R**	0.067	0.038	0.062	0.229	-0.036	0.029	0.197	0.415
	P	0.027	0.205	0.030	<0.0001	0.312	0.415	<0.0001	<0.0001
	N	1086	1086	1225	1225	781	781	1162	1162

The table represents continuous data for all the markers. N= number of cases. R= Pearson's correlation, P=Probability value. c. = cytoplasmic, n. = nuclear expression. * Analysis between the marker itself. The cut-off points were as described in table 2.6.

Table 7.1 Pearson's Correlation between all the Markers in this Study Continued.

Markers		KU70/KU80	DNA-PK	CHK1.c	CHK1.n	CHK2	ATR	ATM	γH2AX.c
BRCA1.c	**R**	0.119	0.140	0.240	0.010	0.013	0.091	0.119	0.103
	P	<0.0001	<0.0001	<0.0001	0.747	0.701	0.004	<0.0001	0.001
	N	1055	1090	1081	1081	858	1000	1158	1052
BRCA1.n	**R**	-0.108	0.070	-0.052	0.352	0.317	-0.085	0.234	-0.033
	P	<0.0001	0.020	0.090	<0.0001	<0.0001	0.007	<0.0001	0.281
	N	1055	1090	1081	1081	858	1000	1158	1052
BARD1.n	**R**	0.011	0.054	0.019	-0.007	0.116	-0.032	0.016	-0.020
	P	0.734	0.061	0.558	0.841	0.001	0.304	0.616	0.500
	N	918	1225	939	939	808	1011	940	1146
BARD1.c	**R**	0.063	0.280	0.202	0.071	0.124	0.055	0.038	0.250
	P	0.058	<0.0001	<0.0001	0.030	<0.0001	0.078	0.250	<0.0001
	N	918	1225	939	939	808	1011	940	1146
Rad51.c	**R**	0.164	0.021	0.300	-0.150	-0.081	-0.011	0.022	0.224
	P	<0.0001	0.551	<0.0001	<0.0001	0.051	0.783	0.535	<0.0001
	N	1030	792	1037	1037	580	673	780	773
Rad51.n	**R**	0.159	0.077	0.011	0.457	0.208	-0.042	0.157	0.027
	P	<0.0001	0.031	0.722	<0.0001	<0.0001	0.272	<0.0001	0.459
	N	1030	792	1037	1037	580	673	780	773
SMC6L1.c	**R**	0.143	0.208	0.125	-0.135	0.129	0.235	-0.028	0.391
	P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.394	<0.0001
	N	869	1156	890	890	790	991	906	1167
SMC6L1.n	**R**	0.280	0.504	0.194	-0.004	0.256	0.098	0.044	0.202
	P	<0.0001	<0.0001	<0.0001	0.90	<0.0001	0.002	0.187	<0.0001
	N	869	1156	890	890	790	991	906	1167
KU70/KU80	**R**	*	0.276	0.161	-0.039	0.027	0.076	0.026	0.072
	P		<0.0001	<0.0001	0.181	0.485	0.037	0.446	0.039
	N		844	1173	1173	650	753	863	818
CHK1.c	**R**	0.161	0.205	*	-0.100	0.053	-0.016	0.013	0.195
	P	<0.0001	<0.0001		<0.0001	0.171	0.654	0.70	<0.0001
	N	1173	867		1321	665	779	892	839
CHK1.n	**R**	-0.039	0.078	-0.100	*	0.171	-0.053	0.125	-0.042
	P	0.181	0.021	<0.0001		<0.0001	0.138	<0.0001	0.222
	N	1173	867	1321		665	779	892	839
CHK2	**R**	0.027	0.340	0.053	0.171	*	0.112	0.107	0.016
	P	0.485	<0.0001	0.171	<0.0001		0.001	0.006	0.665
	N	650	758	665	665		912	648	760
ATR	**R**	0.076	0.202	-0.016	-0.053	0.112	*	-0.021	0.084
	P	0.037	<0.0001	0.654	0.138	0.001		0.549	0.009
	N	753	946	779	779	912		789	955
ATM	**R**	0.026	0.051	0.013	0.125	0.107	-0.021	*	0.039
	P	0.446	0.134	0.70	<0.0001	0.006	0.549		0.257
	N	863	871	892	892	648	789		866
γH2AX.c	**R**	0.072	0.167	0.195	-0.042	0.016	0.084	0.039	*
	P	0.039	<0.0001	<0.0001	0.222	0.665	0.009	0.257	
	N	818	1096	839	839	760	955	866	
γH2AX.n	**R**	0.173	0.376	0.114	0.155	0.354	0.116	0.180	0.250
	P	<0.0001	<0.0001	0.001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	N	818	1096	839	839	760	955	866	1298
PIAS4	**R**	0.464	0.370	0.228	0.136	0.162	0.078	0.073	0.085
	P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.024	0.022	0.010
	N	1129	970	1145	1145	732	848	978	937
PIAS1.n	**R**	-0.021	0.065	-0.100	0.794	0.106	-0.077	0.108	-0.081
	P	0.476	0.061	0.001	<0.0001	0.007	0.036	0.001	0.021
	N	1116	844	1197	1197	631	739	870	818
PIAS1.c	**R**	0.151	0.151	0.426	0.008	0.116	0.066	0.045	0.158
	P	<0.0001	<0.0001	<0.0001	0.786	0.004	0.073	0.181	<0.0001
	N	1116	844	1197	1197	631	739	870	818
UBC9.n	**R**	0.135	0.432	0.095	0.179	0.386	0.101	0.105	0.125
	P	<0.0001	<0.0001	0.004	<0.0001	<0.0001	0.001	0.001	<0.0001
	N	917	1221	931	931	848	1063	966	1234
UBC9.c	**R**	0.127	0.317	0.207	-0.061	0.121	0.173	-0.030	0.376
	P	<0.0001	<0.0001	<0.0001	0.063	<0.0001	<0.0001	0.356	<0.0001
	N	917	1221	931	931	848	1063	966	1234
KPNA2	**R**	-0.004	0.103	0.144	-0.148	-0.107	0.106	-0.109	0.143
	P	0.898	<0.0001	<0.0001	<0.0001	0.003	0.001	0.001	<0.0001
	N	865	1163	882	882	778	976	905	1152
NPM	**R**	0.143	0.438	0.067	0.013	0.219	0.188	-0.059	0.055
	P	<0.0001	<0.0001	0.049	0.706	<0.0001	<0.0001	0.082	0.072
	N	846	1133	862	862	753	934	874	1081

The table represents continuous data for all the markers. N= number of cases. R= Pearson's correlation, P=Probability value. c. = cytoplasmic, n. = nuclear expression. * Analysis between the marker itself. The cut-off points were as described in table 2.6.

Table 7.1 Pearson's Correlation between all the Markers in this Study Continued.

Markers		γH2AX.n	PIAS4	PIAS1.n	PIAS1.c	UBC9.n	UBC9.c	KPNA2	NPM
BRCA1.c	**R**	0.018	0.113	0.004	0.157	0.046	0.110	0.060	0.067
	P	0.561	<0.0001	0.893	<0.0001	0.111	<0.0001	0.044	0.027
	N	1052	1198	1047	1047	1184	1184	1113	1086
BRCA1.n	**R**	0.293	0.085	0.324	0.040	0.287	-0.013	-0.309	0.038
	P	<0.0001	0.003	<0.0001	0.197	<0.0001	0.665	<0.0001	0.205
	N	1052	1198	1047	1047	1184	1184	1113	1086
BARD1.n	**R**	0.068	0.001	-0.012	0.044	0.061	-0.032	-0.025	0.062
	P	0.021	0.979	0.720	0.184	0.029	0.255	0.377	0.030
	N	1146	1049	912	912	1298	1298	1250	1225
BARD1.c	**R**	0.284	0.104	0.070	0.256	0.266	0.269	-0.017	0.229
	P	<0.0001	0.001	0.035	<0.0001	<0.0001	<0.0001	0.542	<0.0001
	N	1146	1049	912	912	1298	1298	1250	1225
Rad51.c	**R**	-0.038	0.135	-0.166	0.368	-0.066	0.130	0.194	-0.036
	P	0.298	<0.0001	<0.0001	<0.0001	0.053	<0.0001	<0.0001	0.312
	N	773	1011	1018	1018	863	863	804	781
Rad51.n	**R**	0.263	0.195	0.422	0.138	0.248	0.028	-0.202	0.029
	P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.420	<0.0001	0.415
	N	773	1011	1018	1018	863	863	804	781
SMC6L1.c	**R**	0.042	0.093	-0.161	0.155	0.018	0.284	0.186	0.197
	P	0.151	0.003	<0.0001	<0.0001	0.518	<0.0001	<0.0001	<0.0001
	N	1167	992	868	868	1265	1265	1237	1162
SMC6L1.n	**R**	0.439	0.303	-0.013	0.104	0.325	0.146	0.075	0.415
	P	<0.0001	<0.0001	0.711	0.002	<0.0001	<0.0001	0.008	<0.0001
	N	1167	992	868	868	1265	1265	1237	1162
KU70/KU80	**R**	0.173	0.464	-0.021	0.151	0.135	0.127	-0.004	0.143
	P	<0.0001	<0.0001	0.476	<0.0001	<0.0001	<0.0001	0.898	<0.0001
	N	818	1129	1116	1116	917	917	865	846
CHK1.c	**R**	0.114	0.228	-0.100	0.426	0.095	0.207	0.144	0.067
	P	0.001	<0.0001	0.001	<0.0001	0.004	<0.0001	<0.0001	0.049
	N	839	1145	1197	1197	931	931	882	862
CHK1.n	**R**	0.155	0.136	0.794	0.008	0.179	-0.061	-0.148	0.013
	P	<0.0001	<0.0001	<0.0001	0.786	<0.0001	0.063	<0.0001	0.706
	N	839	1145	1197	1197	931	931	882	862
CHK2	**R**	0.354	0.162	0.106	0.116	0.386	0.121	-0.107	0.219
	P	<0.0001	<0.0001	0.007	0.004	<0.0001	<0.0001	0.003	<0.0001
	N	760	732	631	631	848	848	778	753
ATR	**R**	0.116	0.078	-0.077	0.066	0.101	0.173	0.106	0.188
	P	<0.0001	0.024	0.036	0.073	0.001	<0.0001	0.001	<0.0001
	N	955	848	739	739	1063	1063	976	934
ATM	**R**	0.180	0.073	0.108	0.045	0.105	-0.030	-0.109	-0.059
	P	<0.0001	0.022	0.001	0.181	0.001	0.356	0.001	0.082
	N	866	978	870	870	966	966	905	874
γH2AX.c	**R**	0.250	0.085	-0.081	0.158	0.125	0.376	0.143	0.055
	P	<0.0001	0.010	0.021	<0.0001	<0.0001	<0.0001	<0.0001	0.072
	N	1298	937	818	818	1234	1234	1152	1081
γH2AX.n	**R**	*	0.259	0.171	0.039	0.530	0.085	-0.206	0.330
	P		<0.0001	<0.0001	0.268	<0.0001	0.003	<0.0001	<0.0001
	N		937	818	818	1234	1234	1152	1081
PIAS4	**R**	0.259	*	0.047	0.207	0.246	0.195	0.091	0.228
	P	<0.0001		0.118	<0.0001	<0.0001	<0.0001	0.004	<0.0001
	N	937		1124	1124	1041	1041	993	966
PIAS1.n	**R**	0.171	0.047	*	-0.090	0.182	-0.091	-0.150	-0.050
	P	<0.0001	0.118		0.001	<0.0001	0.006	<0.0001	0.148
	N	818	1124		1278	912	912	858	843
PIAS1.c	**R**	0.039	0.207	-0.090	*	0.077	0.204	0.038	-0.003
	P	0.268	<0.0001	0.001		0.020	<0.0001	0.262	0.921
	N	818	1124	1278		912	912	858	843
UBC9.n	**R**	0.530	0.246	0.182	0.077	*	0.407	-0.160	0.363
	P	<0.0001	<0.0001	<0.0001	0.020		<0.0001	<0.0001	<0.0001
	N	1234	1041	912	912		1481	1259	1213
UBC9.c	**R**	0.085	0.195	-0.091	0.204	0.407	*	0.155	0.203
	P	0.003	<0.0001	0.006	<0.0001	<0.0001		<0.0001	<0.0001
	N	1234	1041	912	912	1481		1259	1213
KPNA2	**R**	-0.206	0.091	-0.150	0.038	-0.160	0.155	*	0.033
	P	<0.0001	0.004	<0.0001	0.262	<0.0001	<0.0001		0.257
	N	1152	993	858	858	1259	1259		1152
NPM	**R**	0.330	0.228	-0.050	-0.003	0.363	0.203	0.033	*
	P	<0.0001	<0.0001	0.148	0.921	<0.0001	<0.0001	0.257	
	N	1081	966	843	843	1213	1213	1152	

The table represents continuous data for all the markers. N= number of cases. R= Pearson's correlation, P=Probability value. c. = cytoplasmic, n. = nuclear expression. * Analysis between the marker itself. The cut-off points were as described in table 2.6.

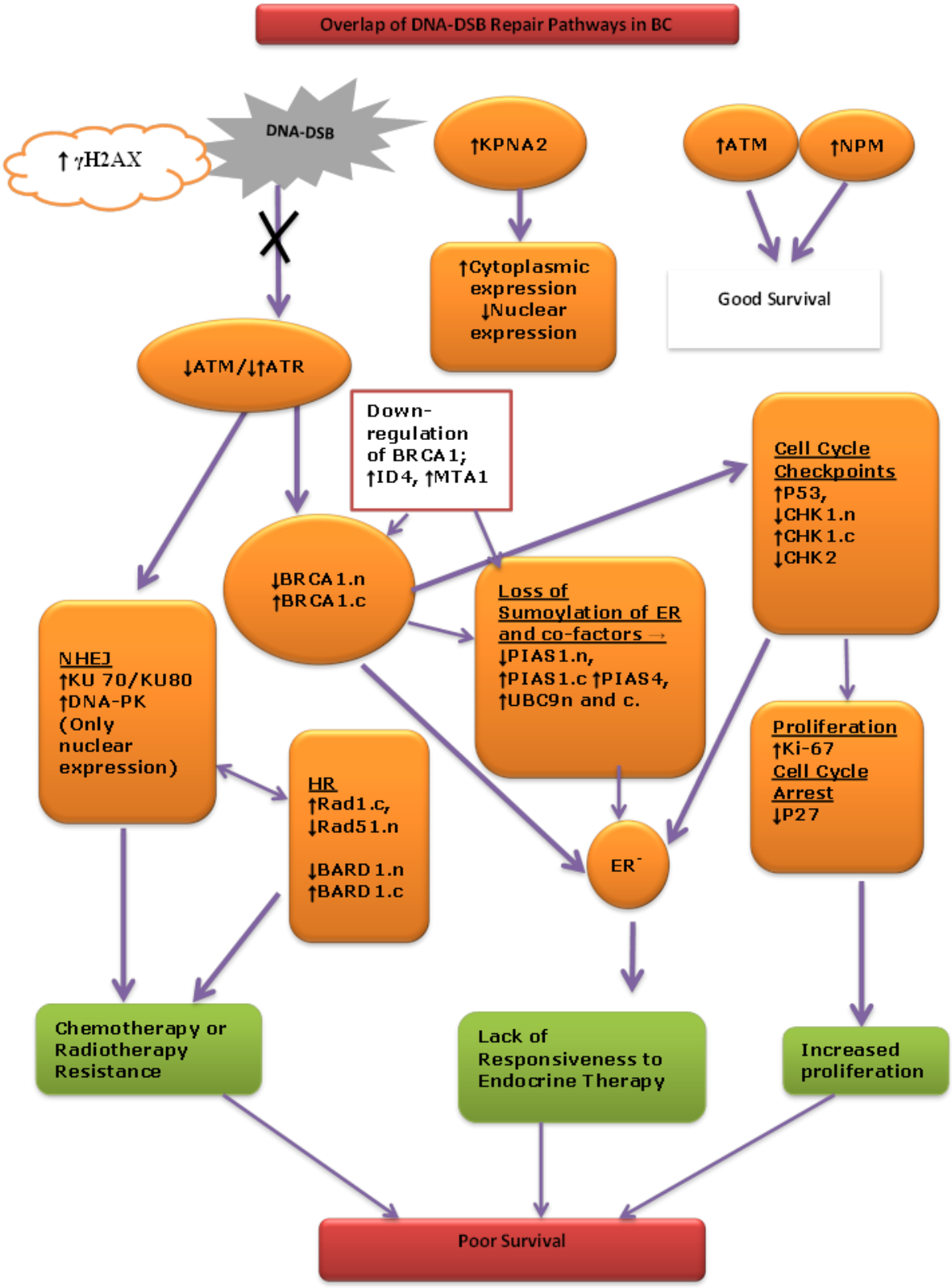

Figure 7.1 A summary of the findings in this thesis showing the pathways that have direct or indirect roles in the repair of DNA-DSB in BC. These pathways are; DNA-damage sensors and signal transducers, DNA-DSB repair pathways (HR and NHEJ), and finally SUMO. In addition, nucleocytoplasmic transport markers (KPNA2 and NPM) are included. **X** represents a defect in the response to DNA damage by showing low level expression of ATM. High expression of SUMO biomarkers showed not only a strong expression of KU70/KU80 (NHEJ), but also a lack of expression of the HR-associated markers. Increased association between MTA1/ID4 and DNA-DSB repair markers may have roles in the low expression of BRCA1.

7.2 Heat Map by Reverse Phase Protein Microarray Analysis

A heat map representing the differential protein expression within studied samples shows the wide range of variation of protein expression is shown in Figure 7.2, which represents the activated DNA repair intermediates molecules using RPPA in the cell lines used in this project (HeLa BRCA1 control (HeLa BRCA1C), HeLa deficient BRCA1, MCF-7 and MDA-MB-436). Each row of the heat map constitutes cell lysates sample (three replicates from each cell line) organised in colour columns red and green; columns represent the different studied target proteins. Red and green denote markers that are present at high and low protein expression, respectively. This was achieved after background subtraction and normalisation to GAPDH. In general most of the markers were highly observed in HeLa BRCA1C and MCF-7 cell lines than HeLa deficient BRCA1 and MDA-MB-436 cell lines.

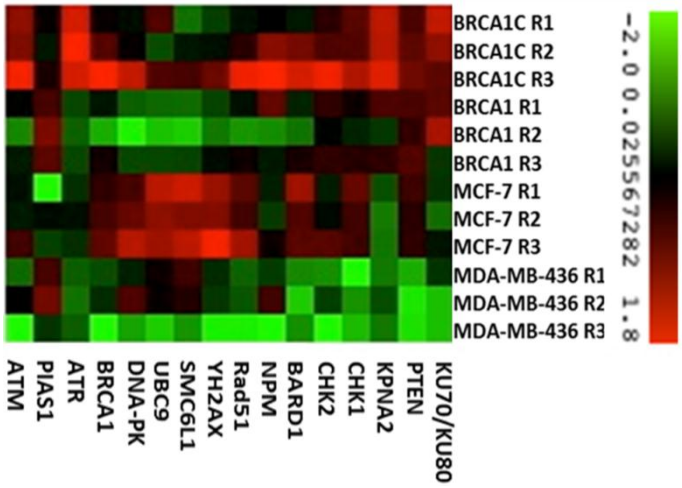

Figure 7.2 Heat map representing the activated DNA repair intermediates molecules using reverse phase protein microarray in the four cell lines used in this project (HeLa BRCA1C, HeLa deficient BRCA1, MCF-7 and MDA-MB-436). Heat map was created using Multi Experiment Viewer (MEV) software. The multiple different proteins of this study are outlined on the vertical axis, and the protein extracts representative of cell lines are on the horizontal axis. Red and green colours indicate high and low protein expression, respectively. BRCA1C= HeLa BRCA1 control. R represents different passage of each sample; therefore, three different passages of each sample were used. HeLa BRCA1; between passage21 and 30, HeLa BRCA1 control; between passage 15 and 20, MCF-7; between passage 25 and 32, and MDA-MB-436; between passage 12 and 20.

7.3 Correlation between BRCA1/BARD1 Complex and DNA Damage Sensors and Signal Transducers

Due to the fact that the BRCA1/BARD1 complex has an important role in DNA-DSB repair through the cell cycle [455], its expression was investigated with markers that have some roles in the cell-cycle control and DNA-damage sensor and signal transducer. However, the selection of the cellular localisation of BRCA1 and BARD1 was based on association with the worst clinico-pathological features or survival of BC. Thus, nuclear BRCA1 and cytoplasmic BARD1 were combined together. In addition, a recent study has further demonstrated that BARD1 shuttles between the nucleus and the cytoplasm: its location in the cell cytoplasm correlates with its apoptotic function, which is significantly decreased by

BRCA1 [453, 454]. BRCA1^{-}/BARD1^{+} showed a high association with P27^{-}, P53^{-}, γH2AXn^{+}.c^{+}, ATM^{-}, Ki-67^{+}, CHK2^{-} and CHK1n^{-}.c^{+} (P<0.0001 for all) (see Table 7.2).

As previously discussed the high value of X^2 can be referred to, a) a bias in the population of patients, b) based on the data here, it does not seem to have any assumption issues, the main problem is normally when one of the expectation values is 5 or less, but in the results presented here a large chi squared value is resulted when the expectations are not less than 5. However, the data just seem to show that it is very likely that the association is not due to chance.

Table 7.2 Correlation between BRCA1/BARD1 Complex and DNA Damage Sensors Proteins/ Cell Cycle Markers

Complex	P27				P21			
	Negative N (%)	**Positive N (%)**	X^2	**P**	**Negative N (%)**	**Positive N (%)**	X^2	**P**
BRCA1n^{-} /BARD1c^{-}	98(42.4)	26(11.8)	74	<0.0001	75(27.2)	50(26.7)	1	0.8
BRCA1n^{+} /BARD1c^{+}	35(15.2)	96(43.6)			85(30.8)	51(27.3)		
BRCA1n^{-} /BARD1c^{+}	96(41.6)	90(40.9)			109(39.5)	82(43.9)		
BRCA1n^{+}/ BARD1c^{-}	2(0.9)	8(3.6)			7(2.5)	4(2.1)		
Complex	**Ki-67**				**P53**			
	Negative N(%)	**Positive N (%)**	X^2	**P**	**Negative N (%)**	**Positive N (%)**	X^2	**P**
BRCA1n^{-} /BARD1c^{-}	29(8.7)	148(21.7)	77.5	<0.0001	72 (10.1)	107 (23.7)	89	<0.0001
BRCA1n^{+} /BARD1c^{+}	171(51)	188(27.5)			334 (46.8)	112 (24.8)		
BRCA1n^{-} /BARD1c^{+}	108(32.2)	322(47.1)			261 (36.6)	222 (49.2)		
BRCA1n^{+}/ BARD1c^{-}	27(8.1)	25(3.7)			46 (6.5)	10(2.2)		

Complex	γH2AX					
	n^{-}c^{-} N (%)	**n^{+}c^{+} N (%)**	**n^{-}c^{+} N (%)**	**n^{+}c^{-} N (%)**	X^2	**P**
BRCA1n^{-} /BARD1c^{-}	7(53.8)	108(14.7)	36(31.9)	12(15)	69	<0.0001
BRCA1n^{+} /BARD1c^{+}	1(7.7)	270(36.7)	17(15)	37(46.3)		
BRCA1n^{-} /BARD1c^{+}	4(30.8)	329(44.8)	58(51.3)	21(26.3)		
BRCA1n^{+}/ BARD1c^{-}	1(7.7)	28(3.8)	2(1.8)	10(12.5)		

c= cytoplasmic and n= nuclear expression and N= number of cases. The selection of the cellular localisation of BRCA1 and BARD1 was based on association with the worst clinico-pathological features or survival of BC. Thus, nuclear BRCA1 and cytoplasmic BARD1 were combined together. Cut-off points were as follows; ≥ 1% for P21, ≥70 H-score for P27, ≥5% for P53 and >34% for Ki-67. In addition, ≥ 40 H-score for BRCA1.c, ≥93 for BRCA1.n and ≥ 130 for BARD1.c.

Table 7.2 Correlation between BRCA1/BARD1Complex and DNA Damage Sensors Proteins/ Cell Cycle Markers Continued.

