The University of Nottingham

# THE BIOLOGICAL HETEROGENEITY OF OESTROGEN RECEPTOR POSITIVE BREAST CANCER AND ITS PHENOTYPIC CHARACTERISATION

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## Abstract

Although global gene microarray studies have demonstrated the molecular heterogeneity of breast cancer (BC) and provided potential for clinical applications, the molecular subclassification of luminal/ER-positive tumours, which is the largest class of BC, remains unclear. Characterisation of luminal/ER-positive subtypes could have important implications in clinical decision-making and patient management.

The patient study cohort is derived from a consecutive series of approximately 1902 cases of primary operable invasive breast carcinoma obtained from the Nottingham Tenovus Primary Breast Carcinoma Series, with patients presenting between 1986 and 1998. This is a well-characterized series of primary breast carcinoma that has been treated in a uniform way and previously used to study a wide range of proteins. Using gene microarray experiments in 128 frozen invasive BC derived from this series, 47,293 gene transcripts were analysed using a number of different bio-statistical models to identify a transcript signature for luminal/ER-positive BC, from which candidate genes were selected and that can be used to characterise ER-positive breast cancer. In addition, other biomarkers with strong relevance in ER-positive breast cancer were studied because the evidence strongly suggests an important role in the biology and molecular classification of ER-positive breast cancer. The selection criteria was based on published literature concentrating mainly on ER related pathways including ER coregulators (CARM1, PELP1), cellular proliferation (p27, TK1, cyclin B1), apoptosis (Bcl2), Akt/PIK3

pathway (FOXO3a), gene expression profiling (FOXA1, XBP1, TFF1) and endocrine resistance (CD71).

Immunohistochemistry and high throughput tissue microarray technology were used to study the protein expression of 16 biomarkers with strong relevance to ER pathways in a well characterised consecutive series of invasive BC (n=1902) in addition to anther 9 markers that were available from the database of the breast cancer research group, University of Nottingham. The data were analysed using different clustering methods including K-means and Partitioning around Medoids. Kaplan Meier plots with Log-rank test (LR) were used to model clinical outcome.

A transcript signature for ER positive BC was identified including RERG, GATA3 and other genes by a supervised classification analysis using 10-fold external cross-validation of the gene microarray data. Immunohistochemical validation studies confirmed their association with ER positive BC.

Through a consensus approach using different clustering techniques applied to protein expression data 25 markers, three biological clusters (patient subclasses) in ER positive breast cancer showing significant difference in clinical outcome (LR= 28.185 & $p<0.001$) have been identified. Importantly, the poor prognosis cluster was significantly characterised by high tumour grade and frequent development of distant metastasis.

In conclusion, our results emphasised the heterogeneity of luminal/ER-positive BC. Molecular profiling of breast cancer using protein biomarkers on TMAs can sub-classify ER-positive tumours into clinically and biologically relevant subgroups.

## Publications

**1. Forkhead-box A1 (FOXA1) expression in breast cancer and its prognostic significance.**

Habashy HO, Powe DG, Rakha EA, Ball G, Paish C, Gee J, Nicholson RI, Ellis IO.

Eur J Cancer. 2008 Jul; 44(11):1541-51.

**2. Transferrin receptor (CD71) is a marker of poor prognosis in breast cancer and can predict response to tamoxifen.**

Habashy HO, Powe DG, Staka CM, Rakha EA, Ball G, Green AR, Aleskandarany M, Paish EC, Douglas Macmillan R, Nicholson RI, Ellis IO, Gee JM.

Breast Cancer Res Treat. 2010 Jan; 119 (2):283-93.

**3. The prognostic significance of PELP1 expression in invasive breast cancer with emphasis on the ER-positive luminal-like subtype.**

Habashy HO, Powe DG, Rakha EA, Ball G, Macmillan RD, Green AR, Ellis IO.

Breast Cancer Res Treat. 2010 Apr;120 (3):603-12.

**4. RERG (Ras-like, oestrogen-regulated, growth-inhibitor) expression in breast cancer: a marker of ER-positive luminal-like subtype.**

Habashy HO, Powe DG, Glaab E, Ball G, Spiteri I, Krasnogor N, Garibaldi JM, Rakha EA, Green AR, Caldas C, Ellis IO.
Breast Cancer Res Treat. 2010 Aug 10 [Epub ahead of print]

**5. FOXO3a nuclear localisation is associated with good prognosis in luminal-like breast cancer**

Habashy HO, Rakha EA, Ball G, Aleskandarany M, Ahmed M, Ellis IO, Green AR, Powe DG
Breast Cancer Res Treat. Accepted

**6. Coactivator-associated arginine methyltransferase-1(CARM1) expression in breast cancer: clinicopathological and prognostic significance**

Habashy HO, Powe DG, Green AR, Rakha EA, Macmillan RD, Ellis IO

In preparation

**7. Prognostic and biological significance of the cell-cycle associated proteins, cyclin B1 and thymidine kinase 1 (TK1) in breast cancer and luminal-like subtype**

Habashy HO, Powe DG, Green AR, Rakha EA, Macmillan RD, Ellis IO

In preparation

**8. A review of the biological and clinical characteristics of luminal-like oestrogen receptor-positive breast cancer**

Habashy HO, Powe DG, Abdel-Fatah TM, Gee MWJ, Nicholson IR, Green AR, Rakha EA, Ellis IO

In preparation

## Presented abstracts at national and international conferences

**1-FOXA1 Expression in Breast Cancer and its Prognostic Significance**

Habashy HO, Powe DG, Rakha EA, Ball G, Paish C, Gee J, Nicholson RI, Ellis IO

Has been presented as a poster presentation

Scientific Meeting of the Pathological Society of Great Britain & Ireland, Leeds, 1-4 July 2008

**2-Transferrin Receptor (CD71) Expression in Breast Cancer and its Prognostic Significance**

Habashy HO, Powe DG, Staka CM, Rakha EA, Ball G, Green AR, Aleskandarany M, Paish EC, Douglas Macmillan R, Nicholson RI, Ellis IO, Gee JM.

Has been presented as a poster presentation

Scientific Meeting of the Pathological Society of Great Britain & Ireland, Leeds, 1-4 July 2008

**3-CARM1 Expression in Breast Cancer and Luminal-like/Oestrogen Receptor Positive Subtype: Clinicopathological and Prognostic Associations**

Hany Onsy Habashy, Desmond G Powe, Emad A Rakha, Claire Paish, Andrew R Green and Ian O Ellis

Has been presented as a poster presentation

Summer Meeting 2009. Scientific Meeting of the Pathological Society of Great Britain & Ireland, Cardiff, 30 June-3 Jul

**4-Luminal Breast Cancer: Identification of Significant Prognostic Patho-biological Indicators**

Hany Onsy Habashy, Desmond G Powe, Emad A Rakha, Claire Paish, Andrew R Green and Ian O Ellis

Has been presented as a poster presentation

Summer Meeting 2009. Scientific Meeting of the Pathological Society of Great Britain & Ireland, Cardiff, 30 June-3 Jul

**5-Oestrogen Receptor-Positive Breast Cancer: Identification of Significant Prognostic Patho-biological Subgroup**

Hany Onsy Habashy, Desmond G Powe' Graham Ball, Ian O Ellis

Has been presented as an oral presentation at the 22nd European Congress of Pathology, 4-9 September, Florence, Italy

**6-Luminal-like oestrogen receptor-positive breast cancer: identification of prognostic biological subclasses**

Hany Onsy Habashy, Desmond G Powe' Graham Ball, Enrico Glaab, Daniele Soria, Jonathan Garibaldi, Natalio Krasnogor, Andrew R Green, Carlos Caldas, Ian O Ellis

Has been presented as a poster presentation at the 7th European Breast Cancer Conference, 24-27 March 2010, Barcelona, Spain. European Journal of Cancer Suppl. 2010, 8(3):91

**7-Oestrogen Receptor Positive Breast Cancer: Prognostic and Biological Significance of Proliferation Assessed by Cyclin B1 and Thymidine Kinase 1 (TK1) Protein Expression**

Hany Onsy Habashy, Desmond G Powe, Graham Ball, Andrew R Green, Emad A Rakha, Ian O Ellis

Has been presented as an oral presentation

Summer Meeting 2010. 198th Scientific Meeting of the Pathological Society of Great Britain & Ireland, 29 June-1 July. Published in the Journal of Pathology, volume 222, Issue S1, Pages S1–S51

**8-(Angiotensin II Receptor, Type 1) Expression and Its Prognostic Implications in the ER-Positive Luminal-Like Breast Cancer**

Hany Onsy Habashy, Desmond G Powe, Graham Ball, Andrew R Green, Emad A Rakha, Ian O Ellis

Has been presented as an oral presentation

Summer Meeting 2010. 198th Scientific Meeting of the Pathological Society of Great Britain & Ireland, 29 June-1 July. Published in the Journal of Pathology, volume 222, Issue S1, Pages S1–S51

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **ANN** | Artificial Neural Network |
| **BC** | Breast cancer |
| **BCSS** | Breast Cancer Specific Survival |
| **CI** | Confidence Interval |
| **CK** | Cytokeratin |
| **DAB** | Diaminobenzidine |
| **DFI** | Disease Free Interval |
| **DMFI** | Distant Metastasis Free Interval |
| **DM** | Distant Metastasis |
| **ER** | Oestrogen Receptor |
| **H&E** | Haematoxylin and Eosin |
| **HR** | Hazard Ratio |
| **IHC** | Immunohistochemistry |
| **GEP** | Gene expression profiling |
| **LR** | Log Rank |
| **LN** | Lymph Node |
| **LOH** | Loss of Heterozygosity |
| **NPI** | Nottingham prognostic Index |
| **PAM** | Partition Around Medoid |
| **PCR** | Polymerase Chain Reaction |
| **PgR** | Progesterone receptor |
| **TBS** | TRIS/HCL Buffered Saline |
| **TDLU** | Terminal Duct Lobular Unit |
| **TMA** | Tissue Microarray |
| **VI** | Vascular Invasion |

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# 1 General Introduction

## 1.1 Introduction

Breast cancer is a major cause of death among middle-aged women and some patients develop relapses despite advances in therapeutic methods. Currently, pathological diagnosis and classification of human neoplasia is based on the pathological features, immunophenotyping and other techniques for distinguishing tumour types. The combination of pathological classification and clinical criteria are mainly used to differentiate distinct subclasses in clinical practice that differ in prognosis. However, there is still marked differences in the clinical behaviour of cancers within this current tumour classification, which makes the prediction of response to treatment and clinical outcomes more difficult. So, breast cancer has to be defined by genetic biomarkers to improve the therapeutic methods and patients follow-up (Ahr et al., 2001). Most breast cancers are derived from the epithelial cells lining the ducts and lobules.

### 1.1.1 Terminal duct lobular unit (TDLU)

The functional unit of the breast is a complex structure that is composed of two major parts: the terminal duct-lobular unit and the large duct system.

The TDLU is formed by the alveoli and the terminal ductule of a lobule and represents the secretory part of the gland. It connects with the subsegmental duct, which in turn leads to a segmental duct and lastly to a collecting duct which empties into the nipple. The TDLU is recognized because of its lobular

arrangement and the presence of a myxoid-appearing connective tissue (Cunha, 1994).

After puberty, this structure forms the major hormone sensitive areas of the mammary epithelium. It also appears to be the site of origin for most mammary cancers. This suggests that it contains the major proliferative stem cell populations that are most sensitive to the effects of somatic cell mutation. Normal breast ducts contain at least three types of epithelial cells: luminal (glandular) cells, basal/myoepithelial cells, and stem cells (**Fig 1.1**). Many theories have been proposed to describe the lining cells and the presence of stem or progenitor cells which give rise to the main lineages, luminal/glandular and myoepithelial.

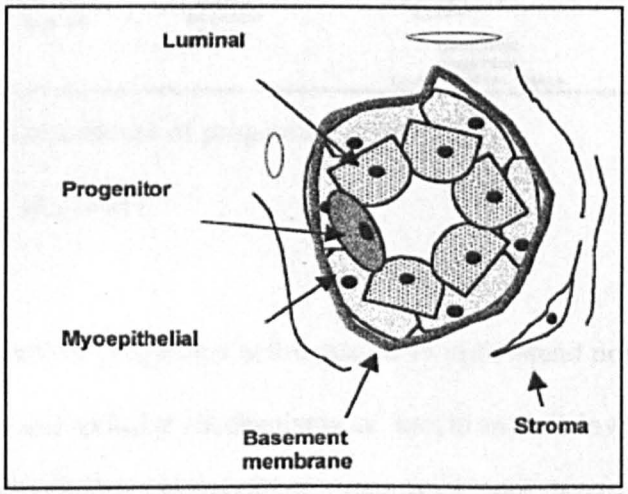


**Figure 1.1:** Cells of the mammary acini

(Birnbaum et al., 2004)

### 1.1.2 Concepts of progenitor cells

**(A)** Stem cells and progenitors are found in the basal or suprabasal position in the acini, in between myoepithelial and luminal layers. Self-renewing pluripotent stem cells enter into a bipotant CK5/6+ progenitor stage and give rise to two main lineages via committed progenitor stages, luminal/glandular CK8/18+ and myoepithelial-restricted CK14+ and smooth muscle actin (SMA)+. New markers are needed to identify the criteria of the different cell types (Birnbaum et al., 2004) **(Fig 1.2)**.

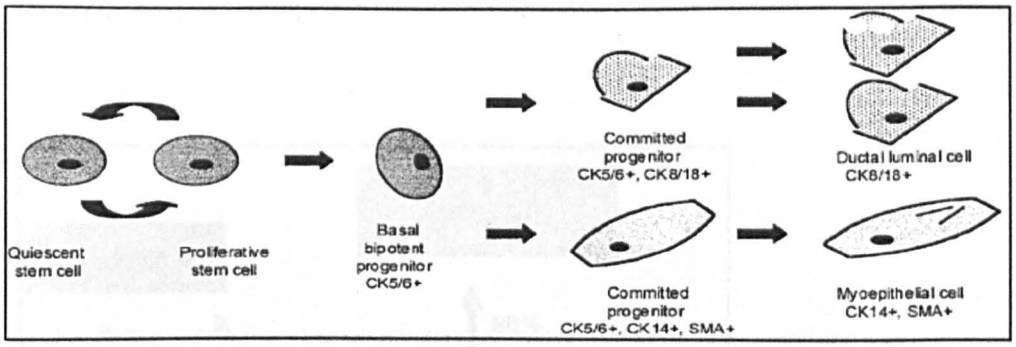


**Figure 1.2:** The concept of progenitor cell

(Birnbaum et al., 2004)

**(B)** The concept of progenitor cell is useful to understand normal physiological regeneration and cellular mechanisms of lactation and involution. Under the effects of hormones produced in pregnancy and lactation, luminal cells differentiate to CK8/18+ secretory cells. In the resting breast, the lobules display cells from progenitors (CK5+) to intermediate glandular (CK5+, CK8/18+) and glandular cells (CK8/18+) (Boecker and Buerger, 2003) (Horwitzt et al., 2008) **(Fig 1.3,4).**

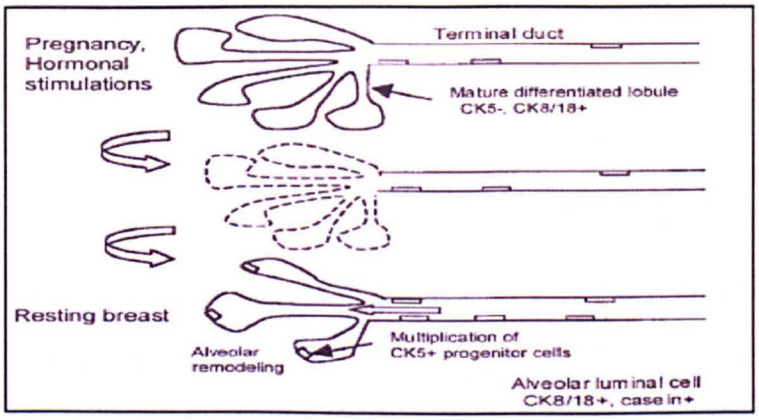


**Figure 1.3:** Another concept of progenitor cell

(Birnbaum et al., 2004)

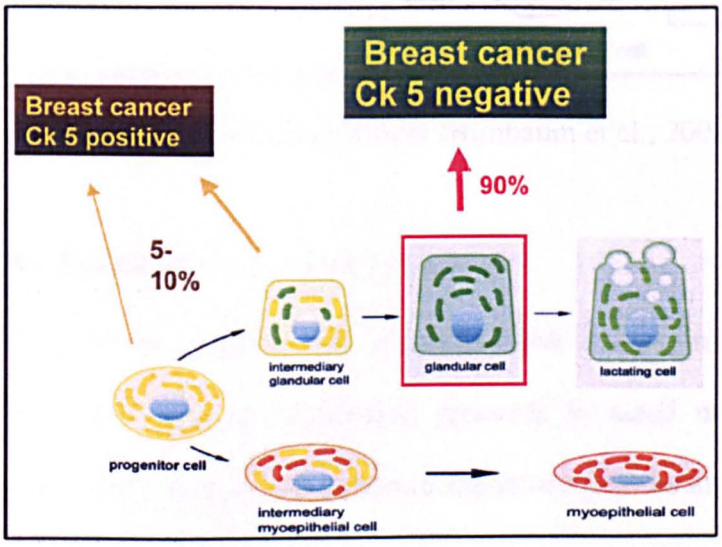


**Figure 1.4:** A cell biology concept model

(Bocker et al., 2002)

A cell biology concept based on CK5 progenitor cells (yellow) give rise to both glandular cells (CK8/18/19; green) and myoepithelial cells (SMA; red) via intermediary cells, which co-express Ck5/6 with the lineage-specific marker (either CK8/18/19 or SMA) (Bocker et al., 2002).

### 1.1.2.1 How do lining cells give rise to cancer?

**(A) The stochastic model**

This model suggests that tumour arises from any cell, despite its stage of differentiation, after a genetic alteration that triggers the transformation; the tumour cell acquires a self-renewing capability without losing its original criteria (Birnbaum et al., 2004) **(Fig 1.5).**

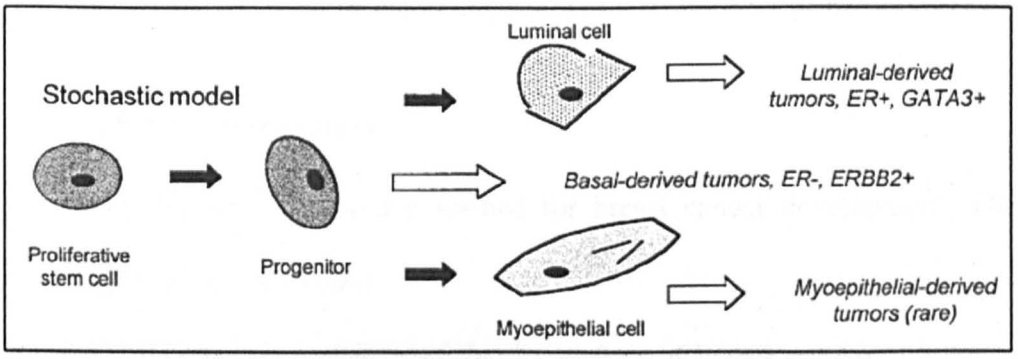


**Figure 1.5:** The stochastic model (Birnbaum et al., 2004)

**(B) The hierarchy or stem cell model.**

This model suggests that transformation occurs in a stem cell, or in a progenitor cell, and expansion proceeds to usual maturation until various stages, depending on the genomic alterations (Birnbaum et al., 2004).

The biology of the tumour could partially reflect the biology of the originally initiated normal epithelial cell stopping the evolution to the developmental stage of the epithelial cell at the time of initiation (Olsson, 2000).

### 1.1.3 Breast Carcinoma

#### 1.1.3.1 Incidence

About 44,100 cases of breast cancer are diagnosed in the UK each year and more than a million women are diagnosed with breast cancer annually worldwide. In 2007, the numbers of new cases of breast cancer in the UK increased to 45,695 cases. (Cancer research UK, UK Breast Cancer statistics)

#### 1.1.3.2 Risk factors

Many risk factors have been described for breast cancer development. The most important are discussed.

**A. Hereditary predisposition and family history of breast cancer**

Mutations in the breast cancer susceptibility genes, BRCA1 and BRCA2, account for the majority of familial breast cancer (Ford et al., 1998). Women carrying this mutation show 50-80% increased incidence of developing breast cancer. A woman with one affected first degree relative has two times the risk of breast cancer in comparison to a woman with no family history of the disease (Clamp et al., 2002).

**B. Hormone replacement therapy (HRT)**

Hormonal replacement therapy (HRT) increases the risk of breast cancer. The risk increases with the use of combined oestrogen and progestin regimen in comparison to the use of oestrogen alone (Schairer et al., 2000).

**C. Diet, alcohol consumption and smoking**

Fat intake, particularly animal fat, may cause a slight increase in breast cancer risk (Bingham et al., 2003). There is a significant association between alcohol intake and breast cancer (Key et al., 2001). Although alcohol and tobacco smoking are closely related social habits, there is no direct association between tobacco and breast cancer (Key et al., 2001).

**D. Radiation exposure**

Ionizing radiation is a well known risk factor for breast cancer. Moderate to high-dose radiotherapy is known to increase the risk of breast cancer. The effect of radiation on the breast is related to age at exposure, the younger the woman is exposed the greater the risk of developing cancer (Berrington de Gonzalez and Darby, 2004).

**E. Benign breast diseases**

The term benign breast disease describes all non-malignant breast conditions. It includes diseases associated with an increased risk of breast cancer and others that have no increase in risk.

**Relative risk for invasive carcinoma associated with benign lesions in a prior breast biopsy** (Fitzgibbons et al., 1998)

**No increased risk**

- Adenosis, other than sclerosing adenosis
- Duct ectasia
- Fibroadenoma lacking complex features

- Fibrosis
- Mastitis
- Hyperplasia without atypia
- Cysts, gross or microscopic
- Simple apocrine metaplasia without associated hyperplasia or adenosis
- Squamous metaplasia

**Slightly increased risk (1.5-2.0)**

- Complex fibroadenoma
- Moderate or florid hyperplasia without atypia
- Sclerosing adenosis
- Solitary papilloma without atypical hyperplasia

**Moderately increased risk (4.0-5.0)**

- Atypical ductal hyperplasia
- Atypical lobular hyperplasia

### 1.1.4 Molecular classification of breast cancer

Wilson and Dering (Wilson and Dering, 2004) proposed that the oestrogen receptor (ER) and the HER-2 gene are central classifiers of breast cancer, the contribution of cell type has emerged as a dominant feature in gene expression profiles that segregate primary human breast cancers **(Fig 1.6)**. For example, ER-negative tumours expressing basal markers exhibit a poor clinical outcome whereas ER-positive luminal cancers are associated with a favourable prognosis and characterized by low frequency of p53 mutation, less lymphocytic infiltration, luminal cytokeratins expression and GATA3 expression.

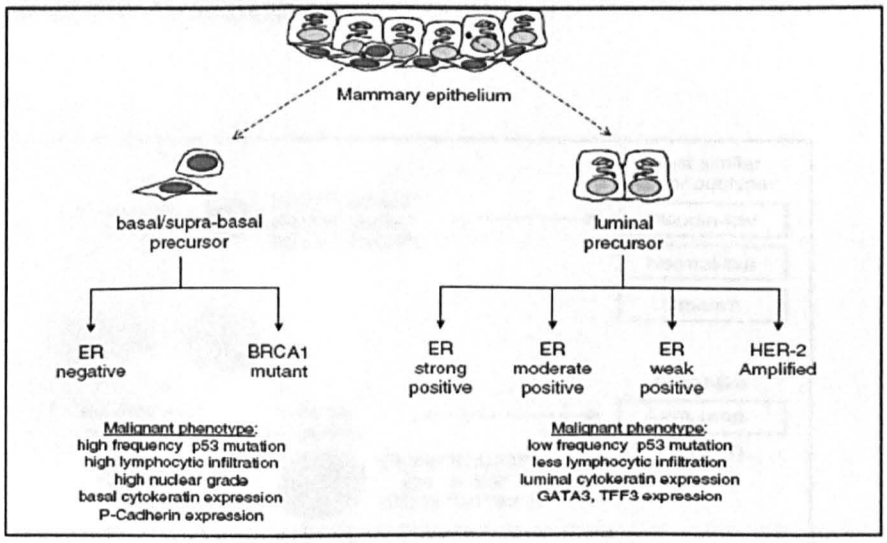


**Figure 1.6:** Cell-type origin model for classification of human breast cancers (Wilson and Dering, 2004)

Based on the expression of EpCAM and CD49f, Lim and colleagues suggested a novel phenotype of breast cancer subtypes. Lim and co-workers found that EpCAM was predominantly expressed on luminal cells, whereas high CD49f expression marked basal cells.

The CD49f hi-EpCAM– subpopulation expressed the basal lineage markers p63, CK14 and vimentin but did not express the oestrogen receptor or progesterone receptor. In contrast, the CD49f–EpCAM+ and CD49f+EpCAM+ subsets expressed luminal lineage markers including CK8 and CK18, CK19, GATA3 and MUC1(Lim et al., 2009) **(Fig 1.7)**.

For many years, ER, PgR and HER2 were extensively used for breast cancer classification but what could be inferred from this study that novels biomarkers could be used in this purpose.

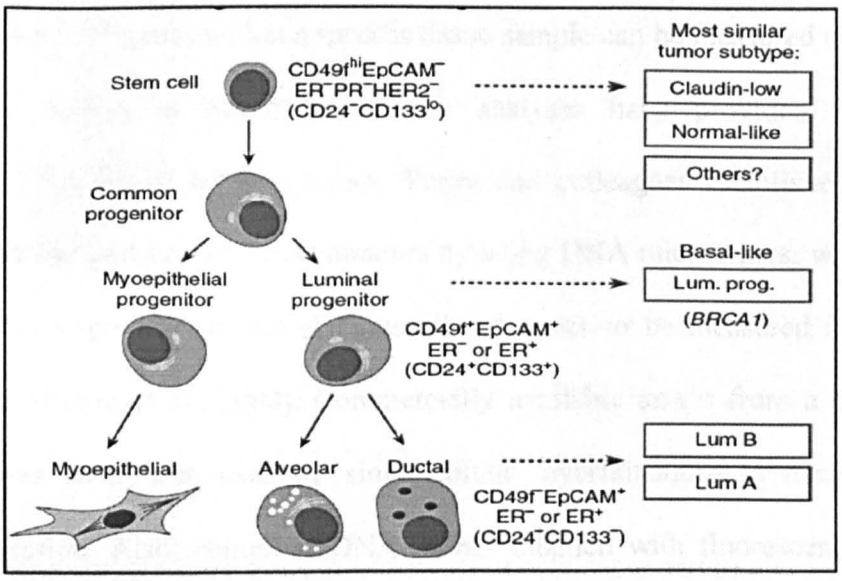


**Figure 1.7**: Model of the cell of origin classification of human breast cancers (Lim et al., 2009)

#### 1.1.4.1 Molecular classification of breast cancer using gene microarrays

The recently developed microarray technologies have created new possibilities to identify gene expression profiles and have provided a better view of the biological processes involved in tumour formation. The identification of cancer subclasses with direct clinical impact has been established, based on gene expression patterns derived from cDNA microarrays or high-throughput reverse transcriptase-polymerase chain reaction (RT-PCR) techniques (van't Veer et al., 2002).

The advances in microarray analysis have been used to explore gene expression in breast tissue on a genome-wide scale, and have shown that different biological subtypes of breast cancer are accompanied by differences in gene expression. With cDNA microarrays, the relative expression levels of thousands of genes within a specific tissue sample can be measured at the same time (Jeffrey et al., 2002). These analyses have provided interesting classifications of breast tumours. Perou and colleagues established the first molecular portraits of breast tumours by using DNA microarrays, which allow mRNA expression levels of thousands of genes to be measured in a single assay (Perou et al., 2000). Commercially available arrays from a number of sources have been used in 'single-colour' hybridizations to measure gene expression. Also, complex cDNA probes labelled with fluorescent dyes are made by performing reverse transcription on the complex mix of mRNAs isolated from a tumour specimen. In contrast to single-colour methods, most

spotted DNA microarray methods consist of a two-colour hybridization method in order to measure gene expression in multiple samples. A mixture of red (Cy5-labelled) cDNA from a test sample and green (Cy3-labelled) cDNA from a known reference sample is hybridized to each cDNA microarray. The relative level of expression for each gene on the array in comparison between multiple samples (e.g. tumour versus normal, or multiple tumours) can be determined by comparing the fluorescent intensity for that gene.

Molecular analysis of breast cancer is used to characterize the breast cancer and has been useful to discover a direct communication between tumour genotype and phenotype and to identify new cancer subtypes and molecular pathways (Reis and Lakhani, 2003). Molecular subgroups may be needed in order to develop the most accurate prediction of treatment response.

To overcome the inherent subjectivity involved in histopathology, a few well-defined molecular biomarkers have been introduced in more recent times to aid tumour classification (Reis-Filho et al., 2005). For instance, hormone receptors (HR) including oestrogen (ER) and progesterone (PR) receptors are used to classify BC into HR-positive and negative categories. In addition, assessment of HER2 status has been used to classify BC into HER2 positive and negative groups (Dowsett et al., 2000). Currently, HR (ER and PR) and HER2 remain the only molecular targets in routine clinical use in BC management.