Complex	CHK1						CHK2			
	n^-c^- N (%)	n^+c^+ N (%)	n^-c^+ N (%)	n^+c^- N (%)	X^2	P	Negative N (%)	Positive N (%)	X^2	P
BRCA1n$^-$ /BARD1c$^-$	8(21.1)	30(17.4)	100(18.2)	2(7.1)	40.5	<0.0001	27(8.1)	19(5.8)	33	<0.0001
BRCA1n$^+$ /BARD1c$^+$	12(31.6)	77(44.8)	194(35.3)	14(50)			114(34)	184(56.3)		
BRCA1n$^-$ /BARD1c$^+$	13(34.2)	56(32.6)	244(44.4)	7(25)			173(51.6)	110(33.6)		
BRCA1n$^+$/ BARD1c$^-$	5(13.2)	9(5.2)	12(2.2)	5(17.9)			21(6.3)	14(4.3)		

Complex	ATM				ATR			
	Negative N (%)	Positive N (%)	X^2	P	Negative N (%)	Positive N (%)	X^2	P
BRCA1n$^-$ /BARD1c$^-$	95(20.7)	50(14.5)	28	<0.0001	17(4.4)	30(7.2)	3	0.3
BRCA1n$^+$ /BARD1c$^+$	131(28.5)	153(44.5)			185(47.8)	192(46.2)		
BRCA1n$^-$ /BARD1c$^+$	219(47.7)	122(35.5)			159(41.1)	171(41.1)		
BRCA1n$^+$/ BARD1c$^-$	14(3.1)	19(5.5)			26(6.7)	23(5.5)		

The selection of the cellular localisation of BRCA1 and BARD1 was based on association with the worst clinico-pathological features or survival of BC. Thus, nuclear BRCA1 and cytoplasmic BARD1 were combined together. c= cytoplasmic and n= nuclear expression and N= number of cases. Cut-off points were as follows; ≥20 and ≥80 H-score for nuclear and cytoplasmic CHK1 respectively, ≥105 H-score for CHK2, ≥18 H-score for ATR and ≥ 75% for ATM. ≥ 40 H-score for BRCA1.c, and ≥93 for BRCA1.n. ≥ 130 for BARD1.c.

Chapter 8

8.1 General Discussion

The diagnosis of DNA repair proteins as markers in BC is a particularly challenging task, as BC is a heterogeneous disease. There are also gaps remaining in our knowledge of susceptible genes, in terms of the repair of DNA-DSBs.

As *BRCA1* dysfunction appears to be related to poor prognostic, aggressive BCs, progressive knowledge of *BRCA1* and its pathways act as an experimental model, leading to the improvement of patient care within this poor prognostic BC group. Currently, little progress has been made in recording cases with larger-scale genetic rearrangements, particularly in terms of amplifications that occur often and are characteristic of tumour cells [603]. These alterations need to be linked with established tumour characteristics, such as tumour subtypes. Considering specific lesions in relation to therapeutic response may also progress a patient's personal stratification, leading to a more accurate prediction of treatment response/resistance. Herein, the results of this thesis combine the power of immunohistochemical staining with the parallel analytic capability of protein microarrays. Protein microarrays have attracted recent attention because of their potential use in high-throughput studies of protein function [604, 605]. Microarrays have been applied in the investigation of the expression profiles of different proteins [606, 607], protein-protein interactions [608] and the diagnosis of diseases such as cancer [609]. It is likely that gene microarray technology and proteomics will be very useful in the diagnosis of repair-deficient somatic cancers in the future [610]. The use of cell lines in RPPA is a possible limitation of this study; for this reason, a cohort of BC cases was required, in order to reliably report on the associations between the levels of the expression of different proteins and various clinico-pathological factors. These were then compared and contrasted with the IHC findings.

BC is a heterogeneous disease, distinguishing the variations between laboratory experiments and *in vivo* studies in humans, where associations between tumour and stroma, three dimensional effects and vascularisation become relevant [603]. Including clinical, radiological, pathological and genomic data in trial populations, with new trial designs, will help us to link and compare conventional markers to and with new technology in different settings [603]. Achievements are dependent upon multi punitive cooperation at an early phase, with the best quality histo-pathological and scientific progress. There are some points that could increase our knowledge of this heterogeneity in BC and, in particular, in cancers with DNA-DSB defects or TNBCs. In terms of gene expression, prognosis of these groups was compared to a better prognosis phenotype (luminal ER positive) and, as a heterogeneous phenotype, it is not surprising to see different levels of survival as a result of particular additional features such as ER positivity, which may respond to endocrine therapy [37].

Gene signatures that are based on biological questions, such as therapy resistance, may be more likely to have an effect on treatment decisions than the prognostic gene method,

as recently reported, although there remains a significant problem with the use of gene expression analysis within the routine setting [611].

The findings in this study suggest the engagement of the pathways of DNA-DSB repair in carcinogenesis of BC and reveal the existence of a BC subtype characterised by a high level of cytoplasmic/low level of nuclear HR markers (Rad51, BRAC1, BARD1) or the nuclear expression only of NHEJ markers (KU70/KU80 and DNA-PK) and negative ER or PgR phenotypes. The determination of the expression levels of the DNA-DSB repair markers in BC has opened up the potential for enhanced molecular classification of mammary tumours, in addition to unlocking new scenarios in the management of BC patients. For example, Rad51 gene expression has been related to response to chemotherapy; thus, a probable exploitation of the expression of Rad51 as a predictive marker of response to anti-cancer treatments should be taken into consideration. Furthermore, the expression of Rad51 may represent an important aspect of new therapeutical strategies that target the pathway of DNA repair [461].

The downregulation of HR leads to rearrangements of chromosomes, caused by the involvement of an alternative mechanism that is an error-prone DSB repair (like NHEJ). In contrast, hyper-recombination triggers various genome instability phenotypes, such as loss of heterozygosity, gene amplification and gene deletion [612]. Although all HR (but not NHEJ), SUMO, CHK1, γH2AX and other tumour markers demonstrated cytoplasmic expression, it is quite difficult to find an explanation for the aberrant cytoplasmic staining through IHC alone. The staining does not appear to represent an artefact; if so, the relevance of the discrepancies between the immunohistochemical results and RPPA must be high. Here, all the markers, with the exception of ATR, KPNA2 and PIAS1 showed similar expressions, in different cohorts (IHC), to the cell lines. It is thus plausible that some tumour markers were present in the cytoplasm. Taken together, these data demonstrate that, in terms of assessing the origin of subcellular localisation, this can possibly be obtained by pairing IHC with molecular analysis; if possible, both techniques should ideally be performed in parallel. In this study, although RPPA was applied to whole cell extracts, the findings were in-line with nuclear expression by IHC. It would be useful if cellular fractionation was conducted, in order to compare cellular localisation for each marker. Nevertheless, IHC may still represent a reasonable technique in screening procedures within laboratories, particularly in those in developing countries, which are not usually equipped for molecular studies.

Both CHK1 and CHK2 are essential kinases for the repair of DNA and are important in the recruitment of the functional associations between BRCA1and Rad51 proteins; thus, they increase the HR-mediated repair of stalled replication forks [613]. CHK1 has been demonstrated to phosphorylate Rad51 and other proteins, such as FANCD2, in order to promote DNA repair pathways [614]. In the present study (Table 7.1), there was a significant direct association between CHK1.n, BRCA1.n and Rad51.n, but negative or no association with cytoplasmic expression, however, CHK2 showed no association with the cytoplasmic expression of both Rad51 and BRCA1. Cases exhibiting low nuclear CHK1 and high

cytoplasmic expression or CHK2 might hypothetically possess a defect in response to DNA damage, leading to a more aggressive tumour. Bahassi et al studied the functional associations between BRCA2 and Rad51, in response to DNA damage and its regulation by CHK1 or CHK2. In UV-treated cells, the depletion of CHK1 from cells through the use of siRNA generates an entire loss of Rad51 localisation to nuclear foci, following block of replication [615]. Conversely, cells that show a truncated and non-functional CHK2 form have no noticeable defect in localisation at the foci of Rad51, suggesting that CHK1 is a participant in managing the interaction of BRCA2–Rad51, in response to block of replication. Cells deficient in CHK2 show an obvious impairment in the localisation of Rad51 immediately after DNA-DSB: this is a direct result of treatment with IR [615].

Fabbro et al used siRNA to deplete BRCA1 or BARD1 in 293T cell lines (human embryonic kidney carcinoma cell line) and revealed that the BRCA1/BARD1 complex is essential for the ATM/ATR mediated phosphorylation of P53, following IR or UV radiation-induced DNA damage [455]. In addition, the inhibition of the phosphorylation of P53 by BRCA1/BARD1 acute suppression compromises the induction of P21 and the checkpoint arrest of the G1/S phases [455]. In the present study, the co-expression of BRCA1.n^-/BARD1.c^+ (the cellular localisation largely associated with poor prognostic features) was highly associated with the negative expression of P27, P53, in addition a strong association with γH2AXn^+.c^+, CHK1n^-.c^+, CHK2$^-$, and ATM$^-$ (Chapter 7, Table 7.1). It is thus possible to hypothesis that the co-expression of BRCA1/BARD1 might decrease the expression of P27 (functions as a regulator of cell cycle progression at G1) [616] and may impair the G1/S cell-cycle checkpoint, yet increase the level of P27 and induce cells to arrest in the G1 phase. To prove this hypothesis, it would be beneficial to utilise siRNA to deplete BRCA1 and BARD1, in order to determine that the BRCA1/BARD1 complex may be required for the cell cycle during the G1 phase, following IR- and UV radiation-induced DNA damage.

This study found that CHK2$^-$ and CHK1.n^- were highly associated with the HR markers Rad51.n^- and BRCA1.n^- and it is possible that the loss of HR markers, resulting from a lack of CHK1, may potentially cause further accumulation of DNA-DSB lesions. A previous study has indicated that loss of DNA-DSB repair pathways may protect cells from apoptosis induction [617]. Hinz et al highlighted how the loss of Rad51,which is required for DNA repair, did not enhance apoptosis levels, following treatment with low levels of camptothecin [617]. However, the interaction of the pathways of DNA-DSB repair with protein checkpoint kinases, such as CHK1 and CHK2, in apoptosis induction after replication stress of DNA remains unclear. DSBs can often arise throughout the S phase, due to the collapse of the replication-fork. CtIP functions cooperatively with the complex of MRN exclusively throughout the S and G2 phases of the cell cycle, in order to enhance the resection of DSB and HR [310, 618]. The activity of CDK encourages the resection stage of the HR reaction and inhibits NHEJ [305, 619]. The KU70/KU80 are able to bind to DSBs, with even more rapidly kinetics, when compared with factors of HR [620]; for this reason, a competition might possibly exist between NHEJ and HR, even throughout the S phase, which proposes that more factors could possibly suppress the binding of KU70/KU80 in favour of

HR proteins. Additionally, the topological complications that occur when two replicons merge with each other at termination are required to become fixed during S–G2, in order to be able to avoid breakage of chromosomes throughout segregation [621, 622]. When DSBs occur during the segregation of chromosomes, during which time chromosomes are remarkably compact and the search for homology is complicated, repair is likely to be achieved by NHEJ in the following G1 phase, if checkpoints or even caretaker genes had not triggered the arrest of the cell-cycle during the G2 and M phases [623-625]. As mentioned earlier in this chapter, ATM and DNA-PK largely act in response to DSBs, whereas ssDNA activates ATR [304]. Karlsson et al stated that, as a result of the inhibition of DNA-PK in the GM16147 cell line (XRCC4 deficient), ssDNA was produced within a repair time of one hour. The formation of ssDNA is actually cell-cycle dependent, as ssDNA ends were not detected in G1-synchronised NHEJ deficient cells. The generation of ssDNA possesses a great influence on the quantification of DSBs by pulsed-field gel electrophoresis, resulting in an underestimation of the amount of un-joined DSBs in cells deficient in NHEJ. These findings propose that the deficiency of NHEJ proteins via an S-phase specific approach are able to access the ends of DSB and, most probably, build long ends of ssDNA, in an attempt to repair the breaks [626]. The results here show that CHK1.n^-.c^+ and $CHK2^+$ are highly associated with at least one of each of the HR and NHEJ markers. Interestingly, ATM showed no association with any of the NHEJ markers, whereas ATR had no or negative associations with the HR marker (nuclear expression), with the exception of the cytoplasmic expression of BRCA1 and BARD1 or nuclear and cytoplasmic SMC6L1 (positive associations), and was positively associated with NHEJ markers. Thus, NHEJ seems to be the major repair pathway in mammalian cells through ATR and HR by ATM. However, this needs further investigation to be confirmed. Real time PCR can be used in the future to detect the accumulation of KU70/KU80 or any markers at the damage site by using fluorescent proteins against the target markers, in order to investigate its expression after DNA damage. The UV Induced DNA Damage Response Antibody Sampler Kit can be used to investigate different proteins involved in the cellular response to UV-induced DNA damage.

There are several important questions remaining, regarding the molecular details of exactly how posttranslational modifications of distinct repair factors have an impact on protein interactions, cellular distribution and efficiency of DNA-DSB repair. For example, the pathway through which SUMO regulates various repair functions or the repair pathway choice remains poorly understood. SUMO usually has an effect on its target, particularly within the area of cellular localisation, and yet are these types of repair activities compartmentalised within the cell? [302] How exactly does BRCA1 increase the function of HR and what are its specific targets in the process of recombination repair? Is the overexpression of NHEJ markers evidence of theirs fundamental role in the DNA-BSB repair and of out-competing their counterpart (HR)? However, homology directed repair studies are usually used on cell lines. A Direct Repeat (DR)-GFP reporter-based mouse model needs to be generated, in order to study HR repair in mice. The cells derived from the mammary epithelium may then be observed undergoing HR repair in I-SceI endonuclease-induced DSBs. In addition, the detection of the HR or NHEJ pathways in BC cells can be analysed

using *in vivo* fluorescent assays. Currently, with a number of genome projects including thousands of sequences of nucleotide to the public databases each day, the exploration of gene function usually starts with a sequence of DNA.

Much of the recorded knowledge on the repair of DNA is the conclusion of biological experiments and clinical trials and there appears to be just a few approaches based on bioinformatics, which creates extra knowledge of DNA repair. An excellent approach in this is the Repair-FunMap, a functional database of the proteins of the human DNA repair pathways [627]: this utilises its knowledge on the list developed by Wood et al for the annotation of DNA repair genes in humans [628]. This list presents accession numbers, in order to ensure that genes can be referenced electronically. It is only lately that some additional repair-related analyses have utilised bioinformatics (for example, to determine phosphorylation sites [629]or to analyse a specific gene [630]).

In terms of the mechanisms through which the activation function of checkpoints organise repair in a cell-cycle dependent-manner, the molecular mechanisms that control the effects of failed DNA-repair attempts and stimulate the arrest of the temporary cell cycle (senescence and apoptosis) may be effective research areas [302]. In the future, a much better understanding of the mechanisms that control DNA-DSB repair and an understanding of the organisation of repair with progression of cell cycle in order to protect genome integrity are required.

The present study demonstrates that a substantial portion of the proteins involved in the emergence and progression of BC possess similar values of protein expression in TMA and cancer cell lines, showing the value of cultured cell lines in BC research. This study revealed the expression of different proteins in cell lines that have markedly different expression patterns, compared to those associated with breast tumour tissue. The composition of the cell culture medium may be precisely why patterns of protein expression differentiate cancer cell lines from breast tumour tissue. However, variations in environmental selection pressures may also clarify the differential patterns of the expression of protein in tumour tissue and the cell lines. Compounds may have a significant and diverse effect on the altered pathways between cell lines and tumour tissue. Regardless of whether cell lines can be made to mimic tumour cell protein expression patterns by simply modifying the conditions of the culture medium, this is yet to be fully investigated [345]. However, expression levels on the nitrocellulose of RPPA showed some variation for the same protein (GAPDH), this was expected since there is variation in the fluorescence intensity between different microarray experiments. Hence, the signals of each marker were normalised to the signals of GAPDH of the same experiment in the same day. However, one of the disadvantages of nitrocellulose is its high intrinsic fluorescence that leads to high levels of background, so limiting the assay sensitivity when fluorescently labelled antibodies are used for detection.

The hypothesis of this thesis was that investigation of alterations in the different pathways of DNA-DSB will help in identifying tumours with impaired DNA-DSB repairs

pathways, improve our understanding of the role of DDR in the different molecular classes of BC, narrow down the number of target proteins and identifying subclasses of sporadic BC with defective DNA-DSB repair can potentially be used to guide targeted systemic therapy. Therefore, alterations of the different pathways of DNA-DSB repair contribute to the aggressive nature of BC, especially in terms of the BRCA1 tumour phenotype and BLBC [178]. The results of this work support this hypothesis by showing an aggressive characteristic of tumours that exhibit a defect (negative/low protein expression) in any pathway with a role in DNA-DSB repair. Herein, this relationship was confirmed, particularly in terms of loss of expression of HR repair-associated proteins and high NHEJ-associated proteins (KU70/KU80 and DNA-PK) in the nucleus and high HR repair-associated proteins expressed in the cytoplasm (Rad51, BARD1). Cytoplasmic rather than nuclear expression was associated with a BLBC, lack of hormonal receptors, and negative/low expression of BRCA1 protein or in known *BRCA1* germline mutations cancers. In addition, the high expression of CHK1.c (rather than nuclear expression) observed in the present study suggests that somehow defects in response to DNA damage in BC patients may be repaired before entering the M phase, which may hypothetically lead to an increase in resistance to chemotherapy (by showing no benefits from chemotherapy); further studies to investigate this hypothesis are warranted. As discussed in chapter 3, genome /exome sequencing can be performed on BC patients who received treatment, and then evaluate the response to the therapy. After sequencing, the data can be analysed and compared with the clinical data for each patient (object response to therapy). In addition, there was a high correlation between SUMO markers and cytoplasmic expression of HR and nuclear expression of NHEJ, but a negative correlation with nuclear expression of HR markers. It is known that SUMO modified proteins in order to function, and its modification plays an important role in the nuclear transport of proteins [631]. It would be better to test the expression of SUMO markers on cells that have a damaged DNA in order to test the effectiveness of these markers.

This is the first study highlighting the importance of and differences in the subcellular localisation of DNA repair associated proteins in BC and the effect on patients' outcomes. Particularly, HR repair markers (but not NHEJ) showed worse features with cytoplasmic location of expression, whereas nuclear expressions were associated with more favourable features. This finding may help in the classification of BC and therefore, targeting this pathway in the development of drugs would enhance better patients' outcomes, in regards to DNA-DSB repair defects (negative/low protein expression) in BC. Major prognostic and predictive variables can be very important in choosing suitable patient treatment plans by identifying the risk of recurrence and classifying patients for clinical trials. However, this is the first study that shows the HR-repair marker Rad51.n, co-expression of Rad51.n.c, complex of HR and NHEJ repair markers (BRCA1.n&KU70/KU80) in non-TNBC, and a complex of NHEJ markers (KU70/KU80&DNA-PK) in TNBC are all independent prognostic markers for BC. The determination of DNA-DSB repair markers expression level alterations in BC opens up the potential for enhanced molecular classification of mammary tumours as well as unlocking new scenarios in the management of BC patients. However, progressive

knowledge of repair pathways act as an experimental model potentially leading to improved patient care of this poor prognostic group of BC.

In conclusion, the DNA repair coordination processes have a critical role in providing the suitable development and survival of organisms. They are responsible for controlling several human diseases including cancer. Following on from the work presented in this thesis, further understanding of the molecular mechanisms through which the DDR works, in combination with the elucidation of the genetic interactions between various pathways of DDR or between DDR pathways and other cellular pathways, will certainly offer therapeutic opportunities for several human diseases.

8.2 Limitation of the Study

1. The number of tumours with known *BRCA1* germline mutations was limited (24 cases).

2. RPPA was applied to cell lines and not to tissue sections from tumours tested using the same markers by IHC.

3. Specificity of the proteins was detected by one method (Western blotting). Although it is reliable and a commonplace technique for detecting the specificity of antibodies, other techniques such as peptide blocking could be used to confirm the specificity.

4. Although, there is strong evidence confirming the effectiveness and reliability of TMA for a large scale assessment of molecular markers with a high concordance rates between TMA and full face sections [279, 632], it may be worth using multiple cores per case. TMA is not very accurate for detecting a biomarker in a small subgroup of tumours. Previous studies by our research group have assessed the expression of different biomarkers such as ER, PgR, Ki67 and hypoxia related marker using TMA and compared the results to full face sections[633, 634] and this showed less sensitivity of TMA in subgroup analysis. In these studies, it was stated that TMA is more likely to produce false-negative results rather than false-positive ones. However, despite the fact that, these discordances between TMA and full face sections might not significantly change the overall association of these biomarkers with the other prognostic variables particularly in large scale studies, it is important to acknowledge these limitations of TMA method, in order to avoid misinterpretation of their results.

5. For this historical patient cohort, relatively old paraffin blocks were used to study the expression of tissue marker. These tissues could possibly be potentially different from recently fixed and prepared tissue specimens, for example, therapeutic routines used at the time when patients of this study were treated tended to be less aggressive than those at present in practice and numerous methods are constantly growing.

8.3 Suggestions and Future Investigations

1- A larger series of known *BRCA1* germline mutations carrier cases to be investigated.

2- Investigate more relevant and key markers that characterise different pathways of DDR such as base and nucleotide excision repair mechanisms to provide a comprehensive profiling of DDR in BC.

3- Subcellular fractionation will be helpful in investigating the subcellular localisations. The Thermo Scientific Subcellular Protein Fractionation Kit can be used on tissue samples and then each extract (e.g. cytoplasmic extract, membrane extract, nuclear extract, chromatin-bound extract, and pellet extract) can be analysed by Western blotting.

4- Pathway analysis and system biology techniques are very promising and informative techniques for assessment of complex biological process such as DDR. The complexity of analysis will be emphasised by the fact that subcellular localisation in addition to the level of expression and interaction between different known and unknown genes may affect the function of any specific genes and subsequently determination of pathways. Methods that combine different protein characteristics include expert systems [635], k-nearest neighbor [636-638], SVM [639], ANN [640], support description of vector data [641], as well as Bayesian networks [642], to be utilised.

5- Investigate further mechanisms that have a role in nuclear transport, for example *in vitro* system, digitonin-permeabilised vertebrate cells can be developed in order to investigate biochemical events in the macromolecules transport across the envelope of the nucleus.

6- In terms of RPPA, a single series of tumour tissue samples representing invasive tumours is required, in order to reliably report on the associations between protein expression levels and various clinico-pathological parameters.

7- In this study, is remains unknown whether the cases suffer from a defect in repair or that the cancer was mainly due to DNA-DSBs thus, further studies are therefore warranted to investigate that. For instance, the Comet assay in individual cells of extremely small tissue samples, is a standard technique for evaluation of damage or repair or DNA, biomonitoring and genotoxicity testing [551, 552], which could be utilised *in vivo* and *in vitro* samples.

8- Real time PCR can be used in the future to detect the accumulation of KU70/KU80 or any markers at the damage site by using fluorescent proteins against the target markers, in order to investigate its expression after DNA damage. The UV Induced DNA Damage Response Antibody Sampler Kit can be used to investigate different proteins involved in the cellular response to UV-induced DNA damage.