Moreover, ER status of BC is used in determining the postoperative therapeutic strategies regarding use of adjuvant hormonal therapy. ER-positive tumours comprise the majority of breast cancers, accounting for up to 70% of all cases and are generally expected to show good response to hormonal therapy and are

associated with better clinical outcome (Murphy and Watson, 2002). It is recognised that most ER-positive tumours have characteristic morphological features: they are frequently of low histological grade displaying glandular differentiation, low degree of nuclear pleomorphism, low mitotic activity, and include most of the good prognostic special histological types (e.g. tubular, invasive cribriform, mucinous and lobular types) in comparison to ER negative tumours (Putti et al., 2005). **Fig 1.8** shows the diversity of BC with respect to ER expression and illustrates the morphological heterogeneity of this disease. Although ER protein expression is a predictor of hormonal treatment response, its effectiveness is impaired because of the existence of a proportion of ER-positive cancers that do not respond to hormonal treatment (Osborne, 1998). Furthermore, it is documented that a proportion of ER-negative tumours respond to hormonal therapy (Esserman et al., 2005). This demonstrates that ER-positivity per se defines a heterogeneous group of tumours with respect to their clinical behaviour and biology.

Improved understanding of the molecular features of ER positive BC and identifying the key oncogenes and tumour suppressor genes involved in defining this molecular heterogeneity could lead to better prediction of tumour behaviour and treatment response (Albertson, 2003, Nessling et al., 2005).

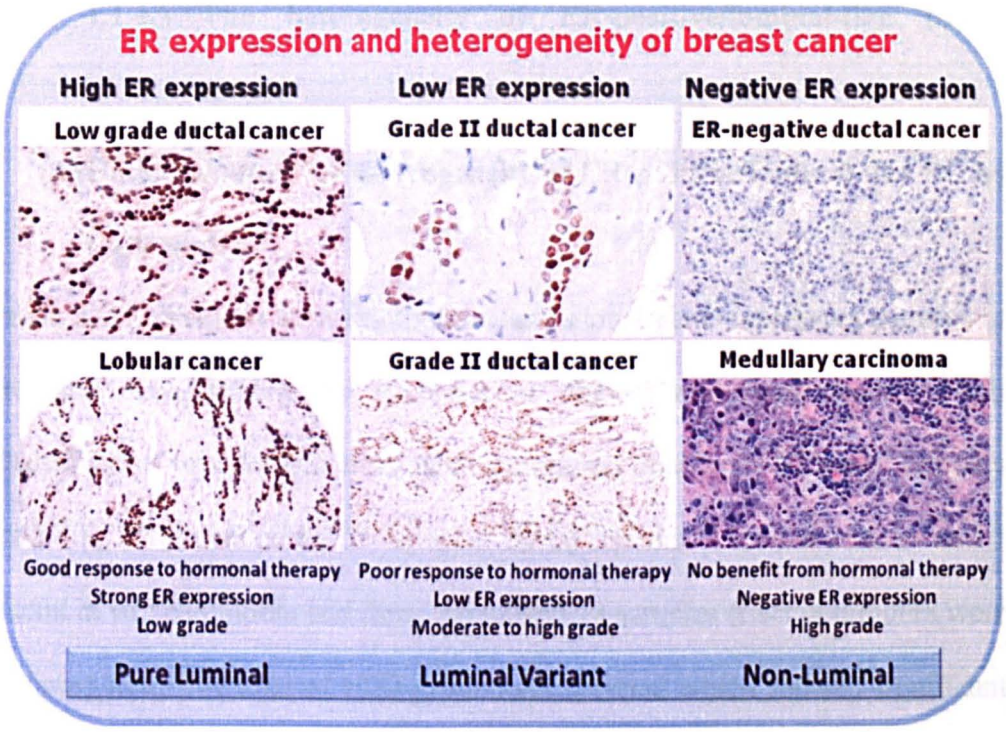


**Figure: 1.8:** The diversity of BC in relation to ER expression

### 1.1.4.2 Identification of the ER-positive luminal-like class of breast cancer

The gene expression microarray studies have provided an alternative view of the complex biological processes involved in tumour development, creating new methods for identifying distinct molecular tumour classes based on gene expression profiles (van't Veer et al., 2002). Importantly, these studies have shown that ER is the main differentiating marker of molecular signature classification, supporting the fact that breast cancer is heterogeneous and that ER-positive and negative breast carcinomas are biologically separate entities (Gruvberger et al., 2001, Sorlie et al., 2001).

#### 1.1.4.3 The heterogeneity of ER-positive/luminal-like breast cancer

**(A) Understanding the heterogeneity of ER-positive/luminal-like BC by GEP studies**

Perou and colleagues were the first to study global gene-expression patterns of BC and demonstrated the existence of distinct molecular classes. Their classification was derived from gene expression data from 40 breast tumours including 1 fibroadenoma, 36 invasive ductal cancers, 2 lobular cancers and 1 ductal in situ carcinoma and three normal breast samples (twenty tumours were sampled twice). A total of 1753 genes were selected which showed significant expression variation between samples from different tumours. Data was subsequently analysed using hierarchical clustering producing a dendogram with two main branches. One branch, called the 'luminal-like' class, was characterised by the expression of ER and other markers of normal luminal glandular epithelial cells of the breast (ER-responsive genes, luminal cytokeratins (CKs) and other luminal associated markers). The other branch, which was mainly ER-negative, was subdivided into three distinct clusters termed 'basal-like' (characterized by HR-negativity and basal CKs positivity), 'HER2-positive', and 'normal-like' class, which showed a gene expression pattern similar to that of normal breast with relatively high expression of genes characterising fat cells and other mesenchymal cell types but decreased expression of luminal epithelial cell genes (Perou et al., 2000).

Initially, although Perou and colleagues recognised the importance of ER and ER-related genes as key markers in the molecular clustering and classification of BC, they did not attempt to subclassify this large ER-positive luminal-like class of tumours. This followed in a subsequent study of 78 cases and seven non-malignant breast samples where the gene list was modified to 456 cDNA clones, with the identification of three luminal-like subgroups: Luminal A, Luminal B, and Luminal C (Sorlie et al., 2001). The latter subclass was distinguished from the other luminal subclasses by high expression of a novel gene set including transferrin receptor (CD71), MYB, nucleolar protein p40, SQLE, GGH and others. In a third study by the same investigators, a modified intrinsic gene list of 534 genes was used and resulted in only two luminal-like subclasses being observed: Luminal A and B. Interestingly, some of the genes that previously clustered in the Luminal C subclass were clustered in the Luminal B subclass and Basal-like class (Sorlie et al., 2003).

Importantly, characterisation of luminal-like cancer varies between studies. Some of the variance between different studies can be explained by the identification and use of different intrinsic gene sets for cluster analysis, leading to the view that a standard intrinsic gene set should be adopted to minimise discordant results (Andre and Pusztai, 2006). In this respect, Hu and colleagues evaluated a novel 1300 intrinsic gene set, sharing 108 genes in common with that of Sorlie's intrinsic set, on an independent combined dataset consisting of 315 breast samples (311 tumours and 4 normal breast samples) (Hu et al., 2006). The combined dataset from 315 breast samples was created by combining the gene array data from Sorlie et al. (2001 and 2003; cDNA

microarrays), van't Veer et al. (2002; oligo-microarrays) and Sotiriou et al. (2003; cDNA microarray). Genes common to all three microarray data sets (2800 genes) were identified and Distance Weighted Discrimination (DWD) was used to fuse the datasets together; DWD compensates for systematic bias between different datasets (Benito et al., 2004). The authors found that 306 of the 1300 intrinsic genes were present in the combined dataset and when analysed by hierarchical clustering, two main luminal-like subclasses were identified corresponding to the previously defined Luminal A and Luminal B in addition to HER2-positive, basal-like, and normal-like tumour groups identified in the earlier studies **(Fig 1.9)**.

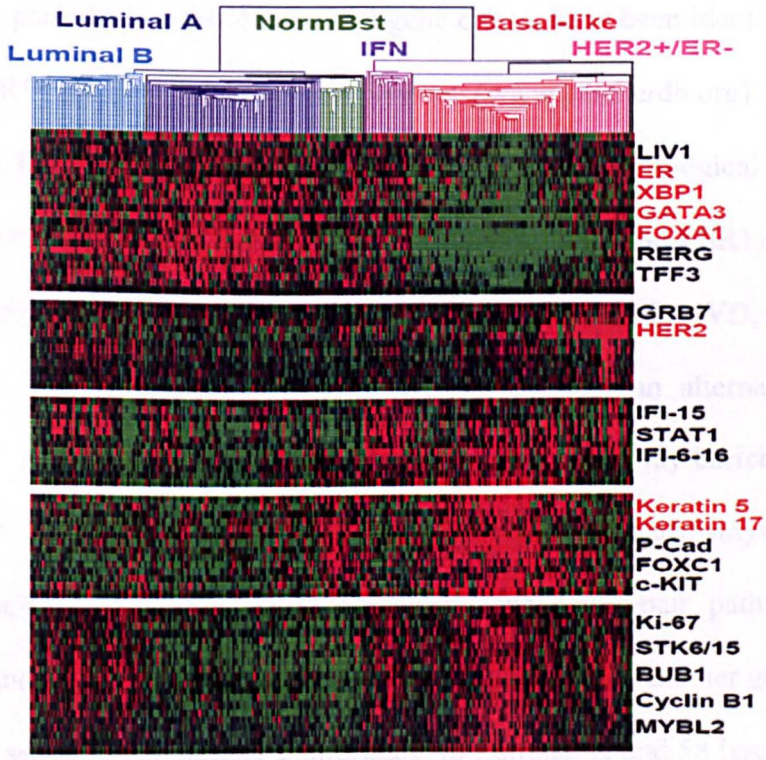


**Figure 1.9:** Dendogram of the molecular breast cancer subtypes (Hu et al., 2006)

Most subsequent studies supported the existence of at least two luminal-like subclasses (A & B). Luminal A tumours are characterised by high expression of luminal epithelial CKs and other luminal associated markers including oestrogen receptor 1 (ESR1), and genes associated with ER function such as LIV1, hepatocyte nuclear factor 3 alpha (FOXA1), X-box binding protein 1 (XBP1) and GATA-binding protein 3 (GATA3) (Sotiriou et al., 2003). Whereas the Luminal B group is characterized by low to moderate expression of the Luminal A genes mentioned above, but is further distinguished by high expression of additional genes, mainly related to proliferation such as v-MYB, GGH, LAPTMB4, NSEP1 and CCNE1 (Sorlie et al., 2001).

Molecular pathways within the luminal gene cluster have been identified using PANTHER™ Protein Classification System (www.pantherdb.org) (Sorlie et al., 2006). For Luminal A the most common represented biological pathways included steroid hormone signalling (e.g. CRABP2, AR, and ESR1) and fatty acid metabolism (e.g. PGDS, ACOX2, PTE2B, CROT, IVD, DECR2, FLJ20920, SLC27A2, ELOVL5, and MCCC2). Using an alternative tool, PathArt™ pathways, revealed that the most common pathway enriched in the Luminal A subclass is ER signalling. The other represented pathways included retinoic acid signalling pathway, nucleotide excision repair pathway, IL6 pathway and EGF signalling pathway (Sorlie et al., 2006). Another group have profiled a series of 138 tumour comprising 80 Luminal A and 58 basal cancers using whole-genome DNA microarrays and identified 5621 genes differentially expressed between the two subclasses. Luminal A tumours overexpressed genes involved mainly in fatty acid metabolism, TGFβ signalling, and

oestrogen receptor (ER) signalling (Bertucci et al., 2009). To the best of our knowledge, there is no published data concerning the Luminal B subclass pathways. In addition, there is no evidence supporting or refuting the progression of Luminal A into the Luminal B subclass.

Improved understanding of the biology of the luminal-like class of BC is clearly required and should translate into more effective methods of diagnosis and management. Despite the fact that these observations demonstrate that the luminal-like class of breast cancers exhibits ER related pathway activity and is ER-positive, it is widely recognised that it comprises a large and heterogeneous group of BC which cannot consistently be subclassified into biologically and clinically distinct subgroups. This has raised the following question: Are there alternative approaches which could help to provide a solution?

Some investigators have applied gene expression profiling technology to ER-positive tumours alone in an attempt to identify distinct molecular and biological subclasses. Oh and co-workers have developed a gene expression signature for outcome prediction of ER-positive BC patients (Oh et al., 2006). The authors used the ER-positive MCF-7 BC cell line treated with 17β-estradiol and a hierarchical clustering method to identify oestrogen-regulated genes. Subsequently, the gene set identified in MCF-7 cells was used to subclassify a training set of 65 breast tumours into two groups with significant clinical outcome differences. Subsequently, the investigators validated this gene expression predictor of outcome on three independent published data sets and found that the good prognosis group had significantly better outcome than the poor prognosis group and were characterised by high expression of

GATA3, XBP1, and PR which are all known ER related genes. The poor prognosis group was characterised by high expression of cell proliferation and anti-apoptosis genes and increased expression of a cluster of oestrogen-related genes that included CTPS, E2F6, and FANCA, demonstrating the heterogeneity and the biological and clinical importance of ER and ER related genes even within the ER-positive tumours.

In another approach, some researchers used a gene expression grade index (GGI), which defines the tumour histologic grade on the basis of their gene expression characteristics to assign a grade index to ER-positive BC in an attempt to refine their molecular classification (Loi et al., 2007). The authors have assigned ER-positive BC to either high or low GGI subgroups and compared these with the molecular classification of ER-positive tumours. The two subclasses were associated with a distinct clinical outcome in both tamoxifen-treated and untreated patients. The authors concluded that the use of genomic grade can identify two different ER-positive molecular subgroups in multiple data sets. They identified 97 genes associated with histologic grade; many of these genes were highly expressed in the luminal-like ER-positive cluster like ESR1, XBP1, FOXA1 and GATA3. In Tamoxifen-only treated populations of more than 650 patients, GGI appeared to be a strong predictor of clinical outcome, indicating the prognostic importance of proliferation genes in ER-positive subgroups, as previously reported by others (Ivshina et al., 2006, Dai et al., 2005). These prognostic classes as defined by genomic grade were an improvement over standard stratification by quantitative ER expression

levels, which correlate well to immunohistochemical protein values (Sotiriou et al., 2006)

Other authors identified distinct biological and clinical subgroups within the ER-positive BC using standard histopathological data and hierarchical clustering analysis (Webster et al., 2008).

### (B) Understanding the heterogeneity of ER-positive / luminal-like BC by genomic DNA profiling

Genomic analysis has been used in the characterization of breast lesions to investigate the relationship between their genotypic and phenotypic characteristics and for providing new prognostic parameters (Reis and Lakhani, 2003).

The identification of genome copy number abnormalities (CNAs) has been used for finding important chromosomal loci for gene identification and more specific characterisation of ER-positive BC. Bergamaschi and colleagues applied array comparative genomic hybridisation (aCGH) to 89 invasive breast cancers with locally advanced disease that were previously classified by expression arrays to determine whether different gene expression subclasses were associated with distinct CNAs (Bergamaschi et al., 2006) . The authors found Luminal A group tumours were associated with gain at 1q12-q41 and 16p12-p13 whereas Luminal B tumours exhibited more frequent loss at 3q12, gain at 8q11-q24 and 20q13, and high-level amplification at 7p22, 8q11-24, 19q13, and 20q13. High level amplifications were more prevalent in Luminal

B tumours compared to Luminal A suggesting that distinct mechanisms of genomic alterations might be behind their pathogenesis.

Chin and colleagues explored the roles of CNAs in BC by identifying associations between recurrent CNAs, gene expression, and clinical outcome in a set of aggressively treated early stage breast tumours. Their study showed that the recurrent CNAs differed between tumour subclasses defined by their gene expression, and the prognostic subclassification of patients can be improved by determining both expression and the associated copy number changes (Chin et al., 2006).

Using high resolution aCGH analysis with BAC clones, Han and colleagues investigated genomic alterations in ER-positive breast cancers showing tumour recurrence within 5 years. The investigators reported loss of 11p15.5, 11p15.4, 1p36.33, 11q13.1, and 11p11 in the recurrence group (Han et al., 2006).

The concept of relating genomic and gene expression data to identify subgroups of BC was further explored (Wang et al., 2004). In this study, loss of heterozygosity (LOH) was determined by single nucleotide polymorphism (SNP) arrays. LOH on 1p and 16q occurred in a subclass of ER positive breast cancers. The authors used 672 gene probes that showed highest variation of expression across samples in clustering and identified two large clusters named 'cluster I' and 'cluster II'; cluster II was characterized by ER positivity and further subdivided into 2 clusters (A and B). They found distinct LOH patterns in two chromosomal regions which are more associated with the ER-positive cancers, 1p34 and 16q23–24, being affected frequently in cluster II. Allelic

imbalance at 16q23–24 and 1p34 was common in cluster IIB; loss at 16q occurred in 88% of IIB tumours compared to only 50% of IIA tumours. Furthermore, loss of 1p34 was seen in 50% of IIB tumours, but not in any of cluster IIA tumours (Wang et al., 2004).

In summary, the genomic studies of ER positive breast cancers support the existence of common characteristics within the luminal-like class. Importantly in future studies it will be necessary to interrogate these findings to assist discovery of new candidate genes with relevance to the biology of ER-positive BC, the causes behind resistance to therapy, and new candidate biomarkers useful in prognosis and prediction. For example, gain on 8q is more frequent in Luminal B subclass tumours and is known to harbour the MYC oncogene. MYC plays a key role in promoting cell proliferation (Adhikary and Eilers, 2005) and this association may explain the higher proliferation rates of Luminal B tumours and their resistance to tamoxifen in some cases compared to the good prognosis luminal subgroup (Bergamaschi et al., 2006). Further genomic studies are needed to understand the relevance of alterations in chromosome 16 and chromosome one in ER-positive BC.

### (C) Understanding the heterogeneity luminal-like BC by immunohistochemical (IHC) identification

In 2005, Abd El-Rehim and colleagues applied semi-quantitative morphometric IHC to tissue microarray (TMA) sections of a large series of invasive BC (1076 tumours) using a panel of 25 tumour relevant biomarkers.

The IHC results were analyzed using hierarchical clustering and artificial neural network (ANN) methods, comparable to the GEP studies described above, to categorize cases into groups and to examine the biomarkers responsible for driving group membership. Two luminal groups (termed group 1 and group 2) were identified, characterised by the expression of oestrogen receptor, luminal CKs, MUC1, absence of basal epithelial phenotype characteristics and lack of HER2 protein overexpression. Group 1 showed relatively stronger combined expression of HER3 and HER4 compared to group 2. In addition, the mean expression of BRCA1 protein was lower in group 1 than in group 2 (Abd El-Rehim et al., 2005).

To date there is no internationally accepted single definition for luminal-like cancers although the majority of these cancers appear to have lower grade or more differentiated morphological features and are ER-positive. As a consequence, ER positivity has been used as the most important feature for a tumour to be classified as luminal-like. In their attempt to define basal-like tumours, Nielsen and co-workers classified all HER2 positive tumours in the HER2 subclass and of the remaining cases, ER-positive tumours were considered as luminal-like (Nielsen et al., 2004). Other investigators recognised the important association between lack of HER2 amplification and ER expression in determining a good prognosis associated with Luminal A tumours (Carey et al., 2006). The authors identified five tumour subclasses, Luminal A (ER-positive and/or PgR-positive and HER2-negative), Luminal B (ER positive and/or PgR-positive and HER2-positive), basal-like (ER-negative, PR-negative, HER2-negative, and CK5/6-positive, and/or HER1-positive),

HER2-positive (ER-negative, PgR-negative, and HER2-positive), and unclassified (negative for all 5 markers) subclasses. Recently, Hugh and colleagues used the proliferation marker Ki67 expression in defining the luminal-like tumours in addition to ER, PgR, and HER2. Luminal A was defined as (ER-positive and/or PR-positive and not HER2-positive or Ki67 high) while Luminal B was defined as (ER-positive and/or PgR-positive and either HER2-positive and/or Ki67 high) (Hugh et al., 2009). The importance of Ki67 in defining the poor prognosis variant luminal form has been studied by many authors in distinguishing it from the good prognosis subclass (Cheang et al., 2009).

Regarding HER2 expression, although GEP studies have shown that some luminal-like tumours express HER2 and some authors include HER2 positivity as a feature of Luminal B tumours, others argue against that and include HER2-positive tumours, regardless of the expression of ER, with the HER2-positive subgroup (Bhargava and Dabbs, 2008). Supporting this, ER-positive HER2 positive tumours are candidates to receive specific systemic therapy targeting HER2 overexpression and so differ in management from ER-positive HER2 negative tumours. Furthermore, the amplified HER2 positive cases have similar genetic changes regardless of their ER status (Marchiò et al., 2008).

In some instances the molecular class of an individual case contradicts its immunophenotypic characteristics. Rouzier and colleagues found that only 80% of the HER2+ molecular class had HER2 gene amplification by *in situ* hybridization analysis and 5% of basal-like tumours were ER-positive (Rouzier et al., 2005). In other studies, 12% of luminal-like tumours as defined by

expression arrays were reported to be ER-negative by IHC (Sotiriou et al., 2003) and ER IHC expression is reported to be found in 5-45% of basal-like cancers and 20-30% of HER2 positive cancers as defined by expression arrays (Sotiriou et al., 2003, Calza et al., 2006). The discordance between molecular and immunophenotypic criteria of BC can be partially explained by differences in methods used including the use of different monoclonal and polyclonal antibodies or different intrinsic gene sets in protein and gene expression studies, respectively. This wide disagreement about the definition of the luminal-like cancer indicates a need for additional protein markers to be used to identify the major tumour subgroups using IHC provided that they are clearly associated with prognosis and distinct biological pathways.

### (D) Additional clinical and biological features of ER-positive / luminal-like classes

There are no specific morphological features that can identify these breast tumours apart from ER positivity and low histological grade and even using such criteria, there are notable exceptions as a small percentage (9%) of luminal-like tumours defined by molecular characteristics are of high grade (Livasy et al., 2006). In the Carolina Breast Cancer Study, luminal breast cancers represented approximately two thirds of the cases with a number of important observations relating to the ER-positive group being made. Young African American women showed a low frequency of Luminal A subclass in comparison to the basal-like subgroup, possibly accounting for the poor outcome in this group of patients (Carey et al., 2006). Ihemelandu and co-

workers showed that the Luminal A subclass was more common (50%) in comparison to the Luminal B (14%) subgroup in premenopausal African American women. However, when stratified by age, results showed that in the group below 35 years of age, Luminal A and Luminal B were less frequent (Ihemelandu et al., 2007).

The expression of the anti-apoptosis Bcl-2 gene was found to be overexpressed in Luminal A compared to Luminal B cancer. As might be expected, TP53 mutation was more frequent in the basal-like subclass compared to good prognosis luminal-like subclasses (Ihemelandu et al., 2007, Sorlie et al., 2001).

Hoadley and colleagues suggested that the high expression of genes associated with the HER family pathway can predict outcome differences in ER-positive and tamoxifen-treated patients and demonstrated that the difference between Luminal A and Luminal B groups is partially due to the activation of this important pathway in Luminal B tumours. In their study, the Luminal A subclass showed low expression of the genes in the HER pathway with the exception of HER4. In contrast, the Luminal B tumours showed moderate to high expression of the EGFR-associated genes, high H-RAS and MEK2 expression (Hoadley et al., 2007).

Badve and Nakshatri proposed a model of hormonal network between ER, FOXA1 and GATA3 with predictive and prognostic signature for ER-positive breast cancers (Badve and Nakshatri, 2009). Other studies have confirmed the relation between ER and its downstream transcription through FOXA1 (Badve et al., 2007).

The luminal-like subclasses have a good prognosis but within this group of patients, Luminal B and C have been described as having a worse prognosis in comparison to the pure Luminal A cancers (Sorlie et al., 2001). The reasons for this difference in prognosis are still unknown but a possible explanation relates to ER function and signalling differences between Luminal A and Luminal B cancers, which could be attributed to the influence of additional transcription factors, coactivators, and corepressors that modulate ER activity. In addition, overexpression of proliferation and cell cycle genes in BC is well recognised to be associated with poor outcome suggesting that these genes may contribute to the Luminal B subgroup's poorer prognosis. Also, it has been proposed that abnormal apoptosis function, DNA damage response and PI3K/Akt pathways may be additional factors influencing prognosis (Bertucci et al., 2009). Supporting this concept, Dai and colleagues reported a cell proliferation signature as the key marker of poor outcome in a population of young women below 55 years whose breast cancers had high expression of ER for their age (Dai et al., 2005).

**(E) Therapeutic implications of ER-positive / luminal-like classes**

Recently, gene signatures derived from microarray studies have also been reported to predict outcome in women with ER-positive breast cancers after tamoxifen treatment better than the traditional pathological factors (Klijn et al., 2005). Jansen and co-workers identified 81 genes that predicted response to tamoxifen using a set of 46 tumours from ER-positive women with advanced

disease on cDNA microarray. They further refined the signature to 44 genes and validated it on a set of 66 tumours. It predicted the response to tamoxifen treatment in 27 out of 35 cases with disease progression. Interestingly, pathway analysis of these genes showed that they were mainly involved in oestrogen function and apoptosis which support the view that prognostic heterogeneity within ER-positive tumours in respect of resistance to hormonal therapy and outcome might be related to abnormal apoptosis function (Jansen et al., 2005).

The performance of this signature was confirmed using 44K oligomicroarray platform on a set of 69 independent patient tumours series treated with tamoxifen and the profile included 78 genes (Kok et al., 2009). Other authors have developed a molecular signature of 36 genes for detection of a subgroup of patients who did not respond to tamoxifen treatment that correctly classified 78% of patients with relapse. Among this prognostic signature, many genes are related to DNA replication and proliferation such as TK1, CCNB2, CDC2 and AURKB (Chanrion et al., 2008).

Previous studies indicated that Luminal A tumours can be treated effectively with hormonal therapy, while Luminal B tumours are more resistant and may benefit from combined endocrine treatment and chemotherapy (Hugh et al., 2009).

Goldhirsch and colleagues reported two categories of ER-positive BC based on their response to endocrine therapy, those that are highly endocrine responsive expressing high level of both ER and PgR, and those that are incompletely endocrine responsive expressing low levels of either/both receptors. A third

group called endocrine non-responsive was reported in tumours having negative expression for ER and PgR. (Goldhirsch et al., 2007). Comparing these finding to the recent molecular subclasses and their response to different types of adjuvant therapy, the highly endocrine responsive category being characterised by high ER expression seems similar to Luminal A, while incompletely endocrine responsive appears closer to Luminal B in term of their ER status and response to adjuvant therapy discussed above.

Generally, ER-positive BC is resistant to chemotherapy (Rouzier et al., 2005) and there is a need to develop prognostic assays to predict chemo-response in ER-positive tumours. Subsequently, an assay based on polymerase chain reaction (PCR) has been developed using a signature of sixteen genes and five reference genes for prediction of recurrence in tamoxifen treated ER-positive lymph node negative patients producing the 'Recurrence Score' (RS) or the 21-gene Oncotype Dx (TM) (Paik et al., 2004). Patients with a low RS were mostly found not to benefit from adjuvant chemotherapy, whereas those with high RS had a high rate of pathological response after chemotherapy (Paik et al., 2004). The low RS group of patients are probably Luminal A because these cases are strongly ER-positive characterised by low tumour grade and low proliferative activity and expected to respond to hormonal therapy better than the high RS group which are characterised by high proliferation (Hugh et al., 2009). The highly proliferative cancers may respond better and show improved survival after chemotherapy (Levack et al., 1999). It is also important to mention that patients with low RS responded well to tamoxifen alone and no

significant survival differences were found if they had given chemotherapy in addition to endocrine therapy (Paik et al., 2004).

Estimation of cell proliferation pathways could have significant clinical benefit in predicting behaviour and subclassification of the luminal-like subclasses, and their potential for response to systemic therapy. There remains a need for further identification of additional biomarkers by their relationship to biological pathways, outcome or therapeutic implications to improve the classification and clinical management of luminal-like BC especially the non-Luminal A subtypes.

The biological and behavioural criteria should be studied in depth for better characterisation of ER-positive breast cancer with respect to prognosis, biology and response to therapy. Subsequently, more research efforts are needed to study the characteristic features of these molecular subclasses and their reliability in diagnosis and prognosis of breast cancers.

### 1.1.5 Estrogen receptor alpha (ESR1)

Oestrogen receptor alpha (ESR1) is a dominant regulator of breast cancer aetiology and progression and the main discriminator marker of molecular classification (Schiff et al., 2005).

#### 1.1.5.1 Structure

ER consists of multiple domains which include a DNA-binding domain (DBD) found in the core of the protein and two major transcriptional activation

function (AF) domains: Ligand-independent AF-1 and the ligand-dependent AF-2, found in the ER amino- and carboxyl-termini, respectively **(Fig 1:10).**

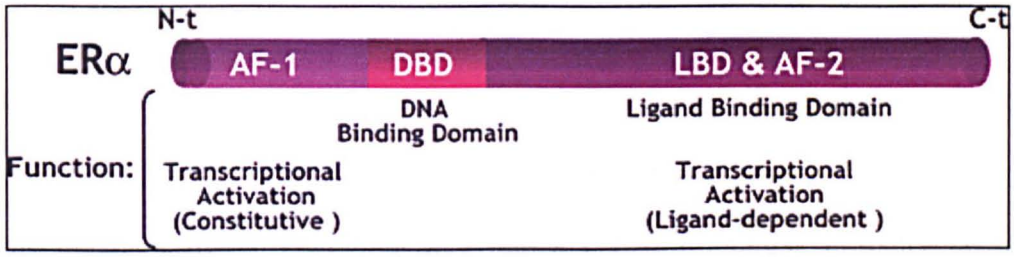


**Figure 1.10**: Structure of ER alpha.

(Schiff et al., 2005)

### 1.1.5.2 Function

**(A) Genomic function**

ER is the key member in the aetiology, pathogenesis and progression of breast cancer. Many genetic and histological studies have confirmed this role. ER is a nuclear protein and shares a common structural and functional organization with all other nuclear receptors. It acts as a ligand-dependent transcription factor and regulates the expression of a variety of genes. Many of these genes promote breast cancer proliferation like the insulin-like growth factor 1 receptor (IGFR) and the cell cycle regulator cyclin D1(Nemere et al., 2003). Others are proteins involved in tumour progression including factors involved in tumour invasiveness and metastasis or in the activation of tumour stromal components such as the angiogenic vascular endothelial growth factor (Klinge, 2001).