Chapter 9

9.1 References

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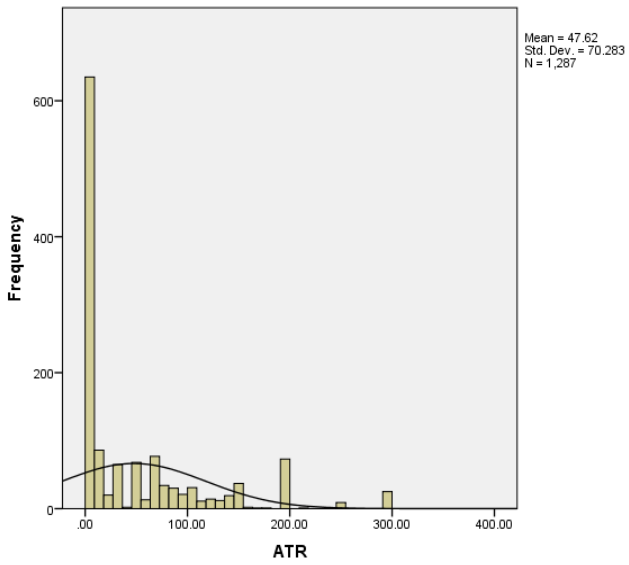
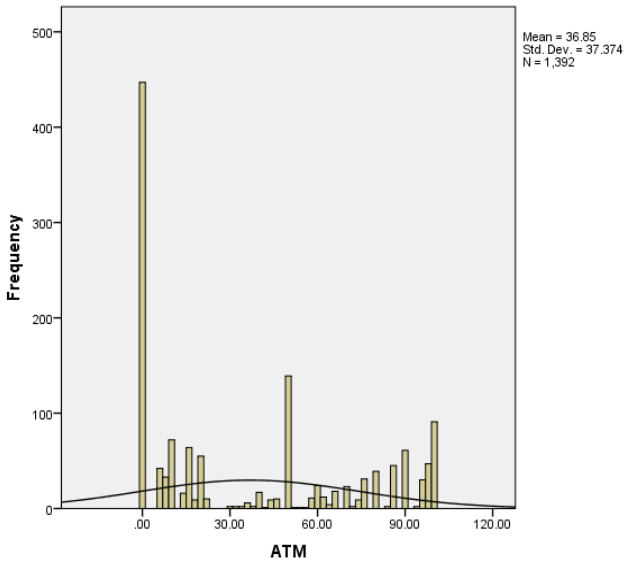
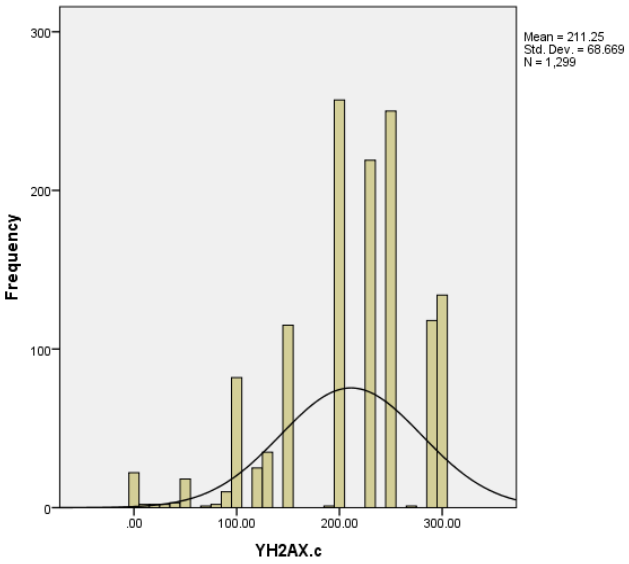
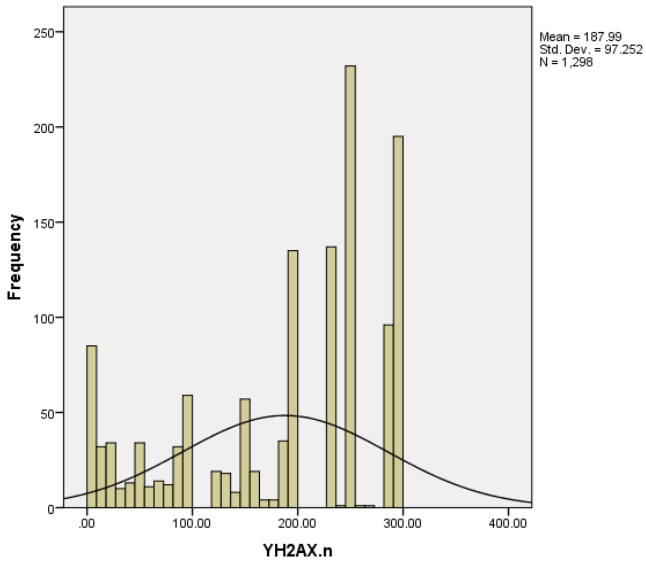
Chapter 10

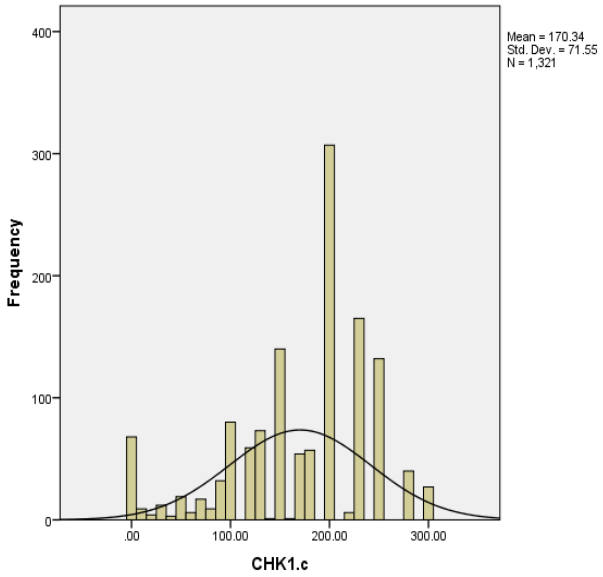
Appendix 1







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Frequency
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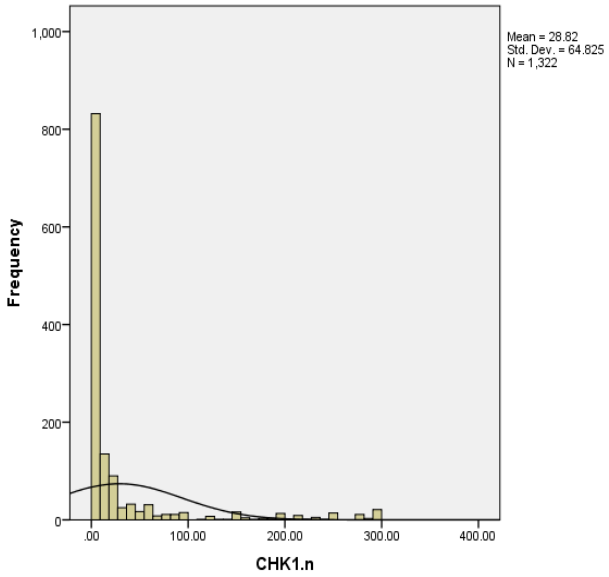
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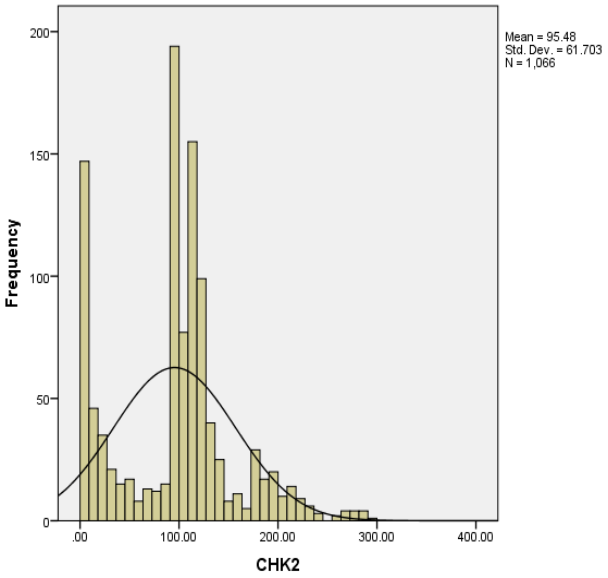
Frequency
CHK1.n


Mean = 95.48
Std. Dev. = 61.703
N = 1,066
Frequency
CHK2

Relationship between DNA Damage Sensors Proteins with Clinico-Pathological Features

Parameters		CHK1						CHK2			
		c^-n^- N(%)	c^+n^+ N(%)	c^+n^- N(%)	c^-n^+ N(%)	X^2	P	Negative N(%)	Positive N(%)	X^2	P
Age	<50	24(31.2)	97(33)	311(35.2)	19(32.2)	1	0.8	165(31.7)	202(37.3)	4	0.05
	>50	53(68.8)	197(67)	573(64.8)	40(67.8)			356(68.3)	340(62.7)		
Size	≤ 1.5cm	31(40.3)	111(37.9)	257(29.3)	24(40.7)	12	0.008	150(28.8)	176(32.5)	2	0.2
	>1.5cm	46(59.7)	182(62.1)	620(70.7)	35(59.3)			371(71.2)	366(67.5)		
Stage	1	50(64.9)	194(66)	511(57.9)	38(64.4)	8.5	0.2	314(60.3)	346(63.8)	1	0.5
	2	19(24.7)	75(25.5)	279(31.6)	18(30.5)			159(30.5)	150(27.7)		
	3	8(10.4)	25(8.5)	92(10.4)	3(5.1)			48(9.2)	46(8.5)		
Grade	1	14(18.2)	60(20.4)	84(9.5)	12(20.3)	59	<0.0001	63(12.1)	81(14.9)	6	0.046
	2	33(42.9)	83(28.2)	237(26.8)	29(49.2)			145(27.8)	176(32.5)		
	3	30(39)	151(51.4)	563(63.7)	18(30.5)			313(60.1)	285(52.6)		
Tubules	1	3(4.1)	19(6.8)	27(3.1)	3(5.1)	16	0.01	18(3.6)	22(4.2)	0.4	0.8
	2	28(38.4)	97(34.5)	241(28)	18(30.5)			155(30.6)	164(31.2)		
	3	42(57.5)	165(58.7)	593(68.9)	38(64.4)			334(65.9)	340(64.6)		
Pleomorphism	1	2(2.7)	9(3.2)	5(0.6)	3(5.1)	77	<0.0001	7(1.4)	8(1.5)	7	0.03
	2	40(54.8)	112(40)	214(24.9)	30(50.8)			153(30.2)	199(37.9)		
	3	31(42.5)	159(56.8)	641(74.5)	26(44.1)			346(68.4)	318(60.6)		
Mitosis	1	28(38.4)	90(32)	214(24.9)	34(57.6)	39	<0.0001	140(27.6)	170(32.3)	4	0.1
	2	14(19.2)	58(20.6)	156(18.1)	6(10.2)			92(18.1)	103(19.6)		
	3	31(42.5)	133(47.3)	491(57)	19(32.2)			275(54.2)	253(48.1)		
NPI	Excellent	8(10.5)	42(14.4)	55(6.3)	9(15.3)	57	<0.0001	46(8.9)	53(9.9)	4	0.6
	Good	18(23.7)	57(19.6)	119(13.6)	15(25.4)			80(15.5)	100(18.7)		
	Moderate I	27(35.5)	95(32.6)	276(31.4)	18(30.5)			151(29.2)	155(29)		
	Moderate	10(13.2)	59(20.3)	262(29.8)	10(16.9)			141(27.3)	142(26.6)		
	Poor	11(14.5)	24(8.2)	122(13.9)	7(11.9)			76(14.7)	65(12.2)		
	Very Poor	2(2.6)	14(4.8)	44(5)	0			23(4.4)	19(3.6)		
Vascular Invasion	Negative	39(61.9)	130(64)	371(59.7)	30(62.5)	1.3	0.7	275(59.9)	285(61.8)	0.3	0.5
	Positive	24(38.1)	73(36)	250(40.3)	18(37.5)			184(40.1)	176(38.2)		
Tumour Type	Invasive	39(52)	165(57.1)	630(72)	17(29.3)	85	<0.0001	340(66)	333(62.8)	10.8	0.028
	lobular	7(9.3)	15(5.2)	40(4.6)	12(20.7)			19(3.7)	30(5.7)		
	Atypical	2(2.7)	7(2.4)	21(2.4)	2(3.4)			21 (4.1)	10 (1.9)		
	Mixed	22(29.3)	84(29.1)	165(18.9)	25(43.1)			124(24.1)	134(25.3)		
	other	5(6.7)	18(6.2)	19(2.2)	2(3.4)			11(2.1)	23(4.3)		

Parameters		γH2AX					
		c^-n^- N(%)	c^+n^+ N(%)	c^+n^- N(%)	c^-n^+ N(%)	X^2	P
Age	<50	9(45)	351(34.7)	60(42.9)	44(35.8)	4	0.2
	>50	11(55)	660(65.3)	80(57.1)	79(64.2)		
Size	≤ 1.5cm	4(22.2)	292(29.1)	25(18.2)	48(39.3)	14	0.002
	>1.5cm	14(77.8)	713(70.9)	112(81.8)	74(60.7)		
Stage	1	12(60)	602(59.7)	81(57.9)	84(86.3)	12	0.06
	2	3(15)	292(29)	48(34.3)	31(25.2)		
	3	5(25)	114(11.3)	11(7.9)	8(6.5)		
Grade	1	1(5)	118(11.7)	4(2.9)	13(10.6)	58	<0.0001
	2	4(20)	267(26.4)	14(10)	54(43.9)		
	3	15(75)	626(61.9)	122(87.1)	56(45.5)		
Tubules	1	0	39(3.9)	0	6(4.9)	27.5	<0.0001
	2	4(21.1)	305(30.5)	19(13.9)	29(23.8)		
	3	15(78.9)	657(65.5)	118(86.1)	87(71.3)		
Pleomorphism	1	0	15(1.5)	1(0.7)	2(1.6)	48	<0.0001
	2	7(36.8)	270(27)	13(9.5)	57(46.7)		
	3	12(63.2)	715(71.5)	123(89.8)	63(51.6)		
Mitosis	1	1(5.3)	264(26.4)	7(5.1)	49(40.2)	64.5	<0.0001
	2	5(26.3)	180(18)	16(11.7)	25(20.5)		
	3	13(68.4)	557(55.6)	114(83.2)	48(39.3)		
NPI	Excellent	1(6.7)	72(7.2)	2(1.4)	9(7.3)	47.5	<0.0001
	Good	1(6.7)	152(15.2)	4(2.9)	30(24.4)		
	Moderate I	8(53.3)	319(31.8)	50(35.7)	44(35.8)		
	Moderate II	3(20)	276(27.5)	47(33.6)	26(21.1)		
	Poor	1(6.7)	131(13.1)	31(22.1)	11(8.9)		
	Very Poor	1(6.7)	52(5.2)	6(4.3)	3(2.4)		
Vascular Invasion	Negative	5(55.6)	404(58.8)	50(57.5)	62(66)	2	0.5
	Positive	4(44.4)	283(41.2)	37(42.5)	32(34)		
Tumour Type	Invasive Ductal/NST	15(78.9)	699(69.8)	106(76.8)	69(58)	92	<0.0001
	lobular	1(5.3)	24(2.4)	4(2.9)	20(16.8)		
	Atypical Medullary	0	22(2.2)	12(8.7)	2(1.7)		
	Mixed	3(15.8)	221(22.1)	14(10.1)	25(21)		
	other	0	35(3.5)	2(1.4)	3(2.5)		

Relationship between DNA Damage Sensors Proteins with Clinico-Pathological Features Continued.									
Parameters		**ATM**				**ATR**			
		Negative N(%)	**Positive N(%)**	X^2	**P**	**Negative N(%)**	**Positive N(%)**	X^2	**P**
Age	**<50**	274 (36.8)	215 (33.4)	1.7	0.2	219(30.6)	196(34.9)	3	0.1
	≥50	470 (63.2)	428 (66.6)			497(69.4)	365(65.1)		
Size	**≤ 1.5cm**	222(29.9)	241 (38)	10	0.002	245(34.2)	151(26.9)	8	0.005
	>1.5cm	520(70.1)	393 (62)			471(65.8)	410(73.1)		
Stage	**1**	444(59.7)	441(68.7)	12.5	0.002	479(66.9)	314(56)	17	<0.0001
	2	224(30.1)	155(24.1)			185(25.8)	185(33)		
	3	76 (10.2)	46 (7.2)			52(7.3)	62(11.1)		
Grade	**1**	61 (8.2)	135 (21)	55.3	<0.0001	152(21.2)	56(10)	40	<0.0001
	2	195(26.2)	186(28.9)			235(32.8)	163(29.1)		
	3	489(65.6)	322(50.1)			329(45.9)	342(61)		
Tubules	**1**	32 (4.4)	37 (5.9)	30	<0.0001	56(8.2)	19(3.4)	13	0.002
	2	170(23.2)	225(35.7)			216(31.7)	185(33)		
	3	532(72.5)	369(58.5)			410(60.1)	357(63.6)		
Pleomorphism	**1**	11 (1.5)	20(3.2)	31	<0.0001	24(3.5)	4(0.7)	29	<0.0001
	2	187(25.5)	239(38)			281(41.5)	174(31)		
	3	535 (73)	370(58.8)			372(54.9)	383(68.3)		
Mitosis	**1**	168(22.9)	240 (38)	40.5	<0.0001	269(39.4)	144(25.7)	28	<0.0001
	2	129(17.6)	110(17.4)			119(17.4)	103(18.4)		
	3	437(59.5)	281(44.5)			294(43.1)	314(56)		
NPI	**Excellent**	49(6.6)	95(15)	56	<0.0001	105(14.7)	35(6.4)	57	<0.0001
	Good	101(13.7)	114(18)			139(19.5)	88(16)		
	Moderate I	241(32.7)	213(33.6)			229(32.1)	143(26)		
	Moderate	200(27.1)	155(24.4)			161(22.6)	159(28.9)		
	Poor	112(15.2)	39(6.2)			63(8.8)	96(17.5)		
	Very Poor	34(4.6)	18(2.8)			16(2.2)	29(5.3)		
Vascular Invasion	**Negative**	297(59.6)	330(70.2)	11.8	0.001	429(67.5)	269(55.6)	16	<0.0001
	Positive	201(40.4)	140(29.8)			207(32.5)	215(44.4)		
Tumour Type	**Invasive Ductal/NST**	525(71)	383(60.3)	30	<0.0001	397(56.6)	367(66.2)	21	<0.0001
	lobular	44(6)	33(5.2)			41(5.8)	27(4.9)		
	Atypical Medullary	25(3.4)	15(2.4)			25(3.6)	10(1.8)		
	Mixed	114(15.4)	170(26.8)			190(27.1)	136(24.5)		
	other	31(4.2)	34(5.4)			48(6.8)	14(2.5)		

Correlation between DNA Damage Sensors Proteins with other Tumours Markers.																	
Markers		**γH2AX**						**CHK1**						**CHK2**			
		c^-n^- N(%)	**c^+n^+ N(%)**	**c^+n^- N(%)**	**c^-n^+ N(%)**	**X^2**	**P**	**c^-n^- N(%)**	**c^+n^+ N(%)**	**c^+n^- N(%)**	**c^-n^+ N(%)**	**X^2**	**P**	**Negative N(%)**	**Positive N(%)**	**X^2**	**P**
ER	**Negativ**	9(52.9)	426(43)	91(67.4)	30(24.8)	49	<0.0001	16(21.9)	100(35.6)	397(46)	11(18.6)	35	<0.0001	169(33.6)	135(25.9)	7	0.007
	Positive	8(47.1)	564(57)	44(32.6)	91(75.2)			57(78.1)	181(64.4)	466(54)	48(81.4)			334(66.4)	386(74.1)		
PgR	**Negativ**	14(73.7)	509(54.1)	101(75.4)	41(35.7)	43	<0.0001	28(73.3)	119(44.9)	467(56.3)	19(33.3)	26	<0.0001	241(48.6)	210(41.1)	6	0.017
	Positive	5(26.3)	432(45.9)	33(24.6)	74(64.3)			47(62.7)	146(55.1)	362(43.7)	38(66.7)			255(51.4)	301(58.9)		
TN	**Negativ**	10(55.6)	690(72)	61(45.9)	97(84.3)	52	<0.0001	62(86.1)	213(78.6)	581(69.2)	49(84.5)	20	<0.0001	396(78.6)	429(82.3)	2	0.1
	Positive	8(44.4)	268(28)	72(54.1)	18(15.7)			10(13.9)	58(21.4)	258(30.8)	9(15.5)			108(21.4)	92(17.7)		
HER-2	**Negativ**	19(95)	798(82.4)	117(84.8)	104(88.1)	5	0.2	66(93)	241(86.1)	703(82)	55(94.8)	13	0.005	418(82.1)	455(86)	3	0.09
	Positive	1(5)	170(17.6)	21(15.2)	14(11.9)			5(7)	39(13.9)	154(18)	3(5.2)			91(17.9)	74(14)		
CK5	**Negativ**	9(75)	560(76)	62(55.4)	74(83.1)	26	<0.0001	48(92.3)	159(75.4)	543(72.7)	39(92.9)	17.5	0.001	337(83.2)	328(82)	0.2	0.6
	Positive	3(25)	117(24)	50(44.6)	15(16.9)			4(7.7)	52(24.6)	204(27.3)	3(7.1)			68(16.8)	72(18)		
CK17	**Negativ**	13(92.9)	589(80.1)	96(79.3)	70(85.4)	3	0.4	48(90.6)	191(86.4)	585(81.6)	32(91.4)	7	0.08	319(84.6)	322(86.8)	0.7	0.4
	Positive	1(7.1)	146(19/9)	25(20.7)	12(14.6)			5(9.4)	30(13.6)	132(18.4)	3(8.6)			58(15.4)	49(13.2)		
CK14	**Negativ**	17(94.4)	811(87)	107(79.3)	101(87.8)	7	0.06	69(89.6)	227(84.7)	719(86.7)	50(92.6)	3	0.3	451(90.7)	449(89.1)	0.8	0.4
	Positive	1(5.6)	121(13)	28(20.7)	14(12.2)			8(10.4)	41(15.3)	110(13.3)	4(7.4)			46(9.3)	55(10.9)		
BLBC	**Negativ**	9(64.3)	674(78)	63(53.4)	95(86.4)	42.5	<0.0001	56(90.3)	207(81.2)	582(74.8)	51(96.2)	22	<0.0001	384(85)	410(86)	0.1	0.7
	Positive	5(35.7)	190(22)	55(46.6)	15(13.6)			6(9.7)	48(18.8)	196(25.2)	2(3.8)			68(15)	67(14)		
P53	**Negativ**	8(47.1)	594(61.6)	69(50.4)	76(65)	9	0.03	58(81.7)	175(63.6)	516(61)	46(79.3)	19	<0.0001	340(69.1)	360(70.6)	0.3	0.6
	Positive	9(52.9)	370(38.4)	68(49.6)	41(35)			13(18.3)	100(36.4)	330(39)	12(20.7)			152(30.9)	150(29.4)		
ID4. n	**Negativ**	16(80)	751(84.5)	103(76.3)	89(86.4)	6.5	0.09	60(87)	208(79.1)	693(85.3)	45(84.9)	6	0.09	407(90.4)	399(88.9)	0.6	0.4
	Positive	4(20)	138(15.5)	32(23.7)	14(13.6)			9(13)	55(20.9)	119(14.7)	8(15.1)			43(9.6)	50(11.1)		
ID4.c	**Negativ**	7(35)	347(35)	50(35.5)	59(48.4)	8	0.038	46(58.2)	116(39.7)	286(32.5)	29(49.2)	27.5	<0.0001	180(34.4)	197(36.3)	0.4	0.5
	Positive	13(65)	644(65)	91(64.5)	63(51.6)			33(41.8)	176(60.3)	594(67.5)	30(50.8)			343(65.6)	346(63.7)		
PTEN	**Negativ**	10(100)	423(79.7)	64 (94.1)	43 (69.4)	15.6	0.001	36 (83.7)	99 (79.8)	359(82.7)	11 (47.8)	17.5	0.001	328(89.1)	226(72.4)	31	<0.0001
	Positive	0	108(20.3)	4 (5.9)	19 (30.6)			7 (16.3)	25 (20.2)	75 (17.3)	12 (52.2)			40 (10.9)	86 (27.6)		
CHK1.n	**Negativ**	11(84.6)	496(76.5)	83(84.7)	47(58)	19	<0.0001							281(81)	233(73)	6	0.015
	Positive	2(15.4)	152(23.5)	15(15.3)	34(42)									66(19)	86(27)		
CHK1.c	**Negativ**	6(46.2)	47(7.3)	10(10.2)	16(19.8)	34	<0.0001							38(11)	35(11)	0	0.99
	Positive	7(53.8)	600(92.7)	88(89.8)	65(80.2)									308(89)	284(89)		
Ki-67	**Negativ**	6(40)	287(32.8)	21(17.4)	56(52.8)	32.5	<0.0001	32(51.6)	95(39.4)	240(31.9)	29(55.8)	22	<0.0001	155(37)	179(40.6)	1.2	0.3
	Positive	9(60)	588(67.2)	100(82.6)	50(47.2)			30(48.4)	146(60.6)	513(68.1)	23(44.2)			264(63)	262(59.4)		
CHK2	**Negativ**	7(63.6)	274(45.4)	63(84)	35(50)	41	<0.0001	30(62.5)	57(45.2)	251(53.9)	8(32)	9	0.028				
	Positive	4(36.4)	330(54.6)	12(16)	35(50)			18(37.5)	69(54.8)	215(46.1)	17(68)						
ATM	**Negativ**	26(66.7)	80 (40.4)	368(59.8)	19 (47.5)	26	<0.0001	26(66.7)	84(42.4)	371(60.3)	19(47.5)	22.5	<0.0001	203(59.9)	147(47.6)	10	0.002
	Positive	13(33.3)	118(59.6)	247(40.2)	21 (52.5)			13(33.3)	114(57.6)	244(39.7)	21(52.5)			136(40.1)	162(52.4)		
ATR	**Negativ**	26(45.6)	109(67.3)	264(50.6)	19(51.4)	16	0.001	26(45.6)	109(67.3)	264(50.6)	19(51.4)	16	0.001	257(57.1)	219(47.6)	8	0.004
	Positive	31(54.4)	53(32.7)	258(49.4)	18(48.6)			31(54.4)	53(32.7)	258(49.4)	18(48.6)			193(42.9)	241(52.4)		
MTA1.n	**Negativ**	15(75)	294(31.7)	105(75.5)	36(31.9)	113	<0.0001	35(66)	73(38.8)	264(42.2)	14(37.8)	13	0.004	187(46.2)	123(31.1)	19	<0.0001
	Positive	5(25)	634(68.3)	34(24.5)	77(68.1)			18(34)	115(61.2)	361(57.8)	23(62.2)			218(53.8)	272(68.9)		
MTA1.c	**Negativ**	9(45)	172(17)	19(13.5)	65(52.4)	139	<0.0001	26(49.1)	26(13.8)	70(11.2)	11(29.7)	63	<0.0001	85(20.9)	41(10.4)	16.7	<0.0001
	Positive	11(55)	841(83)	122(86.5)	59(47.6)			27(50.9)	162(86.2)	556(88.8)	26(70.3)			322(79.1)	354(89.6)		
P21	**Negativ**	8(61.5)	217(56.5)	43(64.2)	27(60)	1.5	0.6	26(61.9)	83(58)	195(62.1)	21(63.6)	0.8	0.8	91(59.5)	95(68.3)	2.5	0.1
	Positive	5(38.5)	167(43.5)	24(35.8)	18(40)			16(38.1)	60(42)	119(37.9)	12(36.4)			62(40.5)	44(31.7)		

Correlation between DNA Damage Sensors Proteins with other Tumours Markers Continued.