**(B) Nongenomic function**

Oestrogen and other steroid hormones, in addition to their role as direct modulators of gene transcription mediated by their classic nuclear receptors, can also perform rapid stimulatory effects on a variety of signal transduction pathways (Nemere et al., 2003). The significance of nongenomic ER activity in mediating oestrogen signalling to promote cell proliferation and survival in breast cancer cells has also been documented. A large number of studies using biochemical, immunohistochemical and genetic methods have further proved the existence and function of ER in breast tumours cells (Levin, 2002).

Several mechanisms by which ER interacts with components of signalling complexes and triggers their responses have been proposed. ER, in response to oestrogen, can directly and indirectly interact with several growth factors and tyrosine kinase receptors such as HER2 and IGFR and thereby activate their kinase/phosphorylation cascades. ER also directly associates with key signal transduction adaptors and kinases. Kinase cascade signalling induced by nongenomic ER activity can phosphorylate and activate various components of the ER pathway as well as other components of the transcriptional machinery such as ERK and PI3 kinase (Kelly and Levin, 2001), resulting in potentiation of nuclear ER transcriptional function (Sun et al., 2001).

## 1.2 AIM OF WORK

This thesis has several aims:

1) Identification of putative biomarkers for better characterisation of the ER-positive luminal-like subclass using gene expression analysis, novel bioinformatic approaches, conventional statistical methods and a literature search which could be used in prognosis and phenotypic characterisation of ER-positive luminal-like breast cancer.

2) Investigation of selected ER related genes on the basis of their biological function and their potential ability to distinguish different prognostic subclasses within the luminal-like group.

3) Validation of selected biomarkers using high throughput proteomic tissue microarrays (TMAs) and immunohistochemistry applied to a well characterised clinical patient series with long term follow-up.

4) Identification of ER-positive subgroups using a consensus of clustering algorithms applied to protein expression data of selected biomarkers.

# 2 General Material and Methods

## 2.1 Material and Methods

### 2.1.1 Study group

The breast cancer samples for this study derived from a consecutive series of 1,942 cases of primary operable invasive breast carcinoma obtained from the Nottingham Tenovus Primary Breast Carcinoma Series **(Table 2.1)**.

In previous studies, these patients have been immunohistochemically characterized using a range of markers of tumour-biological interest; the data has been correlated with survival outcome and prognosis. Previously, tissues from approximately 1942 paraffin processed breast tumours were used to prepare tissue microarrays (TMA) comprising wax blocks containing 150 cores of tissue (Abd El-Rehim et al., 2005). Briefly, formalin fixed paraffin embedded (FFPE) TMAs were prepared from the cases of primary operable (stage I and II) breast carcinoma of patients aged <70 years with tumours of less than 5 cm in diameter presented consecutively to the Nottingham Breast Unit between 1986 to 1998. This well-characterized resource contains patients' clinical and pathological data including patients' age, histologic tumour type (Ellis et al., 1992), primary tumour size, lymph node status, mitotic count and histologic grade, vascular invasion (VI) (Pinder et al., 1994), Nottingham Prognostic Index (Galea et al., 1992), development of recurrence, and distant metastases (DM). Survival data including survival time and disease-free interval (DFI) were maintained on a prospective basis. Breast cancer specific survival (BCSS) was defined as the time (in months) from the date of the primary surgical treatment to the time of death from (or with active) breast

cancer. DFI was defined as the interval (in months) from the date of the primary surgical treatment to the first locoregional or distant metastasis (DMFI).

The available data, slides and blocks were used to:

1- Identification of the oestrogen receptor positive cases in the whole series. Correlations and statistical analysis of the data available on the previously studied biomarkers.

2- Construction of a new TMAs series (n=1,902). TMAs allow large populations of patients' tumours to be rapidly screened to detect overall protein expression in large patient groups, thereby overcoming the weakness of IHC results when using smaller cohorts.

Patients' characteristics of Nottingham primary invasive breast carcinoma series are summarised in **(Table 2.1)**.

**Table 2.1:** Patient characteristics of Nottingham invasive breast carcinoma series

| Variable | Number | Cases (%) |
|---|---|---|
| **Tumour type** | 1961 | |
| No Special Type | | 1089 (56) |
| Lobular | | 219 (11) |
| Mixed NST and special type | | 443 (22) |
| Other | | 210 (11) |
| **Tumour grade** | 1940 | |
| 1 | | 367 (19) |
| 2 | | 648 (33) |
| 3 | | 925 (48) |
| **Tumour size (cm)** | 1943 | |
| <2 | | 1033 (53) |
| 2-5 | | 864 (45) |
| >5 | | 46 (2) |
| **Nodal status** | 1938 | |
| Negative | | 1233 (64) |
| Positive (1-3 nodes) | | 549 (28) |
| Positive (>3 nodes) | | 156 (8) |
| **Nottingham prognostic index** | 1934 | |
| Good prognosis group | | 618 (32) |
| Moderate prognosis group | | 994 (51) |
| Poor prognosis group | | 322 (17) |
| **Oestrogen receptor status** | 1812 | |
| Positive | | 1268 (70) |
| Negative | | 544 (30) |

#### 2.1.1.1 The Nottingham histologic grading system

The Nottingham combined histological grading system (Elston-Ellis modification of Scarff-Bloom-Richardson grading system) is the commonly used system in grading of breast cancer (Elston and Ellis, 1991). The parameters measured are the extent of tubular formation; the extent of nuclear pleomorphism, and mitotic rate. Each of the three elements is assigned a score on a scale of 1 to 3, and the final grade is determined from the sum of the scores. Histological grade is traditionally expressed in three categories: score 3

to 5, well differentiated (grade 1); scores 6 to 7, intermediate (grade 2); and scores 8 to 9, poorly differentiated (grade 3).

#### 2.1.1.2 Nottingham prognostic index (NPI)

NPI is widely used in the UK and was developed for the prognostic management of breast cancer by using multivariate analysis to determine the most important prognostic factors. It includes 3 factors; tumour histologic grade (1-3 using the Nottingham Grading System), lymph node (LN) stage (1-3; 1=LN negative, 2=1-3 positive nodes and 3= more than 3 positive nodes) and primary tumour size (0.2x size in cm). NPI is then categorized into 3 groups: the good prognostic group (score ≤3.4), the moderate group (>3.4 - 5.4) and the poor prognostic group (score > 5.4).

### 2.1.2 Patient management

Patient management was based on the Nottingham Prognostic Index (NPI) score and ER status. The treatment protocol during this time was based on a previous publication (Bianco et al., 1988). Patients within the good prognostic NPI group (≤3.4) did not receive adjuvant systemic therapy. Hormonal therapy (Tamoxifen ± Zoladex if premenopausal) was given to patients with ER-positive tumours and NPI scores of >3.4. Pre-menopausal patients with moderate and poor prognostic NPI groups were given chemotherapy (Cyclophosphamide, Methotrexate, and 5-Flourouracil). ER-positive postmenopausal patients with moderate or poor NPI were offered hormonal therapy, while ER negative patients received CMF if fit to receive these

cytotoxic agents with no concurrent diseases that were considered as potential contraindication to the use of chemotherapy.

### 2.1.3 Gene expression studies

The study population used was derived from the Nottingham Tenovus Primary Breast Carcinoma Series of women aged 70 years or less, who presented with stage I and II primary operable invasive breast carcinomas.

Total RNA was extracted from a total of 128 frozen breast cancers retrieved from Nottingham Hospitals NHS Trust Tumour Bank between 1986 and 1992 in collaboration with Cambridge University (Cambridge dataset). RNA integrity and DNA contamination were analysed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Total RNA was biotin-labelled using the Illumina TotalPrep RNA Amplification kit (Ambion, Austin, TX, USA) according to manufacturer's instructions. Biotin-labelled cRNA (1.5 $\mu$g) was used for each hybridisation on Sentrix Human-6 BeadChips (Illumina, San Diego, CA, USA) in accordance with the manufacturer's protocol. Illumina gene expression data containing 47,293 transcripts were analysed and summarised in the Illumina Bead Studio software. Analyses of the probe level data were done using the beadarray Bioconductor package. The expression data are available at the EBI website (http://www.ebi.ac.uk/miamexpress/) with the accession number E-TABM-576. Transcript expression profiling has been previously described (Chin et al., 2007, Naderi et al., 2006).

#### 2.1.3.1 Histopathological characterisation of the Cambridge dataset

Seventy five percent of patients were postmenopausal, 68.8% were LN stage 1 (no lymph node involvement) and 40.6% were tumour grade 2. During the follow-up period 24.2% developed metastatic disease and 34.4% developed tumour recurrence. Forty four percent of tumours were ductal with no special types. Sixty five percent of patients were positive for oestrogen receptor alpha expression **(Table 2.2)**.

**Table 2.2:** Patient characteristics of Cambridge dataset

| Variable | Frequency | Percentage |
|---|---|---|
| **Menopause** | | |
| Premenopausal | 94 | 75.2 |
| Postmenopausal | 31 | 24.8 |
| **Tumour grade** | | |
| 1 | 33 | 25.8 |
| 2 | 52 | 40.6 |
| 3 | 43 | 33.6 |
| **Lymph node stage** | | |
| 1 | 88 | 68.8 |
| 2 | 28 | 21.9 |
| 3 | 12 | 9.4 |
| **Death** | | |
| Alive | 81 | 63.3 |
| Due to breast cancer | 33 | 23.8 |
| Due to other causes | 14 | 11 |
| **Distant metastasis** | | |
| No | 97 | 75.8 |
| Yes | 31 | 24.2 |
| **Tumour recurrence** | | |
| No | 84 | 65.6 |
| Yes | 44 | 34.4 |
| **Tumour type** | | |
| Ductal NOS | 57 | 44.5 |
| Others | 71 | 55.5 |
| **ER status** | | |
| Positive | 84 | 65.6 |
| Negative | 44 | 34.3 |

### 2.1.4 Gene selection for protein expression studies

One of the aims of this study was to identify a set of specific genes whose expression best identify prognostic subgroups of luminal ER positive tumours. Candidate genes were selected from three sources including:

#### 2.1.4.1 Literature search

A literature search was performed for genes with strong relevance in ER-positive breast cancer, or has been the subject of recently published studies and strongly suggests an important role in the biology and molecular classification of ER-positive breast cancer. The selection criteria was based on the published literature concentrating mainly on ER related pathways such as ER coregulators, cellular proliferation, apoptosis, Akt/PIK3 pathway and endocrine resistance.

#### 2.1.4.2 Bioinformatical analysis of the gene microarray data

**(A)Ensemble classification and cross-validation analysis**

A cross-validation analysis was used in combination with an ensemble sample classification in order to obtain a robust ranking of genes that are differentially expressed between the luminal ER-positive (n=84) cases and the non-luminal cases (all other cases) (n=44) in Cambridge gene microarray data. For this purpose, the 128 patient samples were first partitioned randomly into 10 sub-groups of approximately equal size. For each possible combination of 9 sub-groups differentially expressed genes were selected independently with the "Empirical Bayes moderated t-statistic" (Smyth, 2004) and used to train a machine learning model, while the remaining sub-group was left out as the test set (a procedure known as "external cross-validation"). To classify the samples in these remaining test set groups, the prediction results of four algorithms (Support Vector Machine, Random Forest, kNN and Prediction Analysis for Microarrays) (Tibshirani et al., 2002) were combined to a majority-vote

ensemble classifier. In order to rank the genes based on the cross-validation results, their frequency of occurrence in the list of significantly differentially expressed genes (p-value < 0.05) across different cross-validation cycles was recorded, and genes received higher scores the more often they had been selected (expressed as a z-score significance measure). RERG and GATA3 belonged to the top-ranked genes which were selected in each of the 10 cross-validation cycles. Subsequently they were included in the study.

**(B) Artificial neural networks (ANN) analysis**

ANNs are a form of artificial intelligence inspired by learning in human neuronal systems and have been shown to be capable of modelling complex systems with high predictive accuracies on several large scale datasets (Ball et al., 2002).

We have used the immunohistochemically identified ER-positive cases to develop an ANN model to identify novel genes associated with ER-positive status. Our aim was to identify; using a novel prediction method (ANN), a set of genes that show significant association with ER expression (high expression vs. low expression) and to validate the genes using protein expression. To study this, the ER-positive cases (84 tumours) were categorized according to the level of ER expression into high and low expression using the median of the H-score values (H-score 140). RERG gene was found to be associated with the high ER expression and was included in the study.

The ANN model used a supervised learning approach with multi-layer perceptron architecture and a sigmoidal transfer function, where weights were updated by a back propagation algorithm as previously described (Lancashire

et al., 2010). Data consisted of 84 samples each with 47,293 corresponding variables specifying the Log10 expression ratio of each transcript.

Prior to ANN training, the data were randomly divided into three subsets: 60% for training, 20% for testing (to assess model performance during the training process) and 20% for validation (to independently test the model on data completely blind to the model). This process of random sample cross-validation enabled the generation of confidence intervals for the predictions on a separate blind dataset, and therefore avoided over-fitting of the data. The intensity of each gene was used as an individual input in an ANN model, creating n individual models, where n was the number of transcripts on the array (47,293). These n models were then split into three subsets (described above) and trained. This random resampling and training process was repeated 50 times to generate predictions and associated error values for each sample with respect to the validation (blind) data. Inputs were ranked in ascending order based on predictive error, and the gene that performed with the lowest error was selected for further training. Next, each of the remaining genes was sequentially added to the previous best gene, and was used in combination in a model, creating $n-1$ models each containing two genes as inputs. Training was repeated and performance evaluated. The model with the highest modelling performance was again selected and the process repeated creating $n-2$ models each containing three inputs. This process was repeated until no significant gain was evident from the addition of further inputs. This resulted in a final model containing those transcripts that most accurately classified the patients according to ER status.

Initially we had gene expression data on 112 cases, and we firstly used the definition of luminal cancers [ER (+), HER2 (-), CK5/6(-) and CK14 (-)] in another ANN model to identify the cases with this luminal immunophenotype. Five cases were omitted from the analysis due to missing data. Fifty luminal cases were identified using this definition. The luminal versus non-luminal data was used to divide the gene expression data of the cases into two groups, luminal and non-luminal cases to identify genes that can characterise this luminal phenotype. These data has been bioinfomatically analysed using the ANN analysis in collaboration with Dr Graham Ball from Nottingham Trent University. AGTR1 was found to be significantly associated with this luminal phenotype and was selected for further study.

**(C)Identification of genes with variable expression within the ER-positive cases**

The gene microarray data were analysed to identify the genes with greatest intensity variation in the ER-positive cohort in Cambridge dataset by calculating the mean of the normalised expression values and their standard deviations (500 genes) and these genes were used in genes' selection. BEX1 and TFF3 were selected using this approach **(Table 2.3).**

#### 2.1.4.1 Collaboration with the Tenovus group

Gene lists were provided from our Tenovus group collaborators, University of Cardiff. Tenovus group has Affymetrix (HG-U133A chip) gene expression data for ER (+) MCF7 breast cell lines treated with/without ER antagonists. CD71 was chosen as a result of this collaboration because of its association

with ER-positive endocrine resistant cell lines. The additional methods that have been included in CD71 study including cell culture and growth studies will be discussed in the relevant chapter.

All together, 16 genes from these different sources were selected and grouped in four major groups for discussion purposes. The other remaining biomarkers including (ER, PgR, HER2, MIB1, p53, EGFR, C-MYC, CK18, and CK5/6) have been studied previously with the breast cancer research group. The data from these additional markers was used for statistical analysis and correlation studies with the other selected biomarkers and in the clustering algorithms. The genes selected from the previously mentioned methods were summarised in **(Fig 2.1)**.

**Table 2.3**: The first 40 genes that showed the highest variable expression in the ER-positive cohort in Cambridge dataset

| Gene | Mean normalised expression values | SD |
|---|---|---|
| SCGB1D2 | 9.483224206 | 2.379469 |
| LOC118430 | 7.95417379 | 2.285829 |
| SCGB2A2 | 10.01130869 | 2.043923 |
| LTF | 8.54435204 | 2.039819 |
| **TFF1** | 9.175437669 | 2.013808 |
| CPB1 | 6.971236321 | 2.011272 |
| PIP | 9.189106121 | 1.986287 |
| HLA-DQA1 | 7.159601507 | 1.810792 |
| CLECSF1 | 6.62148258 | 1.720225 |
| HLA-DQA1 | 8.082224755 | 1.708589 |
| HLA-DRB3 | 7.876714799 | 1.705458 |
| S100P | 7.484085096 | 1.675328 |
| CLIC6 | 7.756489436 | 1.630974 |
| HLA-DRB5 | 8.328738367 | 1.591707 |
| LOC374572 | 10.52884356 | 1.571225 |
| CYP4Z1 | 7.574942319 | 1.566103 |
| HLA-DRB1 | 6.647622139 | 1.553243 |
| **BEX1** | 6.815012397 | 1.522082 |
| hmm26383 | 8.237634375 | 1.492765 |
| B7-H4 | 8.062896149 | 1.49053 |
| CALML5 | 6.708645698 | 1.488676 |
| FABP4 | 8.811597417 | 1.486944 |
| IGJ | 8.915041805 | 1.470825 |
| NAT1 | 8.793049646 | 1.459964 |
| SCGB2A1 | 7.451528134 | 1.430942 |
| **TFF3** | 7.699783357 | 1.401232 |
| LOC388978 | 8.372983703 | 1.389522 |
| TCN1 | 6.749026392 | 1.376035 |
| Hs.183902 | 8.609720389 | 1.371672 |
| ALDH3B2 | 8.303825863 | 1.369684 |
| hmm28274 | 8.606773066 | 1.357618 |
| DHRS2 | 6.952377114 | 1.354725 |
| MUC1 | 8.773726712 | 1.339532 |
| hmm28273 | 9.063794915 | 1.33537 |
| EEF1A2 | 7.642618826 | 1.326032 |
| HLA-DRB4 | 8.993471537 | 1.324762 |
| CEACAM6 | 7.007334103 | 1.319888 |
| COL11A1 | 8.599856281 | 1.305509 |
| DCD | 6.179133363 | 1.30549 |
| APOD | 9.369169441 | 1.299178 |

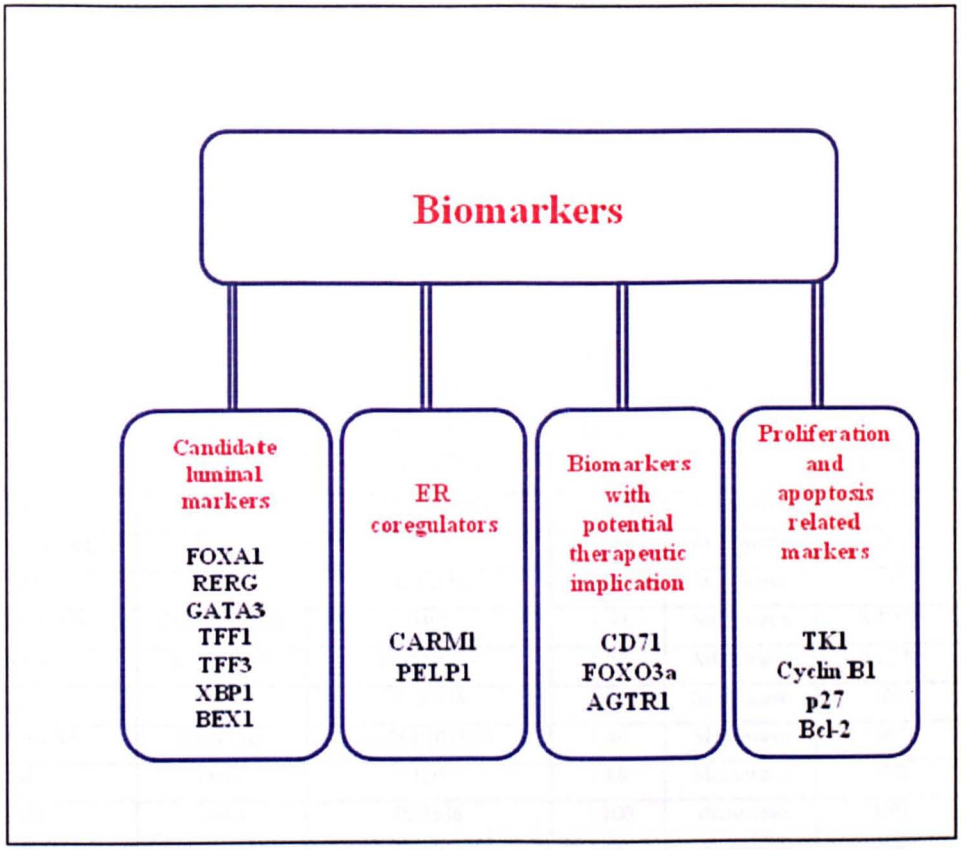


**Figure 2.1:** Biomarkers selected for inclusion in the study

The genes grouped according to phenotypic associations, potential therapeutic implications or their related biological pathways

For the other markers used in the study, the cutoffs were chosen according to the previously published studies of the breast cancer research group (Abd El-Rehim et al., 2005, Rakha et al., 2009). The sources, dilutions, pretreatment of the antibodies used are summarised in (**Table 2.4).**

**Table 2.4:** List of antibodies used in the study

| Antibody | supplier | Cat number/clone | Dilution | Pretreatment | Cut-off |
|---|---|---|---|---|---|
| **FOXA1** | Abcam | Ab40868/2F83 | 1:2000 | Microwave | 10* |
| **TFF1** | Abcam | Ab17829 | 1:2000 | Microwave | 100* |
| **CD71** | Abcam | Ab49517/10F11 | 1:30 | Microwave | 5* |
| **PELP1** | Novus | NB100-1749 | 1:100 | No | 5,170* |
| **CARM1** | Novus | NB100-1817 | 1:300 | Microwave | 30,150* |
| **Bcl-2** | Dako | M0887/124 | 1:100 | Microwave | 10% |
| **BEX1** | Abcam | Ab69032 | 1:3500 | Microwave | 100* |
| **TK1** | Abcam | Ab57757 | 5µg/ml | Microwave | 8% |
| **AGTR1** | Abcam | Ab9391 (1E10-1A9) | 1:100 | No | 30,100* |
| **XBP1** | Novus | NB100-80861 | 0.5µg/ml | Microwave | 0,1,2,3** |
| **Cyclin B1** | Abcam | Ab72 | 0.3µg/ml | Microwave | 0% |
| **TFF3** | Abcam | Ab57752 | 3 µg/ml | Microwave | 90* |
| **FOXO3a** | Cell Signalling | 9467 | 1:50 | Microwave | N/C*** |
| **RERG** | Proteintech | 10687-1-AP | 1:20 | Microwave | 0,1,2** |
| **p27** | Dako | SX53G8 | 1:40 | Microwave | 10% |
| **GATA3** | Santa Cruz | sc-268/HG3-31 | 1:80 | Microwave | 60* |
| **ER** | Dako | 1D5 | 1:80 | Microwave | 10% |
| **PgR** | Dako | PgR636 | 1:100 | Microwave | 10% |
| **AR** | Biogenex | F39.4.1 | 1:30 | Microwave | 10% |
| **E-cadherin** | Zymed | HECD-1 | 1:100 | Microwave | 100 |
| **P-cadherin** | BD | 56 | 1:200 | Microwave | 5% |
| **C-Myc** | Abcam | Ab32/9A10 | 1:100 | Microwave | 0,1,2,3** |
| **p53** | Novocastra | DO7 | 1:50 | Microwave | 10% |
| **Ki67** | Dako | MIB1 | 1:100 | Microwave | 10% |
| **EGFR** | Novocastra | EGFR.113 | 1:10 | Microwave | 10% |
| **HER2** | Dako | cerbB-2 | 1:250 | No | 0,1,2,3**** |
| **BRCA1** | Oncogene Res | MS110 | 1:150 | Microwave | 5% |
| **PIK3CA** | Sigma | HPA009985 | 1:50 | Microwave | 100* |
| **CK14** | Novocastra | LL002 | 1:100 | Microwave | 10% |
| **CK5/6** | Boehringer | D5/16134 | 1:100 | Microwave | 10% |
| **CK7/8** | BD | CAM5.2 | 1:2 | Microwave | 50* |
| **CK18** | Dako | DC10 | 1:50 | Microwave | 50* |
| **CK19** | Dako | DCK 108 | 1:100 | Microwave | 50* |

*Hscore

**Cytoplasmic intensity

***N/C nuclear and cytoplasmic localisation

****Hercep test guidelines (Dako, Cambridge, UK)

### 2.1.5 Tissue microarrays (TMAs) construction

To speed up the analysis of a large number of breast cancers, a high throughput TMA approach was used. 2,000 haematoxylin and eosin stained slides of breast cancer were reviewed for the construction of TMAs. 1,902 cases of paraffin processed breast tumours were used to prepare new tissue microarray blocks, each comprising 150 cores (0.6 mm) of tissue. All the available blocks were marked, in some cases more than one block per cases. Two peripheral tumour core blocks and one central tumour core block were constructed from the whole series in 13 batches (39 TMAs block in total). The new TMA set was used to study the protein expression of the selected candidate genes. The Foxa1 study was conducted using the old TMAs blocks while other studies were performed using the new TMAs. Only the versions of the peripheral cores were selected (invading edge) to avoid the use of central cores which might show areas of necrosis.

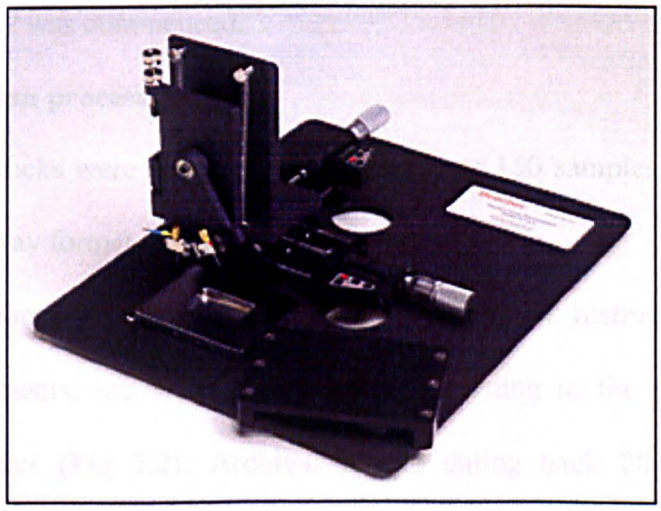

**Figure 2.2:** The manual arrayer used for TMA construction

**Construction**

**1) Preparation of samples to be arrayed**

A representative FFPE block from each patient's tumour sample (donor) was retrieved from the archives, selected for the presence of tumour and adequate thickness ideally 3-4mm. To ensure the presence of invasive tumour tissue a three micron section was cut from each block and stained with haematoxylin and eosin (by Dr Claire Paish) (n=1902). If the block contained tumour, it was then marked on the slide for representative area of the tumour suitable for array sampling. In cases the tumour block did not contain sufficient tumour, we retrieved alternative archive blocks for the patient and repeated the process to ensure that as many patients' samples as possible represented on the new TMAs of Nottingham series.

**2) Preparation of the recipient array block**

5-10 mm deep moulds were used to generate recipient blocks. Once all donor blocks were marked, and the recipient block had been prepared, construction of the tissue array was commenced.

**3) Construction process**

1- The blocks were designed to accommodate 150 samples, using a 3x50 sub-array format.

2- The construction was done using a precision instrument (Beecher Instruments, Inc. San Prairie, USA) according to the manufacturer's datasheet **(Fig 2.2)**. Archival blocks dating back 20–40 years are usually adequate for the construction if they have been fixed in 4% buffered formalin (Kallioniemi et al., 2001).

3- The array construction involved making a hole in the recipient TMA block, acquiring a cylindrical core sample from the donor tissue block and depositing this core into the TMA block.

4- Core tissue biopsies (diameter 0.6 mm; height 3-4 mm) were taken from hundreds of different donor paraffin-embedded tumour blocks and precisely arrayed into a new recipient paraffin block (45 × 20 mm) using the custom-made precision instrument. Samples spaced 1.25 apart and the 50 sub-array spaced 5 mm apart.

5- Kidney cores were used for block orientation.

**3) Preparing the array block**

The surface was smoothed and levelled by incubating the array block in 37°C for 10-15 minutes. This warms the paraffin wax thereby promoting adherence of the tissue cores to the walls of the holes in the array block and makes the wax flexible to handle. After the block has warmed, a clean glass microscope slide was used to apply even pressure on the top of the array block and to push all tissue cores level with the top surface of the array. Using a microtome, 4-5 μm sections were cut from the TMA blocks to generate TMA slides for molecular analyses.

### 2.1.6 Validation of antibodies

We have selected our antibody panel according to the availability of commercially validated antibodies. For confirmation we have applied further validation steps on selected antibodies using WB and peptide blocking.

### 2.1.6.1 Western blotting (WB)

WB was performed on breast cancer cell lysates of the human breast cancer cell line MCF-7 to confirm the specificity of the FOXO3a antibody used in immunohistochemistry.