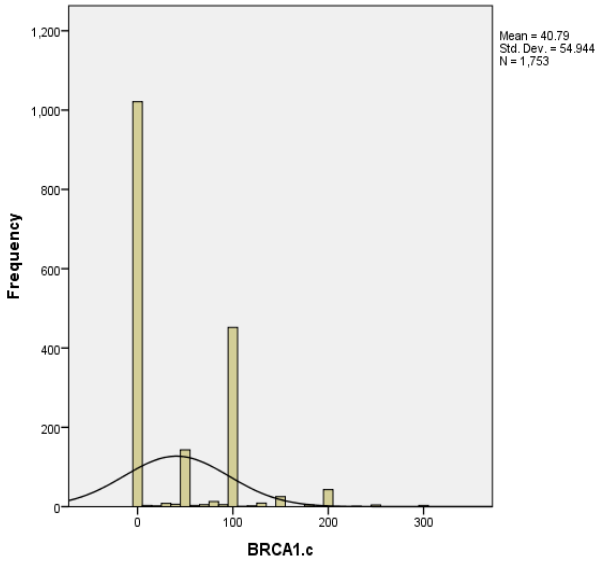
Markers		γH2AX						CHK1						CHK2			
		c^-n^- N(%)	c^+n^+ N(%)	c^+n^- N(%)	c^-n^+ N(%)	X^2	P	c^-n^- N(%)	c^+n^+ N(%)	c^+n^- N(%)	c^-n^+ N(%)	X^2	P	Negative n(%)	Positive n(%)	X^2	P
P27	Negative	7(58.3)	179(46.9)	50(72.5)	18(46.2)	16	0.001	16(37.2)	67(47.5)	156(49.8)	10(31.3)	6	0.1	37(25)	40(29.4)	0.7	0.4
	Positive	5(41.7)	203(53.1)	19(53.8)	21(53.8)			27.8)(6	74(52.5)	157(50.2)	22(68.8)			111(75)	96(70.6)		
γH2AX.n	Negative													70(18.5)	16(4.3)	38.5	<0.0001
	Positive													309(81.5)	365(95.8)		
γH2AX.c	Negative													42(11.1)	39(10.2)	0.1	0.7
	Positive													337(88.9)	342(89.8)		

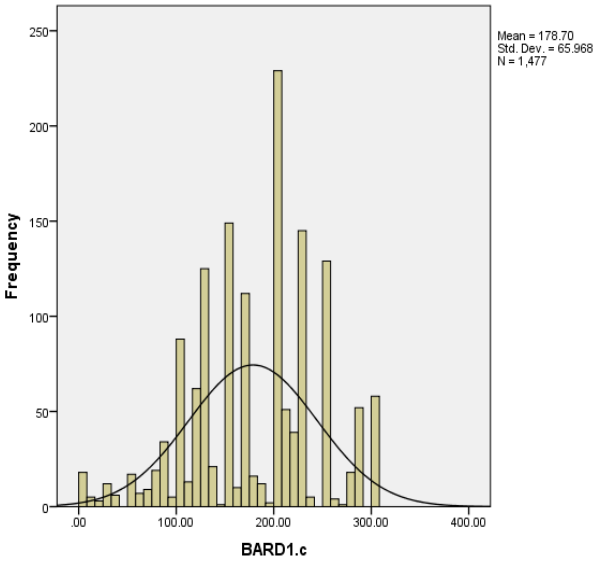
Correlation between DNA Damage Sensors Proteins with Clinico-Pathological Features									
Parameters		**ATM**				**ATR**			
		Negative n(%)	**Positive n(%)**	X^2	**P**	**Negative n(%)**	**Positive n(%)**	X^2	**P**
ER	**Negative**	362 (49.3)	221 (34.9)	29	<0.0001	193(28.3)	141(26)	0.8	0.3
	Positive	372 (50.7)	413 (65.1)			488(71.7)	401(74)		
PgR	**Negative**	415 (58.7)	265 (44.1)	27.7	<0.0001	303(45)	217(41.3)	2	0.2
	Positive	292 (41.3)	336 (55.9)			370(55)	309(58.7)		
Triple Negative	**Negative**	475 (66.5)	485 (78.9)	25	<0.0001	557(81.4)	447(82.6)	0.3	0.6
	Positive	239 (33.5)	130 (21.1)			127(18.6)	94(17.4)		
HER-2	**Negative**	596 (82.8)	523 (85)	1.2	0.26	607(86.8)	459(83.9)	2	0.1
	Positive	124 (17.2)	92 (15)			92(13.2)	88(16.1)		
CK5	**Negative**	427 (73.7)	338(76.3)	.867	.352	402(82.2)	383(85.3)	2	0.2
	Positive	152 (26.3)	105(23.7)			87(17.8)	66(14.7)		
CK17	**Negative**	451 (81.1)	360(85.1)	2.7	0.1	425(89.9)	314(81.8)	12	0.001
	Positive	105 (18.9)	63 (14.9)			48(10.1)	70(18.2)		
BLBC	**Negative**	478 (74.9)	456 (82.2)	9	0.002	531(86.6)	427(86.4)	0.008	0.9
	Positive	160 (25.1)	99 (17.8)			82(13.4)	67(13.6)		
P53	**Negative**	443 (60.9)	417(66.1)	3.8	0.05	471(70.9)	368(69.2)	0.4	0.5
	Positive	284 (39.1)	214(33.9)			193(29.1)	164(30.8)		
CHK1.n	**Negative**	397(79.2)	257(65.6)	21	<0.0001	290(69.2)	289(80.3)	12	<0.0001
	Positive	104(20.8)	135(34.4)			129(30.8)	71(19.7)		
CHK1.c	**Negative**	45(9)	34(8.7)	0.03	0.9	45(10.8)	49(13.6)	1.5	0.2
	Positive	455(91)	358(91.3)			373(89.2)	311(86.4)		
Ki-67	**Negative**	221 (34.7)	233 (43.8)	10	0.001	278(51.1)	151(31.8)	39	<0.0001
	Positive	416 (65.3)	299(56.2)			266(48.9)	324(68.2)		
ATR	**Negative**	211(51.6)	228(60.2)	6	0.016				
	Positive	198(48.4)	151(39.8)						
P21	**Negative**	191(62)	164(59.4)	0.4	0.5	154(62.1)	84(64.1)	0.1	0.7
	Positive	117(38)	112(40.6)			94(37.9)	47(35.9)		
γH2AX.n	**Negative**	81(16.6)	37(9.8)	8.4	0.004	66(13.4)	37(8)	7	0.007
	Positive	407(83.4)	341(90.2)			427(86.6)	425(92)		
γH2AX.c	**Negative**	45(9.2)	46(12.1)	2	0.2	57(11.6)	50(10.8)	0.1	0.7
	Positive	443(90.8)	333(87.9)			436(88.4)	413(89.2)		

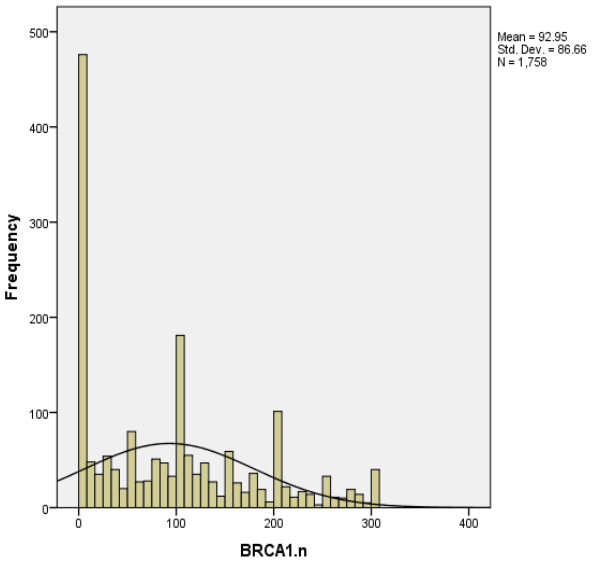
Correlation between DNA Damage Sensors and Signal Transducers Proteins with other Tumour Markers Regardless Co-expression of Cellular Localisation.				
γH2AX.c				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
CHK1.n	**Negative**	58 (61.7)	580 (77.6)	0.001 11.5
	Positive	36 (38.3)	167 (22.4)	
CHK1.c	**Negative**	22 (23.4)	57 (7.6)	<0.0001 24
	Positive	72 (76.6)	689 (92.4)	
PgR				
Markers		**Negative**	**Positive**	**P-value**
CHK1.c	**Negative**	47 (7.4)	85 (14.3)	<0.0001 15
	Positive	586 (92.6)	508 (85.7)	
CHK1.n	**Negative**	495 (78.1)	409 (69)	<0.0001 13
	Positive	139 (21.9)	184 (31)	
γH2AX.c	**Negative**	55 (8.3)	79 (14.5)	0.001 12
	Positive	611 (91.7)	465 (85.5)	
γH2AX.n	**Negative**	115 (17.3)	38(7)	<0.0001 28.7
	Positive	550(82.7)	506 (93)	
P53				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
CHK1.n	**Negative**	574 (72.1)	343 (75.4)	0.2 1.5
	Positive	222 (27.9)	112 (24.6)	
CHK1.c	**Negative**	104 (13.1)	25 (5.5)	<0.0001 18
	Positive	691 (86.9)	430 (94.5)	
ER				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
γH2AX.n	**Negative**	100(18)	52 (7.4)	<0.0001 33
	Positive	456 (82)	655 (92.6)	
γH2AX.c	**Negative**	39 (7)	99 (14)	<0.0001 15.7
	Positive	518 (93)	608 (86)	
CK5				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
γH2AX.c	**Negative**	83 (11.8)	18 (7.3)	0.05 4
	Positive	622 (88.2)	228 (92.7)	
γH2AX.n	**Negative**	71 (10.1)	53 (21.6)	<0.0001 21
	Positive	634 (89.9)	192 (78.4)	
CHK1.n	**Negative**	591 (74.9)	208 (78.8)	0.2 1.6
	Positive	198 (25.1)	56 (21.2)	
CHK1.c	**Negative**	87 (11)	7 (2.7)	<0.0001 17
	Positive	702 (89)	256 (97.3)	
PTEN				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
γH2AX.n	**Negative**	74 (13.7)	4 (3.1)	0.001 11.6
	Positive	466 (86.3)	127 (96.9)	
γH2AX.c	**Negative**	53 (9.8)	19 (14.5)	0.1 2
	Positive	487 (90.2)	112 (85.5)	

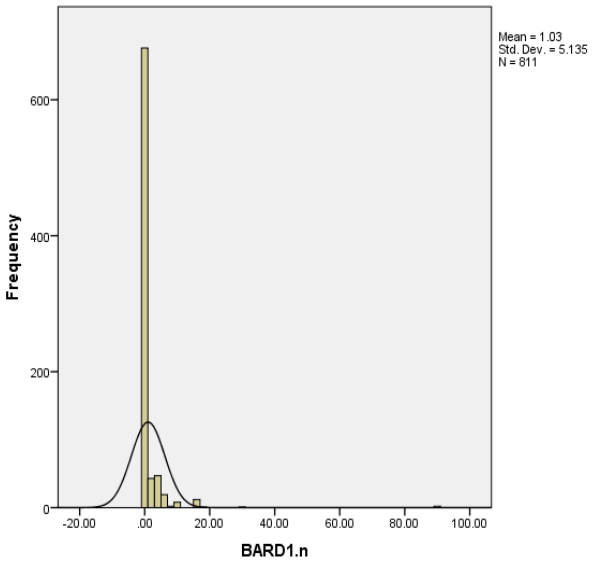
Correlation between DNA Damage Sensors and Signal Transducers Proteins with other Tumour Markers Regardless Co-expression of Cellular Localisation.				
γH2AX.c				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
CHK1.n	**Negative**	58 (61.7)	580 (77.6)	0.001 11.5
	Positive	36 (38.3)	167 (22.4)	
CHK1.c	**Negative**	22 (23.4)	57 (7.6)	<0.0001 24
	Positive	72 (76.6)	689 (92.4)	
PgR				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
CHK1.c	**Negative**	47 (7.4)	85 (14.3)	<0.0001 15
	Positive	586 (92.6)	508 (85.7)	
CHK1.n	**Negative**	495 (78.1)	409 (69)	<0.0001 13
	Positive	139 (21.9)	184 (31)	
γH2AX.c	**Negative**	55 (8.3)	79 (14.5)	0.001 12
	Positive	611 (91.7)	465 (85.5)	
γH2AX.n	**Negative**	115 (17.3)	38(7)	<0.0001 28.7
	Positive	550(82.7)	506 (93)	
P53				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
CHK1.n	**Negative**	574 (72.1)	343 (75.4)	0.2 1.5
	Positive	222 (27.9)	112 (24.6)	
CHK1.c	**Negative**	104 (13.1)	25 (5.5)	<0.0001 18
	Positive	691 (86.9)	430 (94.5)	
ER				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
γH2AX.n	**Negative**	100(18)	52 (7.4)	<0.0001 33
	Positive	456 (82)	655 (92.6)	
γH2AX.c	**Negative**	39 (7)	99 (14)	<0.0001 15.7
	Positive	518 (93)	608 (86)	
CK5				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
γH2AX.c	**Negative**	83 (11.8)	18 (7.3)	0.05 4
	Positive	622 (88.2)	228 (92.7)	
γH2AX.n	**Negative**	71 (10.1)	53 (21.6)	<0.0001 21
	Positive	634 (89.9)	192 (78.4)	
CHK1.n	**Negative**	591 (74.9)	208 (78.8)	0.2 1.6
	Positive	198 (25.1)	56 (21.2)	
CHK1.c	**Negative**	87 (11)	7 (2.7)	<0.0001 17
	Positive	702 (89)	256 (97.3)	
PTEN				
Markers		**Negative N (%)**	**Positive N (%)**	**P-value X^2**
γH2AX.n	**Negative**	74 (13.7)	4 (3.1)	0.001 11.6
	Positive	466 (86.3)	127 (96.9)	
γH2AX.c	**Negative**	53 (9.8)	19 (14.5)	0.1 2
	Positive	487 (90.2)	112 (85.5)	

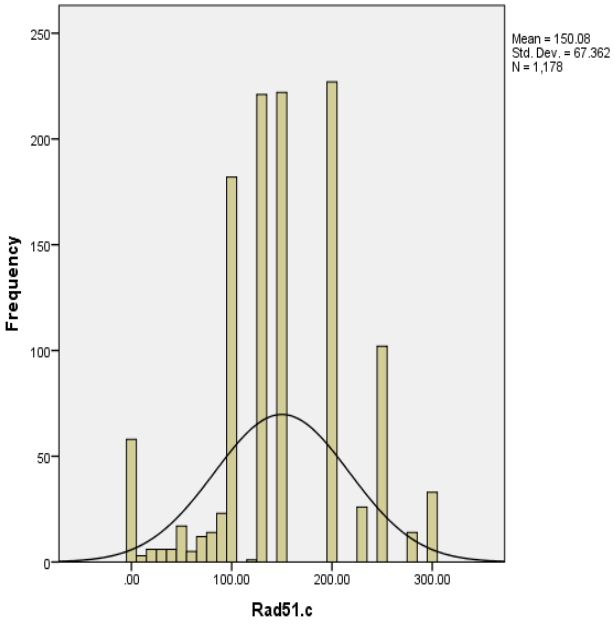
Appendix 2

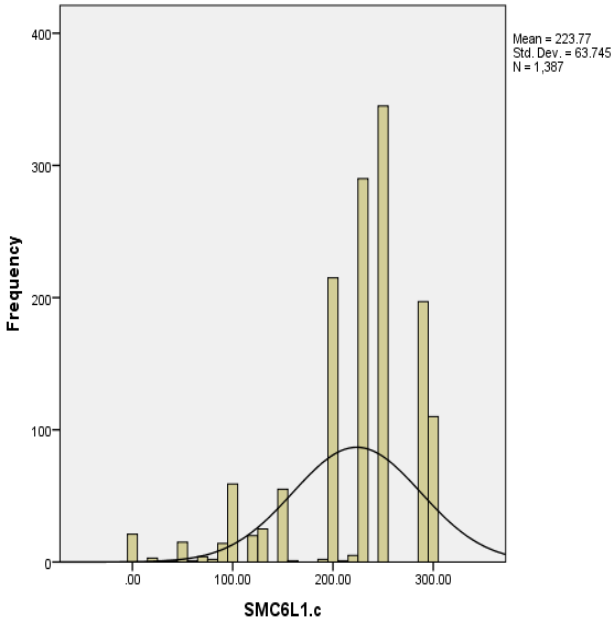

Mean = 40.79
Std. Dev. = 54.944
N = 1,753
Frequency
BRCA1.c

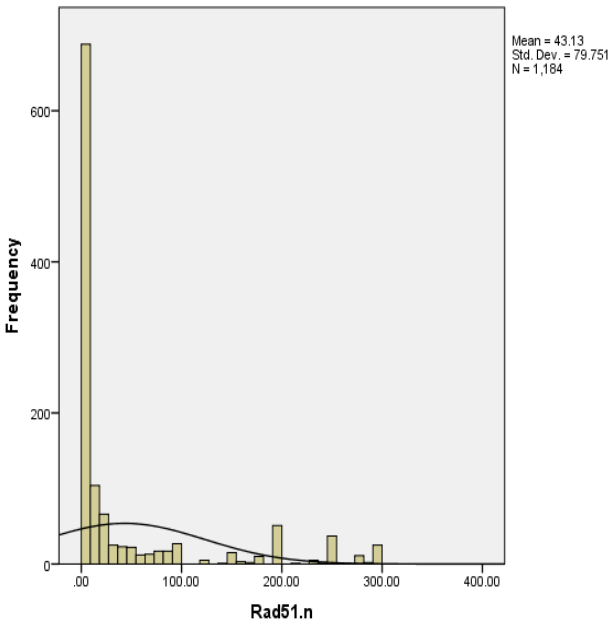

Mean = 178.70
Std. Dev. = 65.968
N = 1,477
Frequency
BARD1.c

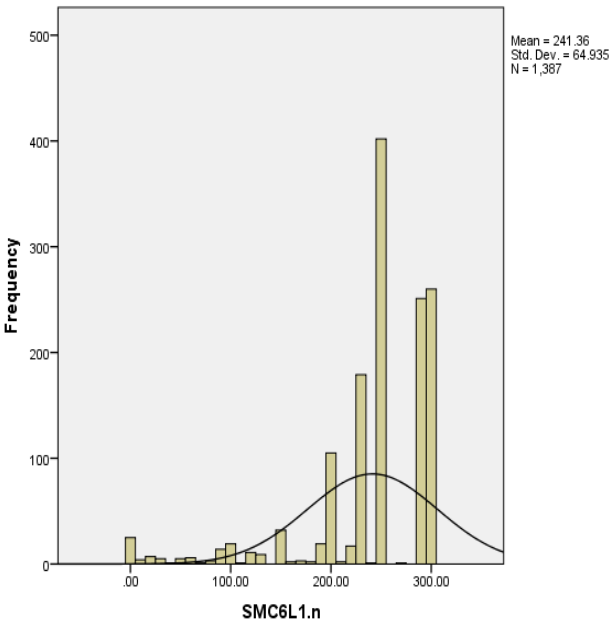

Mean = 92.95
Std. Dev. = 86.66
N = 1,758
Frequency
BRCA1.n

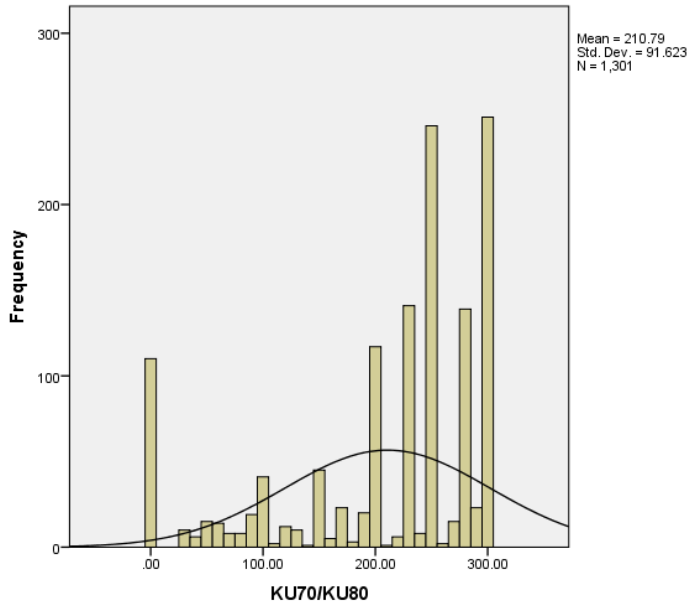

Mean = 1.03
Std. Dev. = 5.135
N = 811
Frequency
BARD1.n

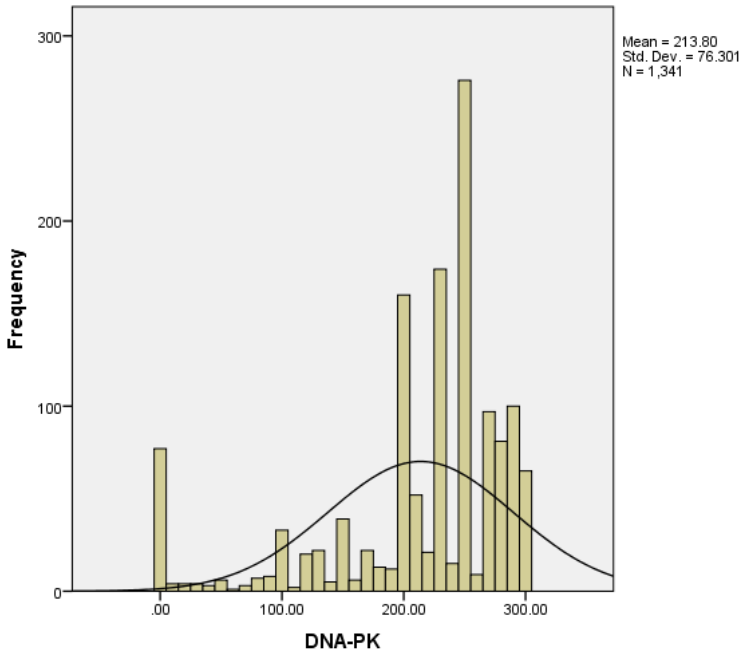

Mean = 150.08
Std. Dev. = 67.362
N = 1,178
Frequency
Rad51.c

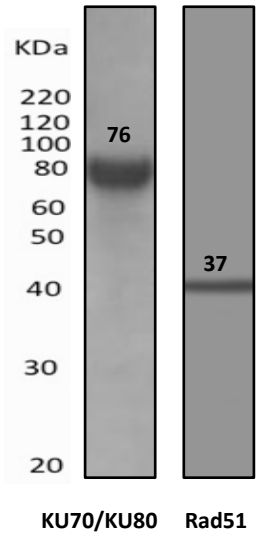

Mean = 223.77
Std. Dev. = 63.745
N = 1,387
Frequency
SMC6L1.c


Mean = 43.13
Std. Dev. = 79.751
N = 1,184
Frequency
Rad51.n


Mean = 241.36
Std. Dev. = 64.935
N = 1,387
Frequency
SMC6L1.n


Mean = 210.79
Std. Dev. = 91.623
N = 1,301
Frequency
300
200
100
0
.00
100.00
200.00
300.00
KU70/KU80


Mean = 213.80
Std. Dev. = 76.301
N = 1,341
Frequency
300
200
100
0
.00
100.00
200.00
300.00
DNA-PK



Detection of Rad51 and KU70/KU80 proteins level by Western blot in a mixture of cell lines MDA-MB-231, MCF-7 and HeLa BRCA1and it's control.