**Method**

*The cell culture experiment was conducted with my colleague Mohamed Ahmed including the western blot experiment using the MCF7 cell lysate.*

MCF-7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI 1640 medium in T75 flasks supplemented with 10% foetal calf serum (FCS), penicillin (100 IU/ml) and streptomycin (100 µg/ ml). The sub-confluent cells were washed with PBS, then 30µl of protease inhibitors (Sigma Aldrich) were added to 470µl of ice-cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS). Western blotting was done on the cell lysates to confirm the specificity of the antibody used in immunohistochemistry. Lysates (20µg) were added to 4X SDS loading buffer with 5% β-mercaptoethanol (Sigma Aldrich, UK) and denatured by heating at 100°C for 10 minutes prior to loading then added for 5 minutes into ice. Samples were subjected to Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) using a 10% resolving polyacrylamide gel and transferred onto a Hybond-P PVDF membrane (Amersham Bioscience, Buckinghamshire, UK). After blocking with 5% milk powder 0.1% TPBS (Tween20 in PBS solution) for 60 minutes, the membrane was then incubated

with 1:1000 dilution of the FOXO3a rabbit polyclonal Antibody (9467) at 4°C overnight. The membrane was washed with 0.1% PBS/Tween20 3 times for 5 minutes each then incubated for 1 hour at room temperature with a horseradish peroxidase-linked secondary antibody (Sigma Aldrich) (1: 4000, anti-rabbit) diluted with 5% milk powder PBS containing 0.1% Tween20. After further 3 washes, the membrane was visualized by enhanced chemiluminescence reagents (Amersham Bioscience, Buckinghamshire, UK). The monoclonal Anti-β-actin antibody (Sigma Aldrich) in a dilution of 1:2000 against the ubiquitous β-actin protein was used.

#### 2.1.6.2 Peptide blocking

It is recommended procedure for confirming the specific reactivity of an antibody. In this protocol, the antibody is incubated with the immunizing peptide that has been used to raise the antibody. The antibody that is bound to the blocking peptide will be no longer available to bind to the peptide antigen in the cell. In comparing the staining from the blocked antibody versus the antibody alone, specific staining should be absent or significantly reduced from the immunostaining performed with the neutralized antibody.

This protocol can be used to prepare "blocked" antibody for use in either western blotting or immunohistochemistry.

After determination of the optimal concentration of antibody that consistently gives a positive result. Using that concentration, we determined how much antibody needed for two experiments. In the first tube, labelled Blocked, the blocking peptide was added to a final concentration of 10 times of the

antibody. In the second tube, labelled Control, an equivalent amount of diluent was added. Both tubes were incubated, with agitation, at room temperature for 30 minutes. The tubes were centrifuged for 15 min at 4°c, and then the staining protocol on the two identical samples, using the blocked antibody for one and the control for the other was performed and the staining was observed. PELP1 antibody was evaluated using this method.

#### 2.1.6.3 Controls

To ensure the correct tissue preparation and staining of the used antibodies, tumours and tissues with known staining patterns were used as positive immunostaining controls and were processed by the same method used to stain the tumour sections and TMAs. Negative controls were obtained by omitting the primary antibodies and were used to evaluate non-specific binding of the secondary antibody to the tissues and to ensure specific detection of the antigen by the primary antibody.

### 2.1.7 Immunohistochemistry and optimization of the antibodies

To determine the optimal staining conditions for each antibody used, full sections and TMAs were used for staining using different antibody concentrations and antigen retrieval methods with different pH and incubation times. Data sheets with each antibody suggested a dilution range for optimisation experiments. If the staining using the suggested dilution was found too intense or weak, further dilutions were used in subsequent experiments to achieve the optimal dilution.

Immunohistochemical staining of the sections was performed using either a DAKO TechMate immunostainer to ensure the consistency between various immunohistochemistry runs or the manual method if the staining requires an overnight incubation with the primary antibody. A set of full face sections for each of the selected biomarkers was stained to assess the staining distribution and to assess its suitability for TMAs.

#### 2.1.7.1 Automatic immunostainer

An indirect labelled streptavidin avidin biotin technique (LSAB©) technique with Diaminobenzidine (DAB) chromogen was performed using a DakoCytomation Techmate 500 Plus (DakoCytomation, Cambridge, UK) automatic immunostainer. IHC was performed on sections of formalin-fixed paraffin embedded tissue (4μm). Prior to staining, the sections were melted on a hotplate for 10 minutes (60°C), dewaxed in two changes of xylene for 10 minutes each, rehydrated in 3 changes of alcohol for 1 minute each. Sections were pre-treated with microwave antigen retrieval (when needed) using 0.01M citrate buffer or EDTA accordingly for 23 minutes at 700w. Then, sections were transferred to the immunostainer and the staining was carried out using Dako LSAB© kit (DakoCytomation, Cambridge, UK). Slides were incubated in buffer 1 (ChemMate) which contains goat serum for 20 minutes and in $H_2O_2$ for 10 minutes to block the endogenous peroxidase. Sections were incubated in the primary antibody for 1 hour followed by incubation in the biotinylated secondary antibody for 30 minutes and HRP horseradish peroxidase streptavidin for 30 minutes. Then, the slides were incubated in DAB for 10

minutes. Slides were washed by buffer 2 and buffer 3 included in the kit between the steps. The sections were counterstained in haematoxylin for 2 minutes, rinsed in tap water, dehydrated in alcohol, cleared in xylene and mounted by DPX.

#### 2.1.7.2 Manual immunostaining

Manual staining methods was used for the RERG study according to the optimisation process; different antibody concentrations and incubation times were tested. Overnight incubation deemed the most optimal method. After microwave antigen retrieval in citrate buffer pH 6, the TMAs sections and control sections were put in a humidity chamber followed by blocking of endogenous peroxidase by applying hydrogen peroxide in methanol for 10 min. The TMA slides were then incubated in primary antibody at 4°C overnight. The immunohistochemical detection of RERG was carried out using a labelled steptavidin biotin technique LSAB® in accordance with the manufacturer's instructions (DakoCytomation, Cambridge, UK). The slides were incubated with the biotinylated secondary antibody for 30 min, followed by HRP-Streptavidin for another 30 min. Tris buffer saline (TBS) were used for washing between steps (3x2 minutes with stirrer). For visualisation of the reaction, the slides were incubated in freshly prepared peroxidase substrate solution (DAB) diluted 1:50 for 10 min.

After application of DAB, the slides were washed in running tap water, counterstained in haematoxylin, dehydrated in alcohols, cleared in xylene and coverslipped using DPX mounting medium. Negative controls were performed

by omitting the primary antibody while positive control BC sections were used in each run.

### 2.1.8 Assessment of protein expression using immunohistochemistry

Sections were examined by light microscope. Positive and negative controls were examined to confirm the appropriate staining. Only the invasive tumour component was evaluated and scored for the intensity and percentage of positive cells accordingly. The distribution of staining was assessed both in whole sections of malignant breast carcinoma and in tissue microarray sections. As the distribution of staining was homogenous in the full section, only one tumour core was stained from each tumour, as previous studies have validated the use of one core to study the expression of tumour markers even for those that have a heterogeneous distribution (Camp et al., 2000). GATA3 and XBP1 were scored using high resolution digital images (NanoZoomer; Hamamatsu Photonics, Welwyn Garden City, UK), at x20 magnification, using a web-based interface (Distiller; Slidepath Ltd, Dublin, Ireland).

H-score (histochemical score) scoring system has been successfully used for TMAs evaluation (Abd El-Rehim et al., 2005). The H-score includes an assessment of both the intensity of staining and the percentage of stained cells. For the intensity, a score of 0, 1, 2 and 3 was used and the percentage of positive cells at each intensity was subjectively estimated. The final score is in the range of 0–300 derived from of multiplying the intensity by the percentage.

### 2.1.9 Statistical analysis

Statistical analysis was performed using SPSS statistical software (SPSS Inc., Chicago, IL, USA). Association between the immunoreactivity and different clinicopathological parameters was evaluated either by Fisher's exact test or chi-squared test. For multiple testing of biomarkers and clinicopathological associations, a conservative p-value of <0.01 was considered to reflect a significance and all were 2-sided. Survival curves were calculated by the Kaplan-Meier method with log rank test to assess significance. Multivariate Cox regression analysis was used to evaluate any independent prognostic effect of the variables on patients' survival with 95% confidence interval and p value of <0.05 was considered.

All factors were used as dichotomous covariates in the statistical analysis with the exception of age, tumour grade, tumour types, lymph node stage, vascular invasion and NPI which were analysed as more than 2 groups.

#### 2.1.9.1 Categorisation of continuous data

1- We used the median of the continuous data when it is abnormally distributed and the mean if the data is normally distributed.

2- X-tile bioinformatic tool. (Camp et al., 2004) developed the graphical method, X-tile plot, to demonstrate the presence of meaningful tumour subpopulations and show the robustness of the relationship between a biomarker and outcome by construction of a two dimensional projection of every possible subpopulation. For further validation of the chosen cut-off point, the X-tile program randomly divides the total patient cohort into two separate

training and validation sets ranked by patient follow up time. Statistical significance is tested by validating the obtained cut points to the validation set (Camp et al., 2004). The approach used was similar to that used by others within the group for existing biomarkers. Where the cut-off was not known, it was determined using the more recently published x-tile technique and compared with the median H-score. The median cut-off value was obtained using the frequency statistics according to the one that produced distinct categorisation and significance with measures of clinical outcome. In the early phases of this research project, we used the median value for stratification of patients' biomarker results with regard to relationships with prognostic and patient outcome variables. This method has been standard practice in the literature. During the project, x-tile software became available which provides a more sophisticated approach to determination of clinically relevant biomarker cut points and was therefore adopted for all subsequent studies. Retrospective use of x-tile was not appropriate as these early studies had been already published using median value stratification.

3-Frequancy distribution histograms were initially used for visualisation of the distribution and for discovery of obvious cutoff points. The histogram is a descriptive figure of frequencies, displayed as adjacent rectangles. Each rectangle is elevated over a certain interval, with an area equal to the frequency of the observations in the interval.

### 2.1.10 Ethical approval

This study was approved by the Nottingham Research Ethics Committee 2 under the title "Development of a molecular genetics classification of breast cancer".

# 3 Study of selected candidate luminal markers and their role in breast cancer prognosis with emphasis on ER-positive luminal-like subtype

## 3.1 Introduction

The recently developed microarray technologies have created new possibilities to identify gene expression profiles and have provided a better view of the involved biological processes. As previously discussed, Perou and colleagues established the first molecular portraits of breast tumours by using DNA microarrays, which allow mRNA expression levels of thousands of genes to be measured in a single assay (Perou et al., 2000). They distinguished two main classes of tumours, one with the characteristics of basal (and/or myoepithelial) cells, the other of luminal cells. The basal tumours expressed CK5 and CK17 mRNAs, while the luminal phenotype was based on the expression of CK8/18, and the oestrogen receptor (ER). Subsequent analyses refined this two-class model and several subclasses of luminal-like (Luminal A, Luminal B and C) tumours were further characterized (Sorlie et al., 2001) then the concept of luminal C became less evident with most of the authors suggesting that luminal breast cancer is better divided into A and B groups (van't Veer et al., 2002, Sorlie et al., 2003)

Luminal A tumours show high expression of oestrogen-regulated and associated genes especially XBP1, GATA3 and FOXA1 while, Luminal B tumours, although still ER positive, expressed lower levels of the genes associated with the ER cluster and also expressed some genes that had previously clustered with some of the HER2 overexpressing and basal tumours. Luminal B tumours in contrast to the Luminal A ER-positive subtype,

produced transcripts encoding the myeloblastosis viral oncogene homologue MYB, gamma-glutamyl hydrolase (GGH), and other enzymes involved in cell signalling and sterol biosynthesis that differentiate them from the better survival group.

In summary, these gene expression array experiments suggest that the luminal-like class of breast cancer is characterised by ER positivity but is heterogeneous with respect to the expression of other genes. While it is recognised that the major luminal-like subclasses differ in terms of prognosis, it can be inferred from the subsequent studies that other genes are responsible for the precise positioning of an individual within the spectrum of luminal-like breast cancer (Sorlie et al., 2003). Importantly, characterisation of luminal-like cancer varies between studies and a consensus of definition is lacking. It is widely recognised that there is a need for identification of new biomarkers that can be used to characterise the ER-positive luminal cancer.

In this chapter we discussed the expression of some candidate luminal genes by IHC and TMAs based method using a large series of patient with long term follow-up.

The selection of biomarkers was based on our bioinformatic analysis of the gene microarray data using the conventional statistical cross validation analysis and ANNs that identified RERG, GATA3, BEX1 and TFF3. TFF1, FOXA1 and XBP1 were selected for further studies due to their potential role in subclassification of breast cancer and as potential makers of the luminal subclass.

## 3.2 FOXA1

### 3.2.1 Introduction

The forkhead-box A1 (FOXA1) gene is a member of the fox family of transcription factors, which is expressed in the breast, liver, pancreas, bladder, prostate, colon and lung and can bind to the promoters of more than 100 genes associated with metabolic processes including regulation of cell signalling and the cell cycle (Lin et al., 2002). It is involved in the pathogenesis of many cancers including lung, oesophageal and prostate cancer (Wolf et al., 2007). In breast cancer however, the role of FOXA1 appears more controversial. Previous studies have shown that FOXA1 can act either as a growth stimulator or repressor. As a stimulator, it functions as a pioneer factor that binds to chromatinized DNA, opens the chromatin and enhances binding of oestrogen receptor-alpha (ERα) to its target genes (Laganiere et al., 2005). Down-regulation of FOXA1 by RNA interference significantly suppressed proliferation of HER2-negative and FOXA1-positive breast cancer cell lines (Yamaguchi et al., 2008). Emphasising its importance, FOXA1 is required for the expression of 50% of ER-regulated genes (Carroll and Brown, 2006, Holmqvist et al., 2005, Laganiere et al., 2005). As a repressor, it has been shown that FOXA1 overexpression can block metastatic progression by influencing expression of the BRCA1 associated cell cycle inhibitor, p27, and promoting E-cadherin expression. This suggests that FOXA1 plays important roles in the upregulation of genes that reduce the growth and motility of breast cancer cells (Williamson et al., 2006, Liu et al., 2005).

Importantly, recent global gene expression studies of breast cancer revealed that high FOXA1 mRNA expression is often found in association with ER positivity, and frequently present in a subset of ER-positive tumours that have favourable outcome. Therefore, FOXA1 expression appears to have potential relevance in sub classification of luminal/ER-positive tumours into subgroups with different biologic behaviour and prognosis.

### 3.2.2 Material and Methods

Tissue microarrays were prepared and the immunohistochemical staining was performed using the streptavidin-biotin complex method using DakoCytomation Techmate 500 plus (DakoCytomation, Cambridge, UK) (General Material and Methods Chapter). To unmask the antigens, the sections were microwaved in citrate buffer pH 6 for 23 minutes.

Mouse monoclonal antibody to FOXA1 (clone 2F83, ab40868; Abcam, Cambridge, UK) that was raised against the recombinant full length human FOXA1 protein was optimized at a working dilution of 1:2000 using full-face sections and TMAs of breast cancer.

H-score was used for immunohistochemical staining assessment. The cutoff point was assigned by using the median of H-score values (H-score $\geq$10).

### 3.2.3 FOXA1 immunohistochemical results

After excluding the uninformative TMA cores from the study, 696 tumours were available. The median age of the patients was 54 years (range 27-70). Sixty seven percent of patients had large tumours greater than or equal to 1.5 cm in size. Twenty one percent of the tumours were grade 1 and 31% showed good NPI. Twenty eight percent of the patients had metastatic disease and 29%

had tumour recurrence. Fifty six percent of the tumours were ductal with no special type. The FOXA1 staining pattern was nuclear with no evidence of cytoplasmic and membranous staining **(Fig 3.1)**.