Relationship between DNA-DSB Repair Markers with Clinico-Pathological Parameters.																	
Parameters		**Rad51**						**BRCA1**						**BARD1.c**			
		c^-n^- N(%)	**c^+n^+ N(%)**	**c^+n^- N(%)**	**c^-n^+ N(%)**	***X²***	**P**	**c^-n^- N(%)**	**c^+n^+ N(%)**	**c^-n^+ N(%)**	**c^+n^- N(%)**	***X²***	**P**	**Negative n(%)**	**Positive n(%)**	***X²***	**P**
NPI	**Excellent**	9(12.5)	45(13.1)	28(4)	7(12.7)	111	<0.0001	28 (6)	37 (13.2)	93 (16.6)	16 (3.8)	148	<0.0001	13(4.5)	89(7.7)	8	0.1
	Good	22(30.6)	80(23.3)	61(8.8)	14(25.5)			50 (10.7)	62 (22.1)	131(23.4)	38 (9)			38(13.1)	202(17.4)		
	Moderate1	16(22.2)	94(27.3)	250(36)	17(30.9)			151(32.2)	75 (26.8)	171(30.5)	146 (34.5)			102(35.1)	367(31.6)		
	Moderae2	16(22.2)	83(24.1)	205(29.5)	16(29.1)			137(29.2)	69 (24.6)	104(18.6)	134 (31.7)			79(27.1)	300(25.8)		
	Poor	6(8.3)	33(9.6)	109(15.7)	1(1.8)			76 (16.2)	26 (9.3)	50 (8.9)	63 (14.9)			46(15.8)	152(13.1)		
	Very poor	3(4.2)	9(2.6)	41(5.9)	0			27 (5.8)	11 (3.9)	11 (2)	26 (6.1)			13(4.5)	51(4.4)		
Vascular Invasion	**Negative**	40(65.6)	186(65.3)	230(55.4)	32(71.1)	10	0.019	182(56.9)	159(62.6)	322(67.8)	137(60.4)	10	0.015	81(64.8)	536(60.4)	0.9	0.3
	Positive	21(34.4)	99(34.7)	185(44.6)	13(28.9)			138(43.1)	95(37.4)	153(32.2)	90(39.6)			44(35.2)	351(39.6)		
Age	**<50**	22(30.6)	104(29.9)	264(37.8)	15(27.3)	8	0.039	176(38.3)	99(34.5)	186(33.2)	165(37.2)	3.5	0.3	113(38)	421(35.9)	0.5	0.5
	≥50	50(69.4)	244(70.1)	435(62.2)	40(72.7)			284(61.7)	188(65.5)	375(66.8)	279(62.8)			184(62)	752(64.1)		
Size	**≤ 1.5cm**	30(41.7)	138(39.8)	177(25.7)	19(34.5)	25.5	<0.0001	128(27.1)	88 (31.1)	229(40.5)	108 (25.9)	31	<0.0001	76(26.6)	356(30.4)	1.5	0.2
	>1.5cm	42(58.3)	209(60.2)	512(74.3)	36(65.5)			345(72.9)	195(68.9)	337(59.5)	309 (74.1)			210(73.4)	816(69.6)		
Stage	**1**	44(61.1)	217(62.4)	402(57.7)	35(63.6)	4.2	0.6	276(60)	184(63.9)	361(64.3)	269(60.9)	5	0.5	188(63.5)	711(60.7)	2	0.4
	2	20(27.8)	102(29.3)	220(31.6)	17(30.9)			140(30.4)	78(27.1)	160(28.5)	126(28.5)			76(25.7)	345(29.4)		
	3	8(11.1)	29(8.3)	75(10.8)	3(5.5)			44(9.6)	26(9)	40(7.1)	47(10.6)			32(10.8)	116(9.9)		
Grade	**1**	14(19.4)	67(19.3)	47(6.7)	9(16.4)	140	<0.0001	41 (8.6)	57 (20.1)	128(22.6)	24 (5.6)	285	<0.0001	27(9.1)	148(12.6)	28	<0.0001
	2	31(43.1)	129(37.1)	139(19.9)	32(58.2)			91 (19.2)	93 (32.9)	255(45.1)	66 (15.3)			50(16.8)	350(29.8)		
	3	27(37.5)	152(43.7)	513(73.4)	14(25.5)			342(72.2)	133 (47)	183(32.3)	340 (79.1)			220(74.1)	676(57.6)		
Tubules	**1**	3(4.5)	21(6.2)	14(2)	1(1.9)	44.5	<0.0001	18 (3.9)	16 (5.8)	38 (6.9)	12 (2.8)	51	<0.0001	12(4.1)	45(3.9)	33	<0.0001
	2	29(43.3)	117(34.6)	160(23.4)	24(44.4)			108(23.5)	105(37.9)	198(35.8)	99 (23.4)			43(14.8)	366(31.9)		
	3	35(52.2)	200(59.2)	509(74.5)	29(53.7)			334(72.6)	156(56.3)	317(57.3)	312 (73.8)			235(81)	738(64.2)		
Pleomorphism	**1**	1(1.5)	9(2.7)	1(0.1)	0	104.5	<0.0001	3 (0.7)	9 (3.2)	14 (2.5)	2 (0.5)	319	<0.0001	5(1.7)	16(1.4)	10	0.007
	2	32(47.8)	142(42.1)	139(20.4)	32(59.3)			97 (21.1)	99 (35.7)	322(58.4)	42 (10)			61(21)	348(30.4)		
	3	34(50.7)	186(55.2)	541(79.4)	22(40.7)			359(78.2)	169 (61)	215 (39)	378 (89.6)			224(77.2)	780(68.2)		
Mitosis	**1**	28(41.8)	135(39.9)	117(17.1)	25(46.3)	104	<0.0001	82 (17.8)	103(37.2)	276(49.9)	58 (13.7)	228.6	<0.0001	57(19.7)	328(28.5)	16	<0.0001
	2	16(23.9)	65(19.2)	120(17.6)	16(29.6)			78 (17)	57 (20.6)	106(19.2)	76 (18)			43(14.8)	215(18.7)		
	3	23(34.3)	138(40.8)	446(65.3)	13(24.1)			300(65.2)	117(42.2)	171(30.9)	289 (68.3)			190(65.5)	606(52.7)		
Tumour Type	**Invasive Ductal/NST**	35(49.3)	188(54.7)	535 (77.3	17 (31.5)	137	<0.0001	355(75.4)	167(60.1)	236(42.1)	353 (83.3)	286	<0.0001	211 (72.5)	782 (67.4)	22	<0.0001
	lobular	8 (11.3)	24 (7)	20 (2.9)	13 (24.1)			10 (2.1)	7 (2.5)	87 (15.5)	6 (1.4)			19 (6.5)	50 (4.3)		
	Atypical Medullary	1 (1.4)	5 (1.5)	21 (3)	0			18 (3.8)	8 (2.9)	6 (1.1)	10 (2.4)			7 (2.4)	29 (2.5)		
	Mixed	21(29.6)	109(31.7)	98 (14.2)	22 (40.7)			70 (14.9)	80 (28.8)	195(34.8)	46 (10.8)			36 (12.4)	265 (22.8)		
	Other	6 (8.5)	18 (5.2)	18 (2.6)	2 (3.7)			18 (3.8)	16 (5.8)	36 (6.4)	9 (2.1)			18 (6.2)	34 (2.9)		

Relationship between DNA-DSB Repair Markers with Clinico-Pathological Parameters Continued.

Parameters		SMC6L1						KU70/KU80				DNA-PK			
		c^-n^- N(%)	c^+n^+ N(%)	c^+n^- N(%)	c^-n^+ N(%)	X^2	P	Negative N(%)	Positive N(%)	X^2	P	Negative N(%)	Positive N(%)	X^2	P
Age	**<50**	87(35.4)	252(35.3)	86(38.4)	71(36.2)	0.7	0.9	55(32.7)	389(34.5)	0.2	0.6	59(29.9)	427(37.6)	4	0.04
	≥50	159(64.6)	462(64.7)	138(61.6)	125(63.8)			113(67.3)	737(65.5)			138(70.1)	709(62.4)		
Size	**≤ 1.5cm**	75(30.9)	198(27.9)	54(24.2)	65(33.7)	5	0.1	57(33.9)	357(32)	0.2	0.6	63(32.1)	313(27.8)	1.5	0.2
	>1.5cm	168(69.1)	512(72.1)	169(75.8)	128(66.3)			111(66.1)	758(68)			133(67.9)	811(72.2)		
Stage	**1**	161(65.4)	384(53.9)	147(65.9)	129(65.8)	24	0.001	115(68.5)	664(59)	6	0.059	134(68)	660(58.1)	7	0.03
	2	68(27.6)	240(33.7)	61(27.4)	61(27.4)			38(22.6)	348(30.9)			46(23.4)	350(30.8)		
	3	17(6.9)	89(12.5)	15(6.7)	15(6.7)			15(8.9)	113(10)			17(8.6)	125(11)		
Grade	**1**	34(13.8)	70(9.8)	21(9.3)	26(13.3)	22	0.001	26(15.5)	139(12.4)	8	0.01	23(11.7)	115(10.1)	1	0.5
	2	72(29.3)	189(26.5)	40(17.8)	65(33.2)			60(35.7)	30(27.1)			56(28.4)	292(25.7)		
	3	140(56.9)	455(63.7)	164(72.9)	105(53.6)			82(48.8)	681(60.5)			118(59.9)	730(64.2)		
Tubules	**1**	8(3.4)	19(2.7)	7(3.2)	9(4.8)	10	0.1	6(3.7)	44(4)	9.5	0.008	4(2.1)	36(3.2)	0.7	0.7
	2	66(27.8)	226(31.9)	49(22.6)	48(25.5)			63(38.7)	295(26.9)			55(28.9)	314(28)		
	3	163(68.8)	463(65.4)	161(74.2)	131(69.7)			94(57.7)	756(69)			131(68.9)	771(68.8)		
Pleomorphism	**1**	8(3.4)	6(0.8)	1(0.5)	4(2.1)	44	<0.0001	2(1.2)	13(1.2)	5	0.09	5(2.6)	10(0.9)	6	0.046
	2	83(35.2)	169(23.9)	40(18.5)	72(38.3)			63(38.6)	330(30.2)			59(31.1)	300(26.8)		
	3	145(61.4)	531(75.2)	175(81)	112(59.6)			98(60.1)	750(68.6)			126(66.3)	809(72.3)		
Mitosis	**1**	61(25.7)	172(24.3)	38(17.5)	68(36.2)	27	<0.0001	53(32.5)	314(28.7)	1.5	0.5	50(26.3)	280(25)	0.2	0.9
	2	48(20.3)	138(19.5)	30(13.8)	27(14.4)			30(18.4)	189(17.3)			32(16.8)	188(16.8)		
	3	128(54)	398(56.2)	149(68.7)	93(49.5)			80(49.1)	592(54.1)			108(56.8)	653(58.3)		
NPI	**Excellent**	22(9.1)	41(5.8)	14(6.3)	14(7.2)	24	0.07	19(11.4)	91(8.2)	7.4	0.2	15(7.7)	70(6.2)	4.3	0.5
	Good	36(14.9)	105(14.9)	23(10.3)	39(20)			33(19.8)	165(14.8)			31(15.9)	166(14.8)		
	Moderate1	92(38)	218(30.8)	77(34.4)	66(33.8)			47(28.1)	357(32)			68(34.9)	351(31.3)		
	Moderate2	58(24)	188(26.6)	69(30.8)	48(24.6)			46(27.5)	294(26.4)			52(26.7)	308(27.4)		
	Poor	26(10.7)	117(16.5)	31(13.8)	21(10.8)			17(10.2)	152(13.6)			20(10.3)	172(15.3)		
	Very poor	8(3/3)	38(5.4)	10(4.5)	7(3.6)			5(3)	56(5)			9(4.6)	56(5)		
Vascular Invasion	**Negative**	126(66)	241(51.6)	109(66.9)	93(66)	21	<0.0001	168(100)	935(83.7)	32	<0.0001	93(61.2)	463(60.4)	0.04	0.8
	Positive	65(34)	226(48.4)	54(33.1)	48(34)			0	182(16.3)			59(38.8)	305(39.6)		
Tumour Type	**Invasive Ductal/NST**	160 (66.7)	506 (71.8)	165 (75.3)	111 (57.2)	49	<0.0001	106(63.5)	738(66.4)	2	0.7	130 (67.4)	791(70.2)	3	0.6
	lobular	14 (5.8)	22 (3.1)	2 (0.9)	19 (9.8)			9(5.4)	77(6.9)			9(4.7)	49(4.3)		
	Atypical Medullary	11 (4.6)	13 (1.8)	9 (4.1)	3 (1.5)			6(3.6)	29(2.6)			8(4.1)	25(2.2)		
	Mixed	45 (18.8)	151(21.4)	37 (16.9)	52 (26.8)			40(24)	226(20.3)			41(21.2)	228(20.2)		
	Other	10 (4.2)	13 (1.8)	6 (2.7)	9 (4.6)			6(3.6)	41(3.7)			5(2.6)	34(3)		

Correlation between DNA- DSB Repair Markers with other Tumour Markers Continued.							
Parameters		**Rad51**					
		c^-n^- N(%)	**c^+n^+ N(%)**	**c^+n^- N(%)**	**c^-n^+ N(%)**	**X^2**	**P**
KU70/KU80	**Negative**	24(36.9)	24(7.5)	58(9.7)	11(23.4)	55	<0.0001
	Positive	41(63.1)	294(92.5)	542(90.3)	36(76.6)		
DNA-PK	**Negative**	13(31)	24(10.5)	82(16.5)	1(4)	15	0.002
	Positive	29(69)	205(89.5)	414(83.5)	24(96)		
SMC6L1.n	**Negative**	17(36.2)	75(31)	171(34.5)	5(17.9)	4	0.2
	Positive	30(63.8)	167(69)	325(65.5)	23(82.1)		
SMC6L1.c	**Negative**	26(55.3)	84(34.7)	131(26.4)	8(28.6)	19.5	<0.0001
	Positive	21(44.7)	158(65.3)	365(73.6)	20(71.4)		
ID4.n	**Negative**	52(81.3)	271(88)	539(82.5)	43(87.8)	5.5	0.1
	Positive	12(18.8)	37(12)	114(17.5)	6(12.2)		
ID4.c	**Negative**	41(57.7)	113(32.3)	236(34.3)	24(43.6)	19	<0.0001
	Positive	30(42.3)	237(67.7)	452(65.7)	31(56.4)		
PTEN	**Negative**	27 (69.2)	151 (76.3)	250 (89)	14 (53.8)	31	<0.0001
	Positive	12 (30.8)	47 (23.7)	31 (11)	12 (46.2)		
CHK1.n	**Negative**	50(73.5)	190(60.5)	518(85.6)	18(35.3)	114	<0.0001
	Positive	18(26.5)	124(39.5)	87(14.4)	33(64.7)		
CHK1.c	**Negative**	18(26.5)	21(6.7)	37(6.1)	15(29.4)	61	<0.0001
	Positive	50(73.5)	292(93.3)	568(93.9)	36(70.6)		
CHK2	**Negative**	20 (52.6)	88 (43.3)	191 (60.8)	8 (32)	20	<0.0001
	Positive	18 (47.4)	115 (56.7)	123 (39.2)	17 (68)		
ATM	**Negative**	20 (64.5)	98 (44.7)	311 (62.8)	13 (37.1)	26.5	<0.0001
	Positive	11 (35.5)	121 (55.3)	184 (37.2)	22 (62.9)		
Ki-67	**Negative**	32(55.2)	139(50)	149(24.1)	22(53.7)	77	<0.0001
	Positive	26(44.8)	139(50)	469(75.9)	19(46.3)		
BARD1.c	**Negative**	12(26.7)	23(9.5)	148(27.9)	3(8.8)	37	<0.0001
	Positive	33(73.3)	218(90.5)	383(72.1)	31(91.2)		
BARD1.n	**Negative**	42(93.3)	211(87.6)	492(92.7)	32(97)	7	0.07
	Positive	3(6.7)	30(12.4)	39(7.3)	1(3)		
MTA1.n	**Negative**	19(46.3)	82(34)	250(48.2)	10(30.3)	16	0.001
	Positive	22(53.7)	159(66)	269(51.8)	23(69.7)		
MTA1.c	**Negative**	15(35.7)	26(10.8)	63(12.1)	9(27.3)	25	<0.0001
	Positive	27(64.3)	215(89.2)	456(87.9)	24(72.7)		
P21	**Negative**	18(64.3)	74(58.7)	199(59.4)	14(63.6)	0.4	0.9
	Positive	10(35.7)	52(41.3)	136(40.6)	8(36.4)		
P27	**Negative**	12(44.4)	34(28.6)	203(59.2)	8(34.8)	36	<0.0001
	Positive	15(55.6)	85(71.4)	140(40.8)	15(65.2)		
γH2AX.n	**Negative**	4(11.1)	8(3.6)	93(19.2)	1(3.7)	34	<0.0001
	Positive	32(88.9)	217(96.4)	392(80.8)	26(96.3)		
γH2AX.c	**Negative**	8(22.2)	24(10.7)	37(7.6)	7(25.9)	17	0.001
	Positive	28(77.8)	201(89.3)	448(92.4)	20(74.1)		

Correlation between DNA- DSB Repair Markers with other Tumour Markers Continued.							
Parameters		**Rad51**					
		c^-n^- N(%)	**c^+n^+ N(%)**	**c^+n^- N(%)**	**c^-n^+ N(%)**	***X²***	**P**
ER	**Negative**	23(33.3)	81(24.4)	387(56.7)	5(9.4)	126	<0.0001
	Positive	46(66.7)	251(75.6)	295(43.3)	48(90.6)		
PgR	**Negative**	30(46.9)	127(38.7)	426(65.4)	6(12.5)	100	<0.0001
	Positive	34(53.1)	201(61.3)	225(34.6)	42(87.5)		
Triple Negative	**Negative**	52(78.8)	284(86.3)	397(60.5)	49(94.2)	88	<0.0001
	Positive	14(21.2)	45(13.7)	259(39.5)	3(5.8)		
HER-2	**Negative**	59(89.4)	289(84.8)	549(81.8)	50(96.2)	9.5	0.02
	Positive	7(10.6)	52(15.2)	122(18.2)	2(3.8)		
CK5	**Negative**	46(86.8)	235(83.9)	376(67.5)	41(95.3)	42	<0.0001
	Positive	7(13.2)	45(16.1)	181(32.5)	2(4.7)		
CK17	**Negative**	51(94.4)	215(84.6)	463(79.3)	36(90)	11.5	0.009
	Positive	3(5.6)	39(15.4)	121(20.7)	4(10)		
CK14	**Negative**	62(93.9)	280(87)	555(84.2)	46(93.9)	8	0.046
	Positive	4(6.1)	42(13)	104(15.8)	3(6.1)		
BLBC	**Negative**	49(87.5)	278(88)	406(68.1)	45(93.8)	58	<0.0001
	Positive	7(12.5)	38(12)	190(31.9)	3(6.3)		
P53	**Negative**	47(75.8)	233(71)	370(55.1)	40(80)	37	<0.0001
	Positive	15(24.2)	95(29)	302(44.9)	10(20)		
Rad51.c	**Negative**						
	Positive						
Rad51.n	**Negative**						
	Positive						

Correlation between DNA- DSB Repair Markers with other Tumour Markers Continued.							
Markers		**BRCA1**					
		c^-n^- N (%)	**c^+n^+ N (%)**	**c^-n^+ N (%)**	**c^+n^- N (%)**	***X²***	**P**
ER	**Negative**	253 (53.9)	68 (24.2)	76 (13.7)	270 (63.2)	325	<0.0001
	Positive	216 (46.1)	213 (75.8)	478 (86.3)	157 (36.8)		
PgR	**Negative**	301 (65.6)	96 (34.3)	155 (28)	269 (67.1)	225	<0.0001
	Positive	158 (34.4)	184 (65.7)	399 (72)	132 (32.9)		
Triple Negative	**Negative**	276 (60)	239 (84.8)	498 (90.2)	240 (60)	179.8	<0.0001
	Positive	184 (40)	43 (15.2)	54 (9.8)	160 (40)		
HER-2	**Negative**	391 (84.6)	237 (85.3)	515 (92.1)	307 (75.2)	52.6	<0.0001
	Positive	71 (15.4)	41 (14.7)	44 (7.9)	101 (24.8)		
CK5	**Negative**	247 (67.5)	194 (87.4)	363 (90.1)	190 (65.3)	95	<0.0001
	Positive	119 (32.5)	28 (12.6)	40 (9.9)	101 (34.7)		
CK17	**Negative**	292 (78.5)	171 (85.9)	319 (91.1)	265 (78.4)	28	<0.0001
	Positive	80 (21.5)	28 (14.1)	31 (8.9)	73 (21.6)		
CK14	**Negative**	396 (86.8)	249 (89.2)	498 (92.1)	330 (81.7)	24	<0.0001
	Positive	60 (13.2)	30 (10.8)	43 (7.9)	74 (18.3)		
BLBC	**Negative**	277 (67.7)	232 (88.5)	491 (94.6)	237 (67.7)	154	<0.0001
	Positive	132 (32.3)	30 (11.5)	28 (5.4)	113 (32.3)		
P53	**Negative**	277 (59.1)	185 (66.1)	449 (81.3)	204 (47.8)	126.9	<0.0001
	Positive	192 (40.9)	95 (33.9)	103 (18.7)	223 (52.2)		
ID4.n	**Negative**	354 (83.3)	194 (85.1)	419 (90.3)	308 (80.8)	16	0.0001
	Positive	71 (16.7)	34 (14.9)	45 (9.7)	73 (19.2)		
ID4.c	**Negative**	181(39.5)	111(38.5)	243(43.3)	155(35.2)	7	0.07
	Positive	277(60.5)	177(61.5)	318(56.7)	285(64.8)		
PTEN	**Negative**	186 (90.3)	126 (81.3)	198 (71.5)	102 (79.7)	26	<0.0001
	Positive	20 (9.7)	29 (18.7)	79 (28.5)	26 (20.3)		

Correlation between DNA- DSB Repair Markers with other Tumour Markers Continued.							
Markers		**BRCA1**					
		$c^- n^-$ N (%)	**$c^+ n^+$ N (%)**	**$c^- n^+$ N (%)**	**$c^+ n^-$ N (%)**	***X*²**	**P**
CHK1.n	**Negative**	259 (80.9)	111 (67.3)	214 (65.6)	210 (78.1)	25.5	<0.0001
	Positive	61 (19.1)	54 (32.7)	112 (34.4)	59 (21.9)		
CHK1.c	**Negative**	32 (10)	12 (7.3)	53 (16.3)	7 (2.6)	33	<0.0001
	Positive	288 (90)	153 (92.7)	272 (83.7)	262 (97.4)		
CHK2	**Negative**	159 (62.6)	69 (40.8)	117 (41.5)	90 (58.8)	34.5	<0.0001
	Positive	95 (37.4)	100 (59.2)	165 (58.5)	63 (41.2)		
Ki-67	**Negative**	109 (26.2)	93 (41.7)	255 (57.7)	102 (25.6)	125	<0.0001
	Positive	307 (73.8)	130 (58.3)	187 (42.3)	297 (74.4)		
ATR	**Negative**	150 (55.8)	97 (51.1)	213 (57.6)	66 (38.8)	17.8	<0.0001
	Positive	119 (44.2)	93 (48.9)	157 (42.4)	104 (61.2)		
ATM	**Negative**	233 (71)	72 (38.3)	159 (47.5)	168 (55.3)	62.8	<0.0001
	Positive	95 (29)	116 (61.7)	176 (52.5)	136 (44.7)		
MTA1.n	**Negative**	159 (48.2)	64 (36.8)	108 (34.7)	126 (39.7)	13	0.004
	Positive	171 (51.8)	110 (63.2)	203 (65.3)	191 (60.3)		
MTA1.c	**Negative**	41 (12.4)	25 (14.3)	68 (21.8)	26 (8.2)	25	<0.0001
	Positive	289 (87.6)	150 (85.7)	244 (78.2)	291 (91.8)		
P27	**Negative**	103 (57.5)	21 (27.6)	45 (25.4)	131 (61.5)	70	<0.0001
	Positive	76 (42.5)	55 (72.4)	132 (74.6)	82 (38.5)		
P21	**Negative**	105(60)	55(66.3)	114(65.5)	126(55.3)	5	0.1
	Positive	70(40)	28(33.7)	60(34.5)	102(44.7)		
γH2AX.n	**Negative**	74 (23.4)	7 (4.5)	17 (6.2)	40 (13.2)	51	<0.0001
	Positive	242 (76.6)	148 (95.5)	258 (93.8)	264 (86.8)		
γH2AX.c	**Negative**	33 (10.4)	20 (12.9)	46 (16.7)	15 (4.9)	21.6	<0.0001
	Positive	283 (89.6)	135 (87.1)	229 (83.3)	290 (95.1)		
BARD1.n	**Negative**	313(91.8)	166(89.7)	294(91.3)	297(90.5)	0.7	0.9
	Positive	28(8.2)	19(10.3)	28(8.7)	31(9.5)		
BARD1.c	**Negative**	97 (28.5)	22 (11.9)	36 (11.1)	83 (25.3)	44	<0.0001
	Positive	243 (71.5)	163 (88.1)	287 (88.9)	245 (74.7)		

Correlation between DNA -DSB Repair Markers with other Tumour Markers Continued.

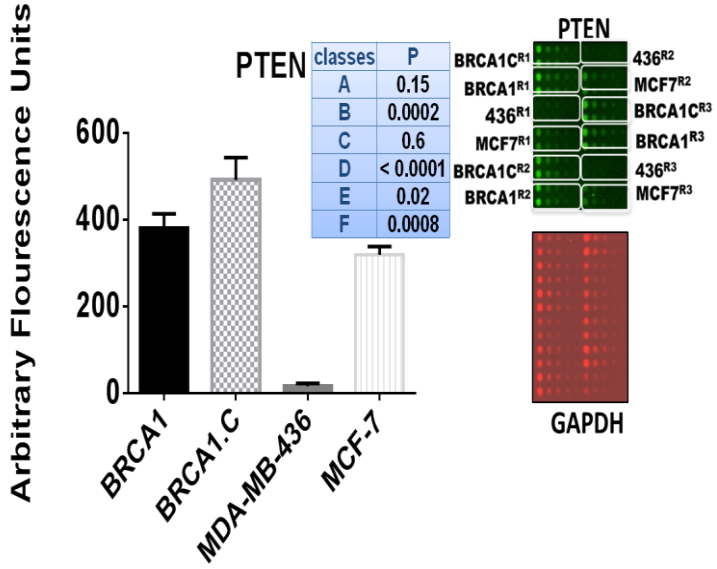
Parameters		SMC6L1						BARD1.c			
		$c^- n^-$ N(%)	$c^+ n^+$ N(%)	$c^+ n^-$ N(%)	$c^- n^+$ N(%)	X^2	P	Negative N(%)	positive N(%)	X^2	P
ER	**Negative**	86(37.9)	299(42.7)	111(50.5)	72(38.1)	9	0.027	188(65.7)	437(38.6)	68	<0.0001
	Positive	141(62.1)	402(57.3)	109(49.5)	117(61.9)			98(34.3)	696(61.4)		
PgR	**Negative**	122(52.4)	345(52.8)	132(60.6)	93(52)	5	0.2	180(69)	552(50.7)	28	<0.0001
	Positive	111(47.6)	308(47.2)	86(39.4)	86(48)			81(31)	536(49.3)		
TN	**Negative**	169(75.4)	498(74.2)	128(59)	132(72.1)	21	<0.0001	137(52.7)	830(75.2)	52	<0.0001
	Positive	55(24.6)	173(25.8)	89(41)	51(27.9)			123(47.3)	273(24.8)		
HER-2	**Negative**	198(83.9)	544(79.9)	190(86.4)	165(88.7)	11	0.01	243(86.8)	922(82.1)	3	0.06
	Positive	38(16.1)	137(20.1)	30(13.6)	21(11.3)			37(13.2)	201(17.9)		
CK5	**Negative**	128(77.1)	397(75.5)	107(63.3)	114(83.8)	18	<0.0001	132(60.3)	676(78.5)	31	<0.0001
	Positive	38(22.9)	129(24.5)	62(36.7)	22(16.2)			87(39.7)	185(21.5)		
CK17	**Negative**	146(86.4)	422(80.2)	129(72.9)	113(86.9)	14	0.003	189(77.5)	683(82.4)	3	0.08
	Positive	23(13.6)	104(19.8)	48(27.1)	17(13.1)			55(22.5)	146(17.6)		
CK14	**Negative**	197(86.4)	581(88.4)	178(83.6)	153(85)	4	0.3	218(83.2)	953(87.4)	3	0.08
	Positive	31(13.6)	76(11.6)	35(16.4)	27(15)			44(16.8)	138(12.6)		
BLBC	**Negative**	160(82.5)	476(79.2)	127(62.6)	135(80.8)	30	<0.0001	138(59.5)	799(80.3)	45	<0.0001
	Positive	34(17.5)	125(20.8)	76(37.4)	32(19.2)			94(40.5)	196(19.7)		
P53	**Negative**	154(67.8)	401(58.9)	126(58.3)	116(64.4)	7	0.06	149(53.4)	706(64.2)	11	0.001
	Positive	73(32.2)	280(41.1)	90(41.7)	64(35.6)			130(46.6)	394(35.8)		
Rad51.c	**Negative**	14(10.6)	33(7.7)	8(5.9)	20(17.1)	12	0.008	15(8.1)	64(9.6)	0.4	0.5
	Positive	118(89.4)	395(92.3)	128(94.1)	97(82.9)			171(91.9)	601(90.4)		
Rad51.n	**Negative**	85(63.9)	284(66)	103(74.6)	72(61.5)	6	0.1	160(85.6)	417(62.2)	36	<0.0001
	Positive	48(36.1)	146(34)	35(25.4)	45(38.5)			27(14.4)	253(37.8)		
KU70/KU80	**Negative**	38(26.8)	41(8.9)	18(12.3)	18(15.3)	31	<0.0001				
	Positive	104(73.2)	422(91.1)	128(87.7)	100(84.7)						
SMC6L1.n	**Negative**							97(38.6)	318(32.3)	4	0.05
	Positive							154(61.4)	668(67.7)		
SMC6L1.c	**Negative**							98(39)	278(28.2)	11	0.001
	Positive							153(61)	708(71.8)		
ID4.n	**Negative**	185(83.7)	531(86.3)	164(81.6)	137(79.7)	6	0.1	214(77)	882(85.9)	13	<0.0001
	Positive	36(6.3)	84(13.7)	37(18.4)	35(20.3)			64(23)	145(14.1)		
ID4.c	**Negative**	101(40.7)	261(37.4)	73(32.7)	84(43.3)	6	0.1	147(51.9)	389(33.4)	33	<0.0001
	Positive	147(59.3)	437(62.6)	150(67.3)	110(56.7)			136(48.1)	774(66.6)		
PTEN	**Negative**	120 (81.6)	284 (79.8)	111 (91)	78 (70.3)	16	0.001	87 (91.6)	558 (79.5)	8	0.005
	Positive	27 (18.4)	72 (20.2)	11 (9)	33 (29.7)			8 (8.4)	144 (20.5)		
CHK1.n	**Negative**	104(70.7)	353(75.6)	121(79.1)	83(66.9)	7	0.08	147(73.1)	550(74.4)	0.1	0.7
	Positive	43(29.3)	114(24.4)	32(20.9)	41(33.1)			54(26.9)	189(25.6)		
CHK1.c	**Negative**	27(18.4)	31(6.6)	16(10.5)	13(10.5)	18	0.001	27(13.4)	62(8.4)	5	0.03
	Positive	120(81.6)	436(93.4)	136(89.5)	111(89.5)			174(86.6)	676(91.6)		
CHK2	**Negative**	93 (60)	160 (40.2)	84 (64.6)	51 (47.7)	32.6	<0.0001	62 (60.8)	333 (47.2)	6.6	0.01
	Positive	62 (40)	238 (59.8)	46 (35.4)	56 (52.3)			40 (39.2)	373 (52.8)		
ATM	**Negative**	74 (50.7)	260 (53.7)	101 (64.3)	69 (58)	7	0.06				
	Positive	72 (49.3)	224 (46.3)	56 (35.7)	50 (42)						
ATR	**Negative**	132 (67.7)	207 (42.2)	79 (49.1)	106 (73.6)	65	<0.0001	63(51.2)	454(51.2)	0	0.99
	Positive	63 (32.3)	283 (57.8)	82 (50.9)	38 (26.4)			60(48.8)	432(48.8)		
Ki-67	**Negative**	83(40.5)	194(31.3)	52(27.4)	73(45.6)	19	<0.0001	81(29)	356(36.7)	6	0.017
	Positive	122(59.5)	426(68.7)	138(72.6)	87(54.6)			198(71)	613(63.3)		
P27	**Negative**	38(39.2)	143(51.1)	42(53.2)	39(47.6)	5	0.2	114(68.3)	170(39.8)	39	<0.0001
	Positive	59(60.8)	137(48.9)	37(46.8)	43(52.4)			53(31.7)	257(60.2)		
BARD1.c	**Negative**	58(28.3)	114(17.5)	39(18.6)	40(23.4)	13	0.006				
	Positive	147(71.7)	537(82.5)	171(81.4)	131(76.6)						
BARD1.n	**Negative**	190(92.7)	587(90.3)	191(91)	159(92.4)	1.5	0.7	297(99.7)	1043(88.6)	35	<0.0001
	Positive	15(7.3)	63(9.7)	19(9)	13(7.6)			1(0.3)	134(11.4)		
MTA1.n	**Negative**	126(58.1)	181(27.3)	150(68.8)	40(23.4)	170	<0.0001	141(54)	365(35.9)	28	<0.0001
	Positive	91(41.9)	482(72.7)	68(31.2)	131(76.6)			120(46)	651(64.1)		
MTA1.c	**Negative**	78(35.9)	34(5.1)	18(8.3)	59(34.5)	185	<0.0001	74(28.4)	111(10.9)	51	<0.0001
	Positive	139(64.1)	630(94.9)	200(91.7)	112(65.5)			187(71.6)	906(89.1)		
γH2AX.n	**Negative**	47(25.3)	28(4.4)	71(37.8)	6(3.7)	180	<0.0001	59(24.5)	90(9.9)	35.5	<0.0001
	Positive	139(74.7)	603(95.6)	117(62.2)	156(96.3)			182(75.5)	815(90.1)		
γH2AX.c	**Negative**	36(19.3)	29(4.6)	7(3.7)	44(27.2)	100	<0.0001	38(15.8)	76(8.4)	11.6	0.001
	Positive	151(80.7)	602(95.4)	181(96.3)	118(72.8)			203(84.2)	830(91.6)		

Correlation between DNA-DSB Repair Markers with other Tumour Markers Continued.

Markers		KU70/KU80				DNA-PK			
		Negative N(%)	Positive N(%)	X^2	P	Negative N(%)	Positive N(%)	X^2	P
ER	**Negative**	51(32.7)	489(44.6)	8	0.005	79(42.7)	496(44.5)	0.2	0.6
	Positive	105(67.3)	607(55.4)			106(57.3)	619(55.5)		
PgR	**Negative**	76(47.8)	568(54.7)	3	0.1	103(56.3)	574(54.3)	0.2	0.6
	Positive	83(52.2)	470(45.3)			80(43.7)	483(45.7)		
TN	**Negative**	124(78.5)	744(70.7)	4	0.04	133(71.9)	749(70.1)	0.2	0.6
	Positive	34(21.5)	309(29.3)			52(28.1)	320(29.9)		
HER-2	**Negative**	136(84)	906(83.7)	0.005	0.9	160(83.8)	903(82.8)	0.1	0.7
	Positive	26(16)	176(16.3)			31(16.2)	187(17.2)		
CK5	**Negative**	110(81.5)	665(73.2)	4.2	0.03	119(78.3)	617(73.7)	1	0.2
	Positive	25(18.5)	244(26.8)			33(21.7)	220(26.3)		
CK17	**Negative**	118(88.1)	734(82.3)	3	0.09	126(86.3)	668(79.8)	3	0.07
	Positive	16(11.9)	158(17.7)			20(13.7)	169(20.2)		
CK14	**Negative**	38(85.2)	896(86.7)	0.26	0.6	165(90.2)	910(85.8)	2	0.1
	Positive	24(14.8)	138(13.3)			18(9.8)	150(14.2)		
BLBC	**Negative**	116(81.7)	746(76.3)	2	0.1	128(79)	735(75.6)	0.9	0.3
	Positive	26(18.3)	232(23.7)			34(21)	237(24.4)		
P53	**Negative**	114(74.5)	653(60.9)	11	0.001	127(68.6)	639(59.1)	6	0.01
	Positive	39(25.5)	420(39.1)			58(31.4)	443(40.9)		
KU70/KU80	**Negative**					37(28.2)	68(9.5)	35.5	<0.0001
	Positive					94(71.8)	645(90.5)		
ID4.n	**Negative**	116(81.1)	857(83.4)	0.5	0.5	140(77.8)	845(84.4)	5	0.028
	Positive	27(18.9)	170(16.6)			40(22.2)	156(15.6)		
ID4.c	**Negative**	82(48)	402(36.2)	9	0.003	82(41.2)	398(35.5)	2	0.1
	Positive	89(52)	709(63.8)			117(58.8)	722(64.5)		
PTEN	**Negative**	7(25.9)	42(12.6)	4	0.05	124 (93.9)	469 (78.7)	16.6	<0.0001
	Positive	20(74.1)	292(87.4)			8 (6.1)	127 (21.3)		
CHK1.n	**Negative**	113(73.4)	766(75.1)	0.2	0.6	100(77.5)	553(74.8)	0.4	0.5
	Positive	41(26.6)	254(24.9)			29(22.5)	186(25.2)		
CHK1.c	**Negative**	40(26)	83(8.1)	45	<0.0001	26(20.2)	53(7.2)	22	<0.0001
	Positive	114(74)	936(91.9)			103(79.8)	685(92.8)		
CHK2	**Negative**	44(47.8)	301(53.9)	1.2	0.3	104 (77.6)	274 (43.9)	50	<0.0001
	Positive	48(52.2)	257(46.1)			30 (22.4)	350 (56.1)		
ATM	**Negative**	45 (51.1)	442 (57)	1	0.3	84 (63.6)	414 (56)	2.60	0.1
	Positive	43 (48.9)	333(43)			48 (36.4)	325 (44)		
ATR	**Negative**	84 (65.1)	325 (52.2)	7	0.007	106 (67.9)	360 (45.7)	25.8	<0.0001
	Positive	45 (34.9)	298 (47.8)			50 (32.1)	428 (54.3)		
Ki-67	**Negative**	42(36.2)	340(35.5)	0.03	0.9	63(40.9)	315(31.8)	5	0.026
	Positive	74(63.8)	619(64.5)			91(59.1)	675(68.2)		
BARD1.c	**Negative**	21(16.8)	179(22.6)	2.1	0.1	67(36.2)	176(16.9)	37	<0.0001
	Positive	104(83.2)	614(77.4)			118(63.8)	864(83.1)		
BARD1.n	**Negative**	113(90.4)	721(90.9)	0.03	0.8	172(93)	935(89.9)	2	0.2
	Positive	12(9.6)	72(9.1)			13(7)	118(9.6)		
MTA1.n	**Negative**	59(52.7)	329(42.4)	4	0.04	129(69)	340(33.8)	82	<0.0001
	Positive	53(47.3)	447(57.6)			58(31)	667(66.2)		
MTA1.c	**Negative**	37(33)	88(11.3)	38	<0.0001	73(39)	88(8.7)	124	<0.0001
	Positive	75(67)	689(88.7)			114(61)	920(91.3)		
P21	**Negative**	38(52.1)	294(63)	3.2	0.07	51(58)	260(59.1)	0.04	0.8
	Positive	35(47.9)	173(37)			37(42)	180(40.9)		
P27	**Negative**	24(32)	233(51.2)	9.5	0.002	32(38.1)	220(49.7)	4	0.05
	Positive	51(68)	222(48.8)			52(61.9)	223(50.3)		
γH2AX.n	**Negative**	18(20.9)	96(13.1)	4	0.048	53(32.9)	92(9.8)	64	<0.0001
	Positive	68(79.1)	636(86.9)			108(67.1)	843(90.2)		
γH2AX.c	**Negative**	16(18.4)	77(10.5)	5	0.029	29(18)	76(8.1)	15.5	<0.0001
	Positive	71(81.6)	655(89.5)			132(82)	860(91.9)		

Correlation between HR Repair Proteins with other Tumour Markers Regardless Co-expression of Cellular Localisation							
Markers		**BRCA1.n**			**BRCA1.c**		
		Negative N (%)	**Positive N (%)**	**P X^2**	**Negative N (%)**	**Positive N (%)**	**P X^2**
ER	**Negative**	526(58.5)	144 (17.2)	<0.0001 312	329(32.2)	338(47.7)	<0.0001 43
	Positive	373(41.5)	693 (82.8)		694(67.8)	370(52.3)	
PgR	**Negative**	572(66.4)	252 (30.1)	<0.0001 223	456(45)	365(53.6)	0.001 12
	Positive	290(33.6)	584 (69.9)		557 (55)	316(46.4)	
CK5	**Negative**	438(66.5)	559 (89.2)	<0.0001 95	610(79.3)	384(74.9)	0.06 3
	Positive	221(33.5)	68 (10.8)		159(20.7)	129(25.1)	
P27	**Negative**	235(59.6)	66(26.1)	<0.0001 70	148(41.6)	152(52.6)	0.005 7.9
	Positive	159(40.4)	187 (73.9)		20 (58.4)	137(47.4)	
SMC6L1.n	**Negative**	219(34.5)	158 (33.5)	0.7 0.1	225(36.5)	152(31.1)	0.06 3
	Positive	416(65.5)	314 (66.5)		392(63.5)	336(68.9)	
MTA1.n	**Negative**	286(44.1)	172 (35.4)	0.003 8.6	267(41.7)	190(38.7)	0.3 1
	Positive	363(55.9)	314 (64.6)		374(58.3)	301(61.3)	
CHK1.c	**Negative**	40 (6.8)	65 (13.2)	<0.0001 12.7	85 (13.2)	19 (4.4)	<0.0001 23
	Positive	551(93.2)	427 (86.8)		560(86.8)	415(95.6)	
CHK1.n	**Negative**	470(79.5)	326 (66.1)	<0.0001 24.7	473(73.2)	321(74)	0.8 0.07
	Positive	121(20.5)	167 (33.9)		173(26.8)	113 (26)	
CHK2	**Negative**	249(61.2)	186 (41.2)	<0.0001 34	276(51.5)	159(49.4)	0.5 0.4
	Positive	158(38.8)	266 (58.8)		260(48.5)	163(50.6)	
γH2AX.n	**Negative**	114(18.3)	24 (5.6)	<0.0001 36	91(15.4)	47(10.2)	0.014 6
	Positive	508 (81.7)	407(94.4)		500(84.6)	412(89.8)	
γH2AX.c	**Negative**	49 (7.9)	66(15.3)	<0.0001 14.5	79(13.4)	35(7.6)	0.003 9
	Positive	574(92.1)	365 (84.7)		512 (86.6)	425(92.4)	
Rad51.n	**Negative**	440(81.5)	195(46.2)	<0.0001 131	348(62.6)	285(70.7)	0.009 6.9
	Positive	100(18.5)	227(53.8)		208 (37.4)	118(29.3)	
BARD1.c	**Negative**	181 (27)	58(11.4)	<0.0001 43.8	133 (20.1)	105(20.5)	0.8 0.03
	Positive	489 (73)	452 (88.6)		530(79.9)	408(79.5)	
Markers		**Rad51.n**			**Rad51.c**		
		Negative N (%)	**Positive N (%)**	**P X^2**	**Negative N (%)**	**Positive N (%)**	**P X^2**
CK17	**Negative**	514(80.4)	255 (85.3)	0.07 3	87 (92.6)	678(80.9)	0.005 7.8
	Positive	125(19.6)	44(14.7)		7 (7.4)	160(19.1)	
P27	**Negative**	216(58.2)	42 (29.6)	<0.0001 33.7	20 (40)	237(51.3)	0.1 2
	Positive	155(41.8)	100 (70.4)		30 (60)	225(48.7)	
SMC6L1.n	**Negative**	188(34.6)	83 (30.3)	0.2 1.5	22 (29.3)	246(33.3)	0.5 0.5
	Positive	356(65.4)	191 (69.7)		53 (70.7)	492(66.7)	

Correlation between HR Repair Proteins with other Tumour Markers Regardless Co-expression of Cellular Localisation Continued.							
Markers		**Rad51.n**			**Rad51.c**		
		Negative N (%)	**Positive N (%)**	**P X^2**	**Negative N (%)**	**Positive N (%)**	**P X^2**
ID4.c	**Negative**	277(36.4)	139 (33.9)	0.4 0.7	65 (51.6)	349(33.6)	<0.0001 16
	Positive	483(63.6)	271 (66.1)		61 (48.4)	689(66.4)	
MTA1.n	**Negative**	269 (48)	93 (33.5)	<0.0001 16	29 (39.2)	332(43.7)	0.4 0.5
	Positive	292 (52)	185 (66.5)		45 (60.8)	428(56.3)	
CHK1.c	**Negative**	56 (8.3)	37 (10.1)	0.3 0.9	33 (27.7)	58 (6.3)	<0.0001 60
	Positive	618(91.7)	331 (89.9)		86 (72.3)	860(93.7)	
CHK2	**Negative**	212(60.1)	97 (41.8)	<0.0001 18.7	28 (44.4)	279 (54)	0.1 2
	Positive	141(39.9)	135 (58.2)		35 (55.6)	238 (46)	
ATM	**Negative**	332 (63)	112 (43.8)	<0.0001 26	33 (50)	409(57.3)	0.2 1.3
	Positive	195 (37)	144 (56.3)		33 (50)	305(42.7)	
MTA1.c	**Negative**	78 (13.9)	36 (12.9)	0.7 0.1	24 (32)	89 (11.7)	<0.0001 24
	Positive	484(86.1)	242 (87.1)		51 (68)	671(88.3)	
γH2AX.n	**Negative**	97 (18.6)	9 (3.5)	<0.0001 33	5 (7.9)	101(14.2)	0.2 2
	Positive	425(81.4)	248 (96.5)		58 (92.1)	609(85.8)	
γH2AX.c	**Negative**	45 (8.6)	33 (12.8)	0.06 3	15 (23.8)	61(8.6)	<0.0001 15
	Positive	477(91.4)	224 (87.2)		48 (76.2)	649(91.4)	
BRCA1.c	**Negative**	348 (55)	208 (63.8)	0.009 7	67 (71.3)	486(56.6)	0.006 7.5
	Positive	285 (45)	118 (36.2)		27 (28.7)	373(43.4)	
DNA-PK	**Negative**	95 (17.6)	25 (9.8)	0.004 8	14 (20.9)	106(14.6)	0.2 1.8
	Positive	444 (82.4)	231 (90.2)		53 (79.1)	619(85.4)	
Markers		**SMC6L1.n**			**SMC6L1.c**		
		Negative N (%)	**Positive N (%)**	**P X^2**	**Negative N (%)**	**Positive N (%)**	**P X^2**
CK5	**Negative**	235 (70.1)	511 (77.2)	0.01 5.8	242 (80.1)	504 (72.5)	0.01 6.5
	Positive	100 (29.9)	151 (22.8)		60 (19.9)	191 (27.5)	
CK17	**Negative**	275 (79.5)	535 (81.6)	0.4 0.6	259 (86.6)	551 (78.4)	0.002 9
	Positive	71 (20.5)	121 (18.4)		40 (13.4)	152 (21.6)	
γH2AX.n	**Negative**	118 (31.6)	34 (4.3)	<0.0001 166	53 (15.2)	99 (12.1)	0.1 2
	Positive	256 (68.4)	759(95.7)		295 (84.8)	720 (87.9)	
BRCA1.n	**Negative**	219 (58.1)	416 (57)	0.7 0.12	158 (47.3)	477 (61.7)	<0.0001 20
	Positive	158 (41.9)	314(43)		176 (52.7)	296 (38.3)	
Ki-67	**Negative**	135 (34.2)	267 (34.2)	0.098 0	156 (42.7)	246 (30.4)	<0.0001 17
	Positive	260 (65.8)	513 (65.8)		209 (57.3)	564 (69.6)	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Arbitrary Flourescence Units
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200
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MCF-7
PTEN
classes P
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B 0.0002
C 0.6
D < 0.0001
E 0.02
F 0.0008
BRCA1C^R1
BRCA1^R1
436^R1
MCF7^R1
BRCA1C^R2
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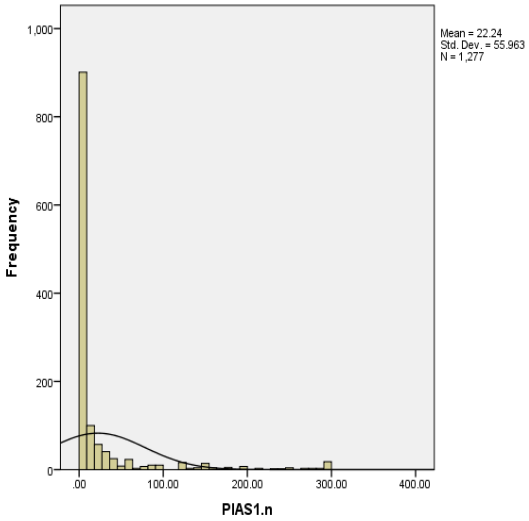
BRCA1^R3
436^R3
MCF7^R3
GAPDH

Appendix 3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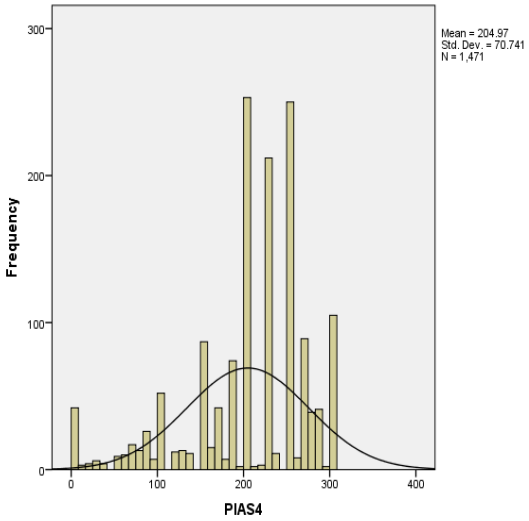
Frequency
PIAS1.n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Std. Dev. = 70.741
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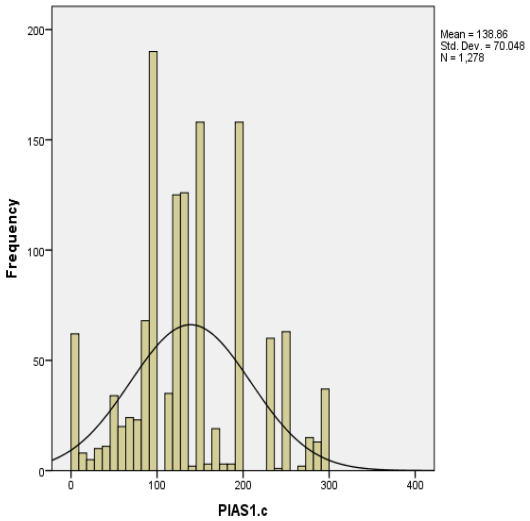
Frequency
PIAS4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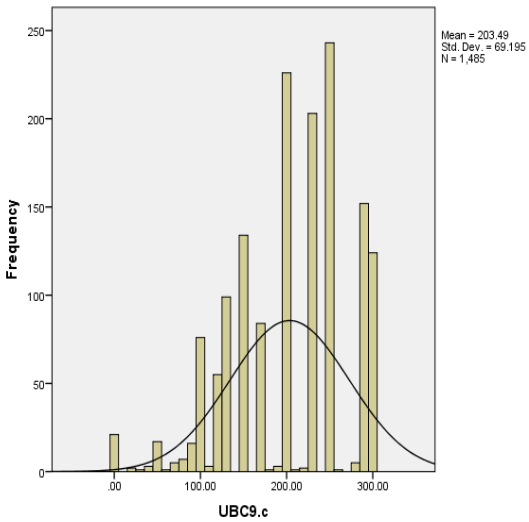
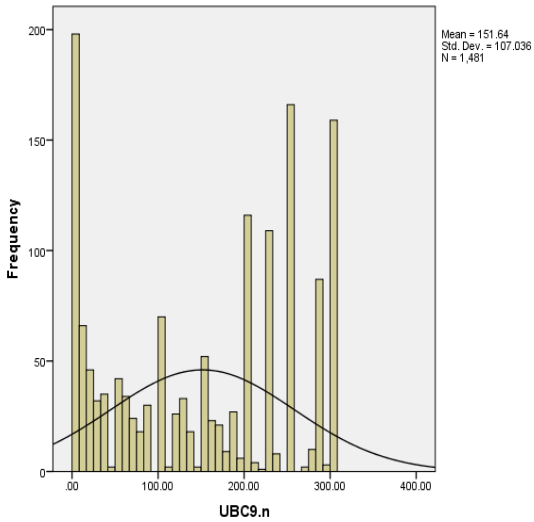
PIAS1.c


Mean = 203.49
Std. Dev. = 69.195
N = 1,485
Frequency
UBC9.c


Mean = 151.64
Std. Dev. = 107.036
N = 1,481
Frequency
UBC9.n

Relationship between SUMO markers with Clinico-Pathological Parameters											
Parameters		**PIAS1**						**PIAS4**			
		c^-n^- N(%)	**c^+n^+ N(%)**	**c^-n^+ N(%)**	**c^+n^- N(%)**	**X^2**	**P**	**Negative N(%)**	**Positive N(%)**	**X^2**	**P**
Age	**<50**	62(31.8)	36(32.4)	22(34.4)	313(35.1)	0.9	0.8	105(33.5)	407(35.4)	0.4	0.5
	≥50	133(68.2)	75(67.6)	42(65.6)	580(64.9)			208(66.5)	743(64.6)		
Size	**≤ 1.5cm**	72(36.9)	47(42.3)	24(37.5)	256(29)	12	0.006	90(29.2)	367(32.1)	0.9	0.3
	>1.5cm	123(63.1)	64(57.7)	40(62.5)	628(71)			218(70.8)	775(67.9)		
Stage	**1**	126(64.6)	77(68.8)	39(60.9)	526(59)	8	0.22	214(68.6)	665(57.9)	13	0.002
	2	46(23.6)	28(25)	20(31.3)	275(30.9)			69(22.1)	367(31.9)		
	3	23(11.8)	7(6.3)	5(6.8)	90(10.1)			29(9.3)	117(10.2)		
Grade	**1**	38(19.5)	33(29.5)	11(17.2)	74(8.3)	90	<0.0001	45(14.4)	129(11.2)	4.1	0.1
	2	64(32.8)	33(29.5)	33(51.6)	228(25.5)			75(24)	330(28.7)		
	3	93(47.7)	46(41.1)	20(31.3)	591(66.2)			193(61.7)	691(60.1)		
Tubules	**1**	12(6.3)	8(7.6)	1(1.7)	25(2.9)	24	0.001	11(3.8)	44(3.9)	0.2	0.9
	2	49(25.9)	45(42.9)	22(37.9)	250(28.7)			81(27.7)	329(29.1)		
	3	128(67.7)	52(49.5)	35(60.3)	595(68.4)			200(68.5)	757(67)		
Pleomorphism	**1**	3(1.6)	2(1.9)	3(5.2)	5(0.6)	84	<0.0001	5(1.7)	11(1)	3.2	0.2
	2	84(44.4)	52(49.5)	29(50)	201(23.2)			96(33)	326(28.9)		
	3	102(54)	51(48.6)	26(44.8)	661(76.2)			190(65.3)	792(70.2)		
Mitosis	**1**	79(41.8)	46(43.8)	32(5.2)	178(20.5)	85	<0.0001	71(24.3)	302(26.7)	2.2	0.3
	2	24(12.7)	20(19)	13(22.4)	168(19.3)			50(17.1)	221(19.6)		
	3	86(45.5)	39(37.1)	13(22.4)	524(60.2)			171(58.6)	607(53.7)		
NPI	**Excellent**	28(14.4)	24(22)	9(14.1)	40(4.5)	80	<0.0001	33(10.6)	78(6.9)	12.4	0.029
	Good	34(17.5)	25(22.9)	13(20.3)	126(14.2)			42(13.5)	181(15.9)		
	Moderate 1	49(25.3)	30(27.5)	21(32.8)	304(34.3)			113(36.3)	357(31.4)		
	Moderate 2	51(26.3)	19(17.4)	18(28.1)	252(28.4)			81(26)	306(26.9)		
	Poor	24(12.4)	10(9.2)	2(3.1)	116(13.1)			28(9)	162(14.2)		
	Very poor	8(4.1)	1(0.9)	1(1.6)	48(5.4)			14(4.5)	53(4.7)		
Vascular Invasion	**Negative**	98(69)	53(65.4)	36(66.7)	352(57.7)	8	0.046	144(63.2)	470(61)	0.3	0.6
	Positive	44(31)	28(34.6)	18(33.3)	258(42.3)			84(36.8)	300(39)		
Tumour Type	**Invasive Ductal/NST**	106(55.2)	58(52.3)	25(39.7)	649(73.5)	92	<0.0001	198(64.7)	774(68)	15	0.006
	lobular	23(12)	5(4.5)	9(14.3)	27(3.1)			12(3.9)	63(5.5)		
	Atypical Medullary	4(2.1)	1(0.9)	1(1.6)	27(3.1)			18(5.9)	23(2)		
	Mixed	48(25)	40(36)	24(38.1)	158(17.9)			65(21.2)	239(21)		
	other	11(5.7)	7(6.3)	4(6.3)	22(2.5)			13(4.2)	40(3.5)		

Relationship between SUMO Markers with Clinic-Pathological Parameters Continued.							
Parameters		**UBC9**					
		$c^- n^-$ N(%)	**$c^+ n^+$ N(%)**	**$c^- n^+$ N(%)**	**$c^+ n^-$ N(%)**	***X^2***	**P**
Age	**<50**	129(33.6)	228(37.6)	40(28.4)	129(37.7)	5.5	0.1
	≥50	255(66.4)	379(62.4)	101(71.6)	213(62.3)		
Size	**≤ 1.5cm**	108(28.6)	190(31.4)	56(40)	81(23.8)	14	0.003
	>1.5cm	270(71.4)	416(68.6)	84(60)	259(76.2)		
Stage	**1**	261(68)	355(58.4)	84(59.6)	197(58.1)	18	0.005
	2	88(22.9)	188(30.9)	37(26.2)	116(34.2)		
	3	35(9.1)	65(10.7)	20(14.2)	26(7.75)		
Grade	**1**	47(12.2)	84(13.8)	20(14.2)	22(6.4)	45	<0.0001
	2	101(26.3)	164(27)	61(43.3)	72(21.1)		
	3	236(61.5)	360(59.66)	60(42.6)	248(72.5)		
Tubules	**1**	14(3.8)	32(5.3)	3(2.2)	8(2.4)	8.5	0.2
	2	106(28.6)	178(29.7)	42(30.7)	89(26.3)		
	3	250(67.6)	390(65)	92(67.2)	242(71.4)		
Pleomorphism	**1**	4(1.1)	7(1.2)	6(4.4)	3(0.9)	47	<0.0001
	2	114(30.9)	183(30.6)	60(43.8)	62(18.3)		
	3	251(68)	409(68.3)	71(51.8)	273(80.8)		
Mitosis	**1**	95(25.7)	169(28.3)	60(43.8)	58(17.1)	55	<0.0001
	2	67(18.1)	115(19.2)	30(21.9)	48(14.2)		
	3	208(56.2)	316(52.7)	47(34.3)	233(68.7)		
NPI	**Excellent**	28(7.3)	48(8)	16(11.6)	16(4.7)	43	<0.0001
	Good	62(16.2)	99(16.6)	35(25.4)	32(9.4)		
	Moderate 1	139(36.4)	186(31.1)	38(27.5)	118(34.7)		
	Moderate 2	98(25.7)	156(26.1)	25(18.1)	101(29.7)		
	Poor	35(9.2)	87(14.5)	19(13.8)	52(15.3)		
	Very poor	20(5.2)	22(3.7)	5(3.6)	21(6.2)		
Vascular Invasion	**Negative**	157(63.6)	262(59.8)	66(68)	139(59.7)	3	0.4
	Positive	90(36.4)	176(40.2)	31(32)	94(40.3)		
Tumour Type	**Invasive Ductal /NST**	253(67.3)	418(69.6)	63(45)	253(74.9)	97.6	<0.0001
	lobular	17(4.5)	18(3)	26(18.6)	4(1.2)		
	Atypical Medullary	13(3.5)	11(1.8)	3(2.1)	11(3.3)		
	Mixed	79(21)	132(22)	42(30)	61(18)		
	other	14(3.7)	22(3.7)	6(4.3)	9(2.7)		

Correlation between SUMO Markers with other Tumour Markers Continued.

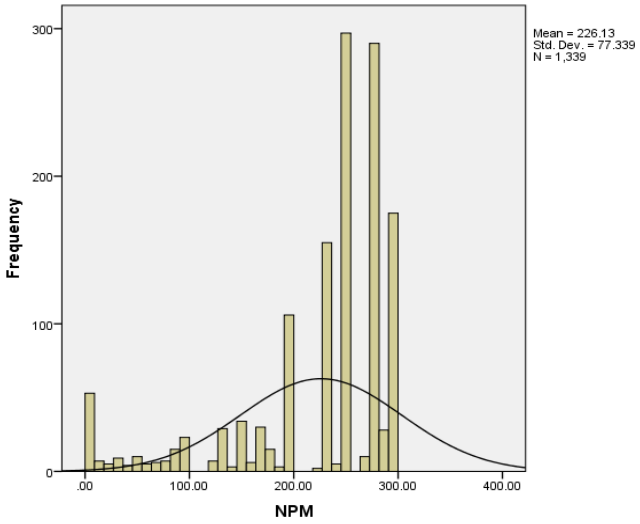
Markers		PIAS1						PIAS4			
		c^-n^- N(%)	c^+n^+ N(%)	c^-n^+ N(%)	c^+n^- N(%)	X^2	P	Negative N(%)	Positive N(%)	X^2	P
ER	**Negative**	72(37.7)	28(26.7)	17(27)	408(46.9)	26	<0.0001	145(48.8)	487(43.3)	3	0.09
	Positive	119(62.3)	77(73.3)	46(73)	462(53.1)			152(51.2)	637(56.7)		
PgR	**Negative**	96(52.7)	41(38.3)	21(36.2)	463(55.4)	17.6	0.001	175(59.7)	546(51.5)	6	0.012
	Positive	86(47.3)	66(6.7)	37(63.8)	373(44.6)			118(40.3)	515(48.5)		
TN	**Negative**	130(71)	86(81.9)	50(82)	596(70.4)	9	0.026	186(64.6)	783(72.6)	7	0.008
	Positive	53(29)	19(18.1)	11(18)	250(29.6)			102(35.4)	296(27.4)		
HER-2	**Negative**	170(92.9)	100(92.6)	59(93.7)	679(79)	35	<0.0001	257(86)	912(82.9)	1.5	0.3
	Positive	13(7.1)	8(7.4)	4(6.3)	181(21)			42(14)	188(17.1)		
CK5	**Negative**	117(74.1)	63(77.8)	41(85.4)	535(72.4)	5	0.2	171(69.5)	654(74.2)	2	0.1
	Positive	41(25.9)	18(22.2)	6(14.6)	204(27.6)			75(30.5)	227(25.8)		
CK17	**Negative**	128(83.1)	66(83.5)	47(97.9)	590(81.6)	8	0.037	203(84.6)	723(82.1)	0.8	0.4
	Positive	26(16.9)	13(16.5)	1(2.1)	133(18.4)			37(15.4)	158(17.9)		
CK14	**Negative**	157(87.7)	91(87.5)	50(84.7)	721(85.8)	0.7	0.8	253(85.5)	913(86.1)	0.08	0.7
	Positive	22(12.3)	13(12.5)	9(15.3)	119(14.2)			43(14.5)	147(13.9)		
BLBC	**Negative**	128(77.6)	81(83.5)	48(85.7)	592(75.6)	6	0.1	184(71.6)	769(77.8)	4.4	0.035
	Positive	37(22.4)	16(16.5)	8(14.3)	191(24.4)			73(28.4)	219(22.2)		
P53	**Negative**	130(71.4)	74(70.5)	39(72.2)	517(60.2)	13	0.005	190(65.3)	668(60.9)	2	0.2
	Positive	52(28.6)	31(29.5)	15(27.8)	342(39.8)			101(34.7)	428(39.1)		
Rad51.c	**Negative**	36(26.5)	13(15.9)	15(34.1)	44(5.9)	81.5	<0.0001	35(16.4)	64(8.1)	13	<0.0001
	Positive	100(73.5)	69(84.1)	29(65.9)	707(94.1)			179(83.6)	731(91.9		
Rad51.n	**Negative**	106(77.4)	28(33.3)	18(40.9)	511(67.9)	60	<0.0001	159(74.3)	453(56.8)	21.5	<0.0001
	Positive	31(22.6)	56(66.7)	26(59.1)	242(32.1)			55 (25.7)	344(43.2)		
KU70/KU80	**Negative**	38(22.4)	12(13.3)	15(27.3)	78(9.8)	30	<0.0001	72(28.5)	69(7.9)	76	<0.0001
	Positive	132(77.6)	78(86.7)	40(72.7)	717(90.2)			181(71.5)	806(92.1)		
DNA-PK	**Negative**	40(30.5)	7(10.6)	4(13.8)	79(12.9)	27	<0.0001	67(35.6)	79(10.1)	77	<0.0001
	Positive	91(69.5)	59(89.4)	25(86.2)	533(87.1)			121(64.4)	702(89.9)		
SMC6L1.n	**Negative**	52(39.7)	23(31.1)	7(20.6)	209(33.5)	5	0.2	99(49)	236(29.9)	26	<0.0001
	Positive	79(60.3)	51(68.9)	27(79.4)	415(66.5)			103(51)	553(70.1)		
SMC6L1.c	**Negative**	55(42)	24(32.4)	18(52.9)	157(25.2)	25	<0.0001	86(42.6)	222(28.1)	15.6	<0.0001
	Positive	76(58)	50(67.6)	16(47.1)	467(74.8)			116(57.4)	567(71.9)		
ID4. n	**Negative**	151(84.8)	72(76.6)	41(77.4)	706(84.8)	6	0.1	237(80.9)	864(83.8)	1.4	0.2
	Positive	27(15.2)	22(23.4)	12(22.6)	127(15.2)			56(19.1)	167(16.2)		
ID4.c	**Negative**	103(52.8)	45(40.5)	39(60.9)	264(29.7)	58	<0.0001	134(42.5)	397(35.1)	6	0.015
	Positive	92(47.2)	66(59.5)	25(39.1)	625(70.3)			181(57.5)	734(64.9)		
PTEN	**Negative**	85(85)	29(65.9)	13 (52)	354(84.9)	26	<0.0001	133(86.9)	414(82)	2	0.15
	Positive	15(15)	15(34.1)	12(48)	63(15.1)			20 (13.1)	91 (18)		
CHK1.n	**Negative**	159(85)	21(20)	9(16.1)	699(82.6)	302	<0.0001	225(86.9)	695(78.4)	9.5	0.003
	Positive	28(15)	83(79.8)	47(83.9)	147(17.4)			34(13.1)	191(21.6)		
CHK1.c	**Negative**	54(28.9)	10(9.6)	16(28.6)	25(3)	157	<0.0001	149(57.5)	352(39.8)	14	<0.0001
	Positive	133(71.1)	94(90.4)	40(71.4)	820(97)			110(42.5)	533(60.2)		
CHK2	**Negative**	60(59.4)	19(39.6)	11(40.7)	239(53.1)	6.7	0.08	109(64.5)	268(47.6)	15	<0.0001
	Positive	41(40.6)	29(60.4)	16(59.3)	211(46.9)			60(35.5)	295(52.4)		
Ki-67	**Negative**	82(47.7)	36(42.4)	29(53.7)	219(29)	34	<0.0001	76(31.8)	350(35.2)	1	0.3
	Positive	90(52.3)	49(57.6)	25(46.3)	535(71)			163(68.2)	643(64.8)		
ATM	**Negative**	84(64.1)	30(38.5)	13(38.2)	360(57.8)	18.4	<0.0001	129(62.3)	422(54.8)	10	0.019
	Positive	47(35.9)	48(61.5)	21(61.8)	263(42.2)			78 (37.7)	348(45.2)		
ATR	**Negative**	68(60.7)	48(70.6)	20(58.8)	259(49.9)	13	0.004	128(66.7)	330(50.5)	15.7	<0.0001
	Positive	44(39.3)	20(29.4)	14(41.2)	260(50.1)			64(33.3)	324(49.5)		
γH2AX.n	**Negative**	18(14.6)	1(1.5)	1(3.4)	93(15.6)	12.6	0.006	50(26.7)	85(11.3)	28.7	<0.0001
	Positive	105(85.4)	65(98.5)	28(96.6)	503(84.4)			137(73.3)	664(88.7)		
γH2AX.c	**Negative**	24(19.5)	11(16.7)	11(37.9)	41(6.9)	44	<0.0001	25(13.4)	73(9.7)	2	0.1
	Positive	99(80.5)	55(83.3)	18(62.1)	556(93.1)			162(86.6)	677(90.3)		
BARD1.c	**Negative**	59(40.7)	14(19.4)	8(22.2)	119(18.2)	35	<0.0001	65(29.7)	168(20.3)	9	0.003
	Positive	86(59.3)	58(80.6)	28(77.8)	535(81.8)			154(70.3)	661(79.7)		
BARD1.n	**Negative**	138(95.2)	67(95.7)	34(94.4)	580(88.7)	9	0.029	203(92.3)	743(89.8)	1.2	0.3
	Positive	7(4.8)	3(4.3)	2(5.6)	74(11.3)			17(7.7)	84(10.2)		

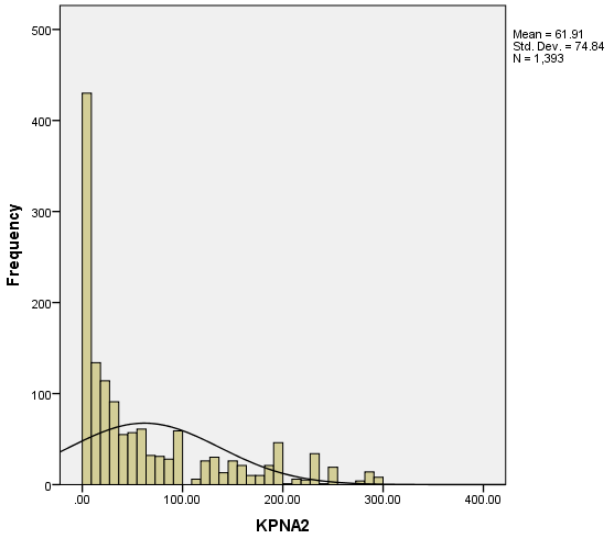
Correlation between SUMO Markers with other Tumour Markers											
Markers		**PIAS1**						**PIAS4**			
		$c^- n^-$ N (%)	**$c^+ n^+$ N(%)**	**$c^- n^+$ N (%)**	**$c^+ n^-$ N (%)**	***X²***	**P**	**Negative N (%)**	**Positive N (%)**	***X²***	**P**
BRCA1.c	**Negative**	119(73.5)	44(51.2)	32(68.1)	414(55.6)	21	<0.0001	170(71.1)	534(55.9)	18	<0.0001
	Positive	43(26.5)	42(48.8)	15(31.9)	331(44.4)			69 (28.9)	421(44.1)		
BRCA1.n	**Negative**	88(54.3)	30(34.9)	13(27.1)	453(60.6)	38	<0.0001	151(63.2)	525(54.7)	5.5	0.019
	Positive	74(45.7)	56(65.1)	35(72.9)	295(39.4)			88(36.8)	439(45.3)		
MTA1.n	**Negative**	72(52.9)	22(30.1)	14(37.8)	266(42.4)	11	0.01	108(51.2)	318(39.9)	9	0.003
	Positive	64(71.1)	51(69.9)	23(62.2)	362(57.6)			103(48.8)	478(60.1)		
MTA1.c	**Negative**	46(33.8)	11(15.1)	9(24.3)	57(9.1)	60	<0.0001	45(21.3)	99(12.4)	11	0.001
	Positive	90(66.2)	62(84.9)	28(75.7)	572(90.9)			166(78.7)	698(87.6)		
P27	**Negative**	32(45.7)	23(41.8)	9(32.1)	182(50)	4.4	0.2	62(40.5)	234(51.1)	5.1	0.02
	Positive	38(54.3)	32(58.2)	19(67.9)	73(46.8)			91(59.5)	224(48.9)		

Correlation between SUMO Markers with other Tumour Markers							
Parameters		**UBC9**					
		$c^- n^-$ N (%)	**$c^+ n^+$ N (%)**	**$c^- n^+$ N (%)**	**$c^+ n^-$ N (%)**	***X²***	**P**
ER	**Negative**	182(49.3)	217(37)	36(26.7)	183(54.8)	48	<0.0001
	Positive	187(50.7)	369(63)	99(73.3)	151(45.2)		
PgR	**Negative**	213(60.2)	268(48)	56(44.4)	205(62.7)	28	<0.0001
	Positive	141(39.8)	290(52)	70(55.6)	122(37.3)		
TN	**Negative**	231(65.3)	440(78)	114(87.7)	198(60.7)	54	<0.0001
	Positive	123(34.7)	124(22)	16(12.3)	128(39.3)		
BLBC	**Negative**	233(72.6)	415(80.7)	111(94.1)	196(68.8)	38	<0.0001
	Positive	88(27.4)	99(19.3)	7(5.9)	89(31.2)		
Rad51.c	**Negative**	23(9.6)	30(9.2)	9(12.3)	16(7.1)	2	0.6
	Positive	216(90.4)	297(90.8)	64(87.7)	208(92.9)		
Rad51.n	**Negative**	184(76.7)	176(53.5)	44(59.5)	181(80.4)	58	<0.0001
	Positive	56(23.3)	153(46.5)	30(40.5)	44(19.6)		
KU70/KU80	**Negative**	50(18.9)	32(9)	12(16.4)	21(9.4)	17	0.001
	Positive	214(81.1)	325(91)	61(83.6)	202(90.6)		
DNA-PK	**Negative**	97(31.8)	22(4.3)	7(7.3)	54(17.8)	122	<0.0001
	Positive	208(68.2)	494(95.7)	89(92.7)	250(82.2)		
SMC6L1.n	**Negative**	140(42.6)	114(22.1)	26(24.8)	151(47.9)	74.5	<0.0001
	Positive	189(57.4)	402(77.9)	79(75.2)	164(52.1)		
SMC6L1.c	**Negative**	149(45.3)	126(24.4)	60(57.1)	66(21)	89	<0.0001
	Positive	180(54.7)	390(75.6)	45(42.9)	249(79)		
CHK1.n	**Negative**	205(79.5)	267(72.8)	45(55.6)	182(80.5)	24	<0.0001
	Positive	53(20.5)	100(27.2)	36(44.4)	44(19.5)		
CHK1.c	**Negative**	38(14.7)	23(6.3)	13(16)	12(5.3)	22	<0.0001
	Positive	222(85.3)	343(93.7)	68(84)	214(94.7)		
CHK2	**Negative**	147(70.3)	117(32.1)	27(37.5)	125(61.9)	97	<0.0001
	Positive	62(29.7)	248(67.9)	45(62.5)	77(38.1)		
Ki-67	**Negative**	122(38.7)	183(35.4)	53(45.7)	68(23.2)	26	<0.0001
	Positive	193(61.3)	334(64.6)	63(54.3)	228(76.8)		
ATM	**Negative**	151 (56.6)	201 (52.9)	37 (44)	150 (63.8)	12	0.007
	Positive	116 (43.4)	179 (47.1)	47 (56)	85 (36.2)		
ATR	**Negative**	181(69.3)	203(43.6)	56(58.3)	116(48.7)	47	<0.0001
	Positive	80(30.7)	263(56.4)	40(41.7)	122(51.3)		
γH2AX.n	**Negative**	62(19.4)	15(2.9)	4(4.1)	77(26.4)	114	<0.0001
	Positive	257(80.6)	511(97.1)	93(95.9)	215(73.6)		
γH2AX.c	**Negative**	53(16.6)	39(7.4)	22(22.7)	8(2.7)	54	<0.0001
	Positive	267(83.4)	487(92.6)	75(77.3)	284(97.3)		
BARD1.n	**Negative**	308(91.1)	485(90.1)	95(91.3)	293(92.1)	0.99	0.8
	Positive	30(8.9)	53(9.9)	9(8.7)	25(7.9)		
BARD1.c	**Negative**	112(33.2)	77(14.3)	19(18.3)	60(18.9)	47	<0.0001
	Positive	225(66.8)	463(85.7)	85(81.7)	258(81.1)		
BRCA1.c	**Negative**	196(65.1)	246(51)	74(66.7)	160(55.6)	19.5	<0.0001
	Positive	105(34.9)	236(49)	37 (33.3)	128(44.4)		
BRCA1.n	**Negative**	202(67.1)	239(49.4)	37(33)	201(69.8)	70	<0.0001
	Positive	99(32.9)	245(50.6)	75(67)	87(30.2)		
MTA1.n	**Negative**	180(53.7)	131(24.6)	13(12)	190(59.7)	166	<0.0001
	Positive	155(46.3)	402(75.4)	95(88)	128(40.3)		
MTA1.c	**Negative**	115(34.2)	32(6)	24(22.2)	24(7.5)	149.2	<0.0001
	Positive	221(65.8)	502(94)	84(77.8)	294(92.5)		

Correlation between PIAS1 and UBC9 with other Tumour Markers Regardless of Co-expression of Cellular Localisation.							
Markers		**PIAS1.c**		**P** X^2	**PIAS1.n**		**P** X^2
		Negative N (%)	**Positive N (%)**		**Negative N (%)**	**Positive N (%)**	
ATM	**Negative**	99(59.3)	391(55.6)	0.4	445(58.9)	44(38.6)	<0.0001
	Positive	68(40.7)	312(44.4)	0.4	311(41.1)	70(61.4)	16.5
BRCA1.c	**Negative**	152(72)	459(55)	<0.0001	534(58.7)	78(57.8)	0.08
	Positive	59(28)	375(45)	20	375(41.3)	57(42.2)	0.45
BRCA1.n	**Negative**	102(48.1)	485(57.9)	0.01	542(59.4)	44(32.4)	<0.0001
	Positive	110(51.9)	352(42.1)	7	370(40.6)	92(67.6)	35
ER	**Negative**	89(34.6)	439(44.8)	0.003	481(45.2)	46(26.9)	<0.0001
	Positive	168(65.4)	540(55.2)	8.6	582(54.8)	125(73.1)	20
KU70/KU80	**Negative**	53(23.2)	91(10.2)	<0.0001	116(12)	27(18.5)	0.03
	Positive	175(76.8)	797(89.8)	27	851(88)	119(81.5)	5
Ki-67	**Negative**	113(49.3)	256(30.4)	<0.0001	302(32.5)	68(47.9)	<0.0001
	Positive	116(50.7)	587(69.6)	28.7	626(67.5)	74(52.1)	13
BARD1.c	**Negative**	67(36.6)	134(18.4)	<0.0001	179(22.3)	22(20.4)	0.6
	Positive	116(63.4)	595(81.6)	28	622(77.7)	86(79.6)	0.2
CHK1.n	**Negative**	170(69.1)	722(75.8)	0.03	860(83.1)	30(18.6)	<0.0001
	Positive	76(30.9)	230(24.2)	5	175(16.9)	131(81.4)	304
DNA-PK	**Negative**	45(27.8)	86(12.6)	<0.0001	119(16)	11(11.6)	0.3
	Positive	117(72.2)	596(87.4)	23	625(84)	84(88.4)	1.2
MTA1.n	**Negative**	88(50)	290(41.2)	0.03	339(44.3)	36(32.7)	0.02
	Positive	88(50)	414(58.8)	4.5	427(55.7)	74(67.3)	5
SMC6L1.n	**Negative**	61(36.3)	233(33.3)	0.4	262(34.6)	30(27.8)	0.2
	Positive	107(63.7)	467(66.7)	0.55	495(65.4)	78(72.2)	2
Markers		**UBC9.n**			**UBC9.c**		
		Negative N (%)	**Positive N (%)**	**P** X^2	**Negative N (%)**	**Positive N (%)**	**P** X^2
BRCA1.c	**Negative**	356(60.4)	320(54)	0.02	270 (65.5)	407(52.8)	<0.0001
	Positive	233(39.6)	273(46)	5	142(34.5)	364(47.2)	18
BRCA1.n	**Negative**	403(68.4)	276(46.3)	<0.0001	239(57.9)	441(57.1)	0.8
	Positive	186(31.6)	320(53.7)	59	174(42.1)	332(42.9)	0.07
ER	**Negative**	365(51.9)	253(35.1)	<0.0001	219(43.4)	400(43.4)	0.99
	Positive	338(48.1)	468(64.9)	41	286(56.6)	522(56.6)	0
PgR	**Negative**	418(61.4)	324(47.4)	<0.0001	270(56.1)	474(53.4)	0.3
	Positive	263(38.6)	360(52.6)	27	211(43.9)	414(46.6)	0.9
Rad51.n	**Negative**	365(78.5)	220(54.6)	<0.0001	228(72.6)	358(64.4)	0.01
	Positive	100(21.5)	183(45.4)	56	86(27.4)	198(35.6)	6
Rad51.c	**Negative**	39(8.4)	39(9.8)	0.5	32(10.3)	46(8.3)	0.3
	Positive	424(91.6)	361(90.3)	0.45	280(89.7)	507(91.7)	0.9
CHK1.c	**Negative**	387(80)	312(69.6)	<0.0001	250(73.7)	449(75.6)	0.5
	Positive	97(20)	136(30.4)	13	89(26.3)	145(24.4)	0.4
SMC6L1.c	**Negative**	215(33.4)	186(30)	0.2	210(48.3)	193(23.1)	<0.0001
	Positive	429(66.6)	435(70)	1.7	225(51.7)	641 (76.9)	83

Appendix 4

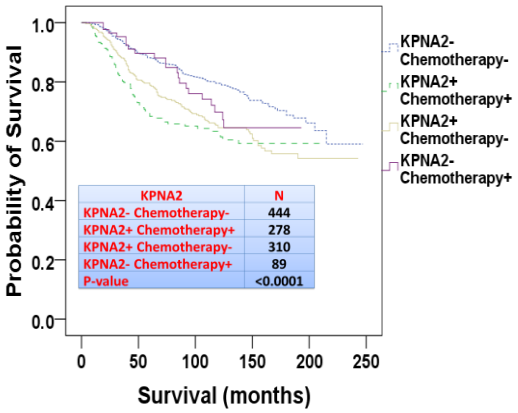

Mean = 226.13
Std. Dev. = 77.339
N = 1,339
Frequency
NPM


Mean = 61.91
Std. Dev. = 74.84
N = 1,393
Frequency
KPNA2

Correlation between Nucleocytoplasmic Transport Markers with other Tumour Markers.									
Markers		NPM				KPNA2			
		Negative N (%)	positive N (%)	X^2	P	Negative N (%)	positive N (%)	X^2	P
ER	Negative	105(46.1)	465(43.6)	0.45	0.5	153(23.9)	447(63.8)	215	<0.0001
	Positive	123(53.9)	601(56.4)			487(76.1)	254(36.2)		
PgR	Negative	133(56.4)	542(54)	0.4	0.5	234(37.7)	473(71.5)	148	<0.0001
	Positive	103(43.6)	461(46)			387(62.3)	189(28.5)		
Triple Negative	Negative	154(67.8)	726(71.2)	1	0.3	533(85.3)	373(55.8)	134	<0.0001
	Positive	73(32.2)	293(28.8)			92(14.7)	296(44.2)		
HER-2	Negative	208(85.2)	851(82.4)	1	0.3	585(90.4)	521(76.6)	45.5	<0.0001
	Positive	36(14.8)	182(17.6)			62(9.6)	159(23.4)		
Ki-67	Negative	78(37.9)	308(33.1)	1.7	0.2	306(54.2)	105(17)	180	<0.0001
	Positive	128(62.1)	622(66.9)			259(45.8)	513(83)		
Rad51.c	Negative	10(6.6)	61(9.7)	1.4	0.2	55(15.2)	25(5.7)	20	<0.0001
	Positive	142(93.4)	568(90.3)			308(84.8)	416(94.3)		
Rad51.n	Negative	107(70.4)	422(66.6)	0.8	0.4	222(60.7)	328(73.9)	16	<0.0001
	Positive	45(29.6)	212(33.4)			144(39.3)	116(26.1)		
Rad51	n-c-	9(5.9)	32(5.1)	5	0.1	35(9.6)	14(3.2)	38	<0.0001
	n+c+	44(28.9)	179(28.5)			121(33.3)	103(23.4)		
	n-c+	98(64.5)	389(61.8)			187(51.5)	313(71)		
	n+c-	1(0.7)	29(4.6)			20(5.5)	11(2.5)		
γH2AX.c	Negative	23(11.6)	89(10.1)	0.4	0.5	82(16)	40(6.2)	29	<0.0001
	Positive	175(88.4)	795(89.9)			429(84)	601(93.8)		
γH2AXn	Negative	62(31.3)	83(9.4)	67	<0.0001	47(9.2)	111(17.3)	16	<0.0001
	Positive	136(68.7)	800(90.6)			463(90.8)	530(82.7)		
γH2AX	n-c-	7(3.5)	9(1)	67	<0.0001	9(1.8)	11(1.7)	46.5	<0.0001
	n+c+	120(60.6)	720(81.5)			390(76.5)	501(78.2)		
	n-c+	55(27.8)	74(8.4)			38(7.5)	100(15.6)		
	n+c-	16(8.1)	80(9.1)			73(14.3)	29(4.5)		
BARD1.c	Negative	68(29.3)	180(18.1)	14.5	<0.0001	122(20.9)	144(21.6)	0.09	0.8
	Positive	164(70.7)	813(81.9)			461(79.1)	522(78.4)		
BARD1.n	Negative	220(94.4)	885(89.3)	6	0.018	536(92.1)	598(89.8)	2	0.1
	Positive	13(5.6)	106(10.7)			46(7.9)	68(10.2)		
BARD1	n-c-	69(29.6)	179(18.1)	19	<0.0001	123(21.1)	143(21.5)	3	0.4
	n+c+	13(5.6)	105(10.6)			46(7.9)	67(10.1)		
	n-c+	151(64.8)	706(71.2)			413(71)	455(68.3)		
	n+c-	0	1(0.1)			0	1(0.2)		
BRCA1.c	Negative	123(63.7)	487(54.7)	5	0.02	308 (59.2)	317(53.7)	3	0.06
	Positive	70 (36.3)	404(45.3)			212 (40.8)	273(46.3)		
BRCA1.n	Negative	118(61.1)	505(56.5)	1.4	0.2	226(43.4)	429(72.5)	97	<0.0001
	Positive	75(38.9)	389(43.5)			295(56.6)	163(27.5)		
BRCA1	n-c-	74 (38.3)	236(26.5)	11.5	0.009	119 (22.9)	218(36.9)	96	<0.0001
	n+c+	26 (13.5)	137(15.4)			105 (20.2)	64 (10.8)		
	n-c+	44 (22.8)	267 (30)			107 (20.6)	209(35.4)		
	n+c-	49 (25.4)	251(28.2)			189 (36.3)	99 (16.8)		
*BARD1 /BRCA1	Negative	23(10.2)	60(5.8)	6	0.016	30(4.9)	60(9)	8	0.005
	Positive	202(89.8)	971(94.2)			577(95.1)	605(91)		

Correlation between Nucleocytoplasmic Transport markers with other Tumour Markers Continued.									
Markers		**NPM**				**KPNA2**			
		Negative N (%)	**positive N (%)**	X^2	**P**	**Negative N (%)**	**positive N (%)**	X^2	**P**
SMC6L1.n	**Negative**	120(54.8)	278(29.5)	50.5	<0.0001	213(36.7)	222(33.8)	1	0.3
	Positive	99(45.2)	665(70.5)			367(63.3)	434(66.2)		
SMC6L1.c	**Negative**	101(46.1)	254(26.9)	31	<0.0001	225(38.8)	153(23.3)	35	<0.0001
	Positive	118(53.9)	689(73.1)			355(61.2)	503(76.7)		
SMC6L1	**n-c-**	68(31.1)	134(14.2)	63	<0.0001	135(23.3)	84(12.8)	39	<0.0001
	n+c+	66(30.1)	545(57.8)			277(47.8)	365(55.6)		
	n-c+	52(23.7)	144(15.3)			78(13.4)	138(21)		
	n+c-	33(15.1)	120(12.7)			90(15.5)	69(10.5)		
CHK1	**n-c-**	20(12.7)	33(4.7)	15.5	0.001	39(9.2)	17(3.7)	27	<0.0001
	n+c+	36(22.8)	145(20.6)			107(25.2)	81(17.7)		
	n-c+	97(61.4)	498(70.7)			259(60.9)	348(76.1)		
	n+c-	5(3.2)	28(4)			20(4.7)	11(2.4)		
CHK1.n	**Negative**	117(74.1))	531(75.3)	0.1	0.7	298(70.1)	365(79.7)	11	0.001
	Positive	41(25.9)	174(24.7)			127(29.9)	93(20.3)		
CHK1.c	**Negative**	25(15.8)	61(8.7)	7	0.007	59(13.9)	28(6.1)	15	<0.0001
	Positive	133(84.2)	643(91.3)			366(86.1)	429(93.9)		
UBC9	**n-c-**	116(49.4)	195(19.9)	112	<0.0001	175(30.1)	157(23.2)	57	<0.0001
	n+c+	40(17)	545(46.4)			238(40.9)	279(41.3)		
	n-c+	70(29.8)	243(24.8)			104(17.9)	217(32.1)		
	n+c-	9(3.8)	86(8.8)			65(11.2)	23(3.4)		
UBC9.n	**Negative**	186(79.1)	438(44.8)	89.5	<0.0001	279(47.9)	374(55.3)	7	0.009
	Positive	49(20.9)	540(55.22)			303(52.1)	302(44.7)		
UBC9.c	**Negative**	125(53)	282(28.8)	50	<0.0001	240(41.1)	181(26.7)	29	<0.0001
	Positive	111(47)	698(71.2)			344(58.9)	496(73.3)		
PIAS1	**n-c-**	27(16.9)	97(14.3)	5.5	0.1	88(22.4)	50(10.9)	44.5	<0.0001
	n+c+	19(11.9)	48(7.1)			46(11.7)	28(6.1)		
	n-c+	107(66.9)	506(74.7)			239(60.8)	373(81.3)		
	n+c-	7(4.4)	26(3.8)			20(5.1)	8(1.7)		
PIAS1.c	**Negative**	35(21.6)	125(18.4)	0.9	0.3	111(28)	58(12.6)	32	<0.0001
	Positive	127(78.4)	556(81.6)			286(72)	403(87.4)		
PIAS1.n	**Negative**	135(83.9)	604(89.1)	3	0.06	327(83.2)	425(92.2)	16.3	<0.0001
	Positive	26(16.1)	74(10.9)			66(16.8)	36(7.8)		

Relationship between Nucleocytoplasmic Transport markers with Clinico-Pathological Parameters.									
Parameters		**NPM**				**KPNA2**			
		Negative N (%)	**Positive N (%)**	X^2	**P**	**Negative N (%)**	**Positive N (%)**	X^2	**P**
Age	**<50**	89(35.5)	389(35.9)	0.02	0.9	205(30.5)	299(41.9)	20	<0.0001
	≥50	162(64.5)	694(64.1)			468(69.5)	414(58.1)		
Size	**≤ 1.5cm**	76(31)	320(29.7)	0.2	0.7	239(35.6)	152(21.6)	33	<0.0001
	>1.5cm	169(69)	757(70.3)			432(64.4)	551(78.4)		
Stage	**1**	170(67.7)	622(57.5)	10	0.006	434(64.4)	404(56.9)	10	0.007
	2	65(25.9)	338(31.3)			183(27.2)	218(30.7)		
	3	16(6.4)	121(11.2)			57(8.5)	88(12.4)		
Grade	**1**	35(13.9)	104(9.6)	4	0.1	145(21.5)	18(2.5)	326	<0.0001
	2	65(25.9)	309(28.5)			263(39)	84(11.8)		
	3	151(60.2)	671(61.9)			266(39.5)	611(85.7)		
Tubules	**1**	8(3.4)	35(3.3)	4	0.1	40(6.2)	7(1)	104	<0.0001
	2	54(22.8)	316(29.4)			251(38.7)	133(18.9)		
	3	175(73.8)	723(67.3)			357(55.1)	565(80.1)		
Pleomorphism	**1**	5(2.1)	14(1.3)	3	0.2	15(2.3)	3(0.4)	220	<0.0001
	2	74(31.4)	288(26.8)			295(45.7)	77(10.9)		
	3	157(66.5)	771(71.9)			335(51.9)	624(88.6)		
Mitosis	**1**	60(25.3)	278(25.9)	0.2	0.9	287(44.3)	57(8.1)	316	<0.0001
	2	40(16.9)	191(17.8)			141(21.8)	85(12.1)		
	3	137(57.8)	605(56.3)			220(34)	563(79.9)		
NPI	**Excellent**	23(9.3)	61(5.7)	11	0.05	88(13.1)	10(1.4)	214	<0.0001
	Good	39(15.9)	168(15.7)			164(24.5)	36(5.1)		
	Moderate 1	91(37)	340(31.7)			209(31.2)	248(35.4)		
	Moderate 2	58(23.6)	296(27.6)			132(19.7)	225(32.1)		
	Poor	29(11.8)	153(14.3)			61(9.1)	136(19.4)		
	Very poor	6(2.4)	54(5)			16(2.4)	46(6.6)		
Tumour Type	**Invasive Ductal /NST**	164(67.2)	738 (68.8)	20	<0.0001	363(55.3)	586(82.9)	169	<0.0001
	lobular	7 (2.9)	60 (5.6)			47(7.2)	11 (1.6)		
	Atypical Medullary	15 (6.1)	17 (1.6)			7 (1.1)	31 (4.4)		
	Mixed	52 (21.3)	223 (20.8)			206(31.4)	68 (9.6)		
	other	6 (2.5)	35 (3.3)			34(5.2)	11 (1.6)		
Vascular Invasion	**Negative**	117(66.1)	429(58.5)	3	0.06	330(63.6)	252(57.7)	3	0.06
	Positive	60(33.9)	304(41.5)			189(36.4)	185(42.3)		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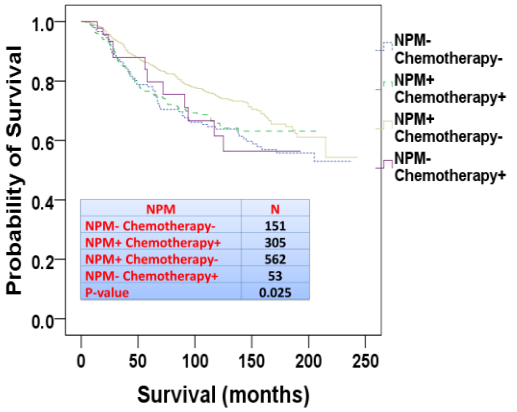
0.2
0.0
0
50
100
150
200
250
Survival (months)
KPNA2- Chemotherapy-
KPNA2+ Chemotherapy+
KPNA2+ Chemotherapy-
KPNA2- Chemotherapy+
KPNA2 N
KPNA2- Chemotherapy- 444
KPNA2+ Chemotherapy+ 278
KPNA2+ Chemotherapy- 310
KPNA2- Chemotherapy+ 89
P-value <0.0001


Probability of Survival
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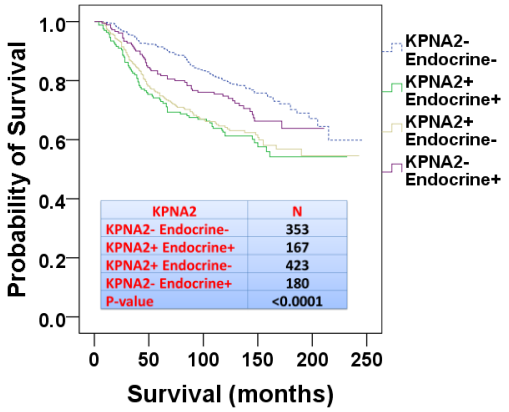
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
250
Survival (months)
NPM- Chemotherapy-
NPM+ Chemotherapy+
NPM+ Chemotherapy-
NPM- Chemotherapy+
NPM N
NPM- Chemotherapy- 151
NPM+ Chemotherapy+ 305
NPM+ Chemotherapy- 562
NPM- Chemotherapy+ 53
P-value 0.025


Probability of Survival
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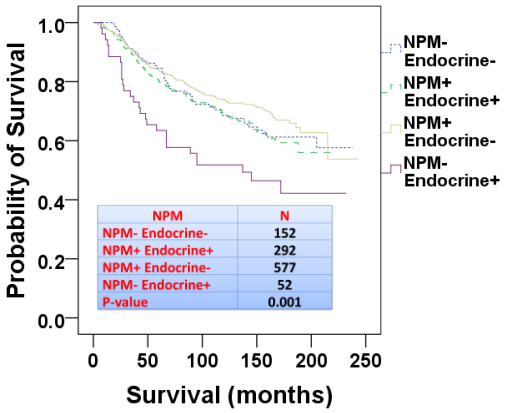
0.6
0.4
0.2
0.0
0
50
100
150
200
250
Survival (months)
KPNA2- Endocrine-
KPNA2+ Endocrine+
KPNA2+ Endocrine-
KPNA2- Endocrine+
KPNA2 N
KPNA2- Endocrine- 353
KPNA2+ Endocrine+ 167
KPNA2+ Endocrine- 423
KPNA2- Endocrine+ 180
P-value <0.0001


Probability of Survival
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0.6
0.4
0.2
0.0
0
50
100
150
200
250
Survival (months)
NPM- Endocrine-
NPM+ Endocrine+
NPM+ Endocrine-
NPM- Endocrine+
NPM N
NPM- Endocrine- 152
NPM+ Endocrine+ 292
NPM+ Endocrine- 577
NPM- Endocrine+ 52
P-value 0.001