The expression was detected in the nuclei of the malignant cells as well as in some luminal ductal epithelial cells of the entrapped normal tissues in the cores.

#### 3.2.3.1 Correlation between FOXA1 expression and other clinicopathological variables

FOXA1 nuclear expression was associated with smaller primary tumour size, lower grade tumours, lower mitotic count ($p<0.001$) and with the good NPI group. It also showed an association with histologic tumour type with frequent expression in invasive lobular and tubular carcinomas and decreased expression in medullary carcinomas ($p<0.001$). No associations were found between FOXA1 protein expression and patients' age, lymph node stage, vascular invasion, development of recurrences or distant metastasis **(Table 3.1)**.

#### 3.2.3.2 Correlation between FOXA1 expression and other biomarkers

There were positive associations between FOXA1 expression and ERα, PgR, AR and BRCA1. In contrast, we found inverse associations between FOXA1 expression and basal CKs expression (CK5/6; $p=0.003$) and P-cadherin ($p=0.002$). No associations were found between FOXA1 and p53, HER2 or EGFR expression **(Table 3.2)**.

When the analysis was repeated only on the cohort of ER-positive (luminal-like) patients, FOXA1 expression retained its significant association with smaller tumour size **(Tables 3.3&3.4)**.

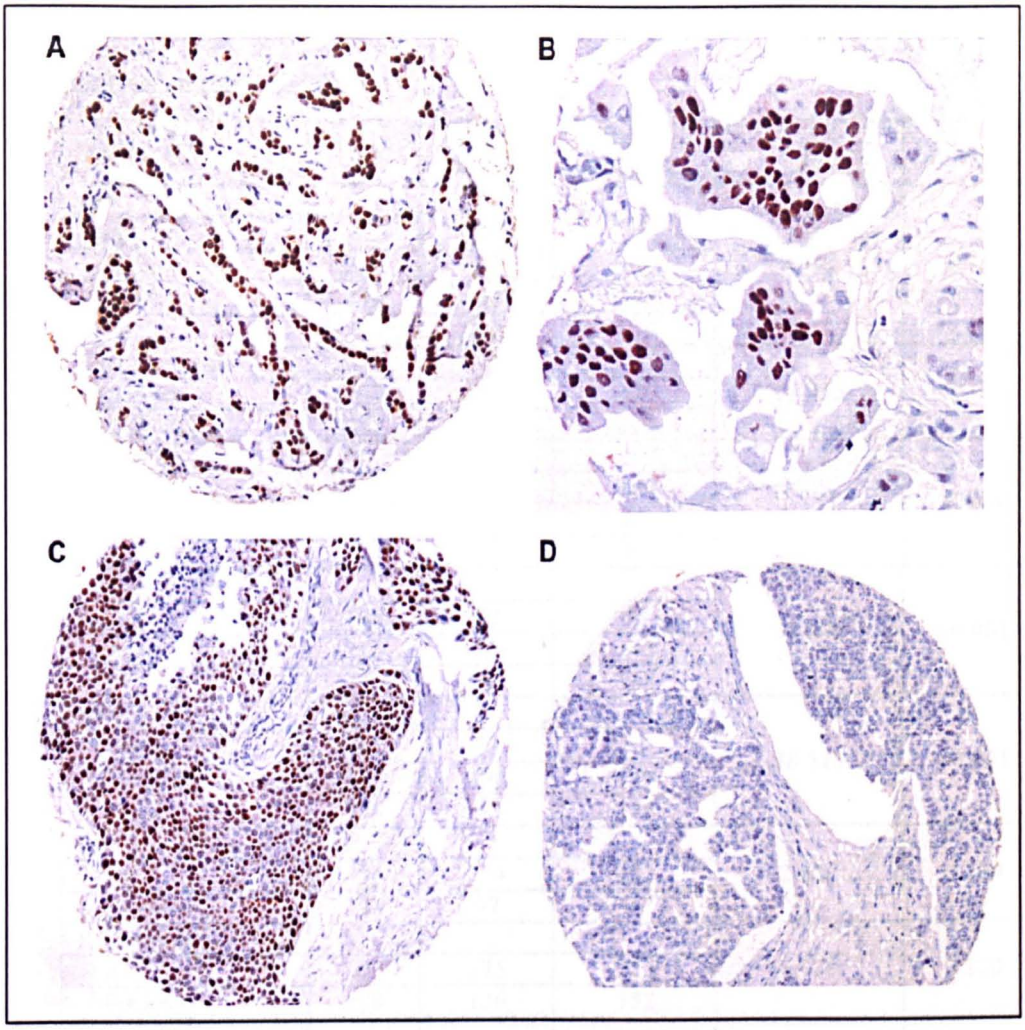


**Figure 3.1:** FOXA1 protein expression in breast cancer

TMA cores of (A) lobular (x100), (B) Grade 2 ductal (x 200) and (C) Grade 3 ductal carcinoma (x100), using immunohistochemistry. (D) Grade 3 ductal carcinoma with negative FOXA1 expression (x100).

**Table 3.1:** Relation of FOXA1 expression to other clinicopathological variables in the whole series

| Variable | Total | Negative FOXA1 | Positive FOXA1 | $\chi^2$ | p- value |
|---|---|---|---|---|---|
| **Age** | | | | 2.822 | 0.420 |
| <40 | 40 | 19 | 21 | | |
| 40-50 | 200 | 97 | 103 | | |
| 51-60 | 246 | 111 | 135 | | |
| >60 | 210 | 85 | 125 | | |
| **Tumour Size** | | | | 14.755 | <0.001 |
| <1.5 cm | 230 | 150 | 80 | | |
| ≥1.5 cm | 460 | 231 | 229 | | |
| **LN Stage** | | | | 1.133 | 0.568 |
| 1(Negative) | 461 | 201 | 260 | | |
| 2(1-3 LN) | 158 | 75 | 83 | | |
| 3(>3 LN) | 72 | 35 | 37 | | |
| **Grade** | | | | 38.209 | <0.001 |
| 1 | 148 | 50 | 98 | | |
| 2 | 216 | 74 | 142 | | |
| 3 | 325 | 187 | 138 | | |
| **NPI** | | | | 38.515 | <0.001 |
| Poor | 96 | 53 | 43 | | |
| Moderate | 377 | 198 | 179 | | |
| Good | 216 | 60 | 156 | | |
| **DM** | | | | 2.647 | 0.100 |
| No | 496 | 214 | 282 | | |
| Positive | 194 | 97 | 97 | | |
| **Recurrence** | | | | 0.979 | 0.320 |
| No | 403 | 175 | 228 | | |
| Positive | 288 | 136 | 152 | | |
| **VI** | | | | 1.501 | 0.470 |
| No | 271 | 125 | 146 | | |
| Probable | 328 | 141 | 187 | | |
| Definite | 82 | 41 | 41 | | |
| **Mitotic counts** | | | | 30.797 | <0.001 |
| **1** | 218 | 69 | 149 | | |
| **2** | 125 | 51 | 74 | | |
| **3** | 309 | 172 | 137 | | |
| **Tumour type** | | | | 33.724 | <0.001 |
| Ductal/NST | 382 | 198 | 184 | | |
| Lobular | 62 | 17 | 45 | | |
| Tubular and Tubular mixed | 163 | 56 | 107 | | |
| Medullary | 21 | 16 | 5 | | |
| Other special types | 14 | 6 | 8 | | |
| Mixed | 40 | 15 | 25 | | |

*Includes Mucoid, invasive cribriform and invasive papillary carcinoma, ** Include ductal/NST mixed with lobular or special type

**Table 3.2:** Relation of the FOXA1 expression to other biomarkers in the whole series

| Variable | Total | Negative FOXA1 | Positive FOXA1 | $\chi^2$ | p-value |
|---|---|---|---|---|---|
| **ERα** | | | | | |
| Negative | 213 | 141 | 72 | 54.677 | <0.001 |
| Positive | 435 | 154 | 281 | | |
| **AR** | | | | | |
| Negative | 245 | 151 | 94 | 42.192 | <0.001 |
| Positive | 359 | 125 | 234 | | |
| **PgR** | | | | | |
| Negative | 302 | 178 | 124 | 35.965 | <0.001 |
| Positive | 340 | 120 | 220 | | |
| **BRCA1** | | | | | |
| Negative | 73 | 47 | 26 | 8.540 | 0.003 |
| Positive | 425 | 195 | 230 | | |
| **HER2** | | | | | |
| Negative | 440 | 205 | 235 | 0.008 | 0.928 |
| Positive | 89 | 41 | 48 | | |
| **p53** | | | | | |
| Negative | 383 | 111 | 494 | 2.715 | 0.090 |
| Positive | 133 | 26 | 159 | | |
| **EGFR** | | | | | |
| Negative | 418 | 194 | 224 | 0.697 | 0.400 |
| Positive | 106 | 54 | 52 | | |
| **CK5/6** | | | | | |
| Negative | 523 | 224 | 299 | 8.987 | 0.003 |
| Positive | 150 | 85 | 65 | | |
| **CK14** | | | | | |
| Negative | 524 | 231 | 293 | 6.080 | 0.01 |
| Positive | 132 | 74 | 58 | | |
| **CK18** | | | | | |
| Negative | 348 | 177 | 171 | 5.121 | 0.024 |
| Positive | 237 | 98 | 139 | | |
| **CK19** | | | | | |
| Negative | 399 | 112 | 155 | 3.087 | 0.08 |
| Positive | 267 | 195 | 204 | | |
| **CK7/8** | | | | | |
| Negative | 352 | 184 | 168 | 11.894 | <0.001 |
| Positive | 318 | 123 | 195 | | |
| **E-cadherin** | | | | | |
| Negative | 280 | 143 | 137 | 5.283 | 0.022 |
| Positive | 360 | 151 | 209 | | |
| **P-cadherin** | | | | | |
| Negative | 185 | 73 | 112 | 9.624 | 0.002 |
| Positive | 312 | 168 | 144 | | |

**Table 3.3:** Relation of FOXA1 expression to other clinicopathological variables in the ER-positive cohort

| Variable | Total | Negative FOXA1 | Positive FOXA1 | $\chi^2$ | p- value |
|---|---|---|---|---|---|
| **Age** | | | | | |
| <40 | 17 | 4 | 13 | 1.782 | 0.619 |
| 40-50 | 109 | 36 | 73 | | |
| 51-60 | 158 | 60 | 98 | | |
| >60 | 151 | 54 | 97 | | |
| **Tumour Size** | | | | | |
| <1.5 cm | 167 | 46 | 121 | 7.318 | 0.007 |
| ≥1.5 cm | 268 | 108 | 160 | | |
| **LN Stage** | | | | | |
| 1(Negative) | 288 | 93 | 195 | 4.161 | 0.125 |
| 2(1-3 LN) | 104 | 45 | 59 | | |
| 3(>3 LN) | 42 | 16 | 26 | | |
| **Grade** | | | | | |
| 1 | 125 | 40 | 85 | 5.261 | 0.072 |
| 2 | 176 | 56 | 120 | | |
| 3 | 134 | 58 | 76 | | |
| **NPI** | | | | | |
| Poor | 48 | 22 | 26 | 14.291 | 0.001 |
| Moderate | 205 | 86 | 119 | | |
| Good | 182 | 46 | 136 | | |
| **DM** | | | | | |
| No | 322 | 109 | 213 | 1.51 | 0.219 |
| Positive | 109 | 44 | 65 | | |
| **Recurrence** | | | | | |
| No | 266 | 94 | 172 | 0.008 | 0.93 |
| Positive | 165 | 59 | 106 | | |
| **VI** | | | | | |
| No | 171 | 58 | 113 | 4.386 | 0.112 |
| Probable | 211 | 72 | 139 | | |
| Definite | 44 | 22 | 22 | | |
| **Mitosis** | | | | | |
| 1 | 187 | 57 | 130 | 4.124 | 0.127 |
| 2 | 92 | 32 | 60 | | |
| 3 | 54 | 76 | 130 | | |

**Table 3.4**: Relation of FOXA1 expression to other biomarkers in the ER-positive cohort

| Variable | Total | Negative FOXA1 | Positive FOXA1 | $\chi^2$ | p-value |
|---|---|---|---|---|---|
| **AR** | | | | | |
| Negative | 104 | 45 | 59 | 4.315 | 0.038 |
| Positive | 297 | 95 | 202 | | |
| **PgR** | | | | | |
| Negative | 116 | 52 | 64 | 5.804 | 0.016 |
| Positive | 307 | 99 | 208 | | |
| **HER2** | | | | | |
| Negative | 297 | 108 | 189 | 0.015 | 0.902 |
| Positive | 34 | 12 | 22 | | |
| **p53** | | | | | |
| Negative | 343 | 129 | 241 | 1.827 | 0.177 |
| Positive | 75 | 22 | 53 | | |
| **EGFR** | | | | | |
| Negative | 300 | 115 | 185 | 0.045 | 0.832 |
| Positive | 57 | 21 | 36 | | |
| **CK5/6** | | | | | |
| Negative | 373 | 134 | 239 | 0.214 | 0.644 |
| Positive | 55 | 18 | 37 | | |
| **CK14** | | | | | |
| Negative | 362 | 130 | 232 | 0.053 | 0.818 |
| Positive | 56 | 21 | 35 | | |
| **CK18** | | | | | |
| Negative | 192 | 65 | 127 | 0.964 | 0.326 |
| Positive | 194 | 75 | 119 | | |
| **CK19** | | | | | |
| Negative | 220 | 74 | 146 | 0.809 | 0.368 |
| Positive | 209 | 130 | 79 | | |
| **CK7/8** | | | | | |
| Negative | 175 | 60 | 115 | 0.124 | 0.724 |
| Positive | 256 | 92 | 164 | | |
| **E-cadherin** | | | | | |
| Negative | 176 | 72 | 104 | 3.343 | 0.06 |
| Positive | 245 | 79 | 166 | | |
| **P-cadherin** | | | | | |
| Negative | 156 | 54 | 102 | 3.005 | 0.08 |
| Positive | 180 | 79 | 101 | | |

#### 3.2.3.3 Correlation between FOXA1 expression and patient outcome

In the whole patient series, an association between loss of FOXA1 expression and shorter breast cancer specific survival (BCSS) was found (Log Rank (LR) =6.987, $p$=0.008). However, multivariate Cox hazard analysis including tumour size, histologic grade, lymph node stage and FOXA1 expression showed that FOXA1 expression was not an independent predictor of survival (Hazard ratio (HR)=0.891, $p$=0.418) **(Table 3.5)**. Interestingly, in a model that included only FOXA1 and ERα expression, FOXA1 did not retain independent significance in contrast to ERα which did. No association between FOXA1 expression and disease free interval (DFI) was found (LR= 1.687, $p$ =0.194).

In the ER-positive group, no association between FOXA1 expression and outcome was found **(Fig 3.2A&B)**. In the group of patients who had not received hormonal therapy, FOXA1 expression was associated with more favourable BCSS (LR=0.5.49, $p$ =0.01).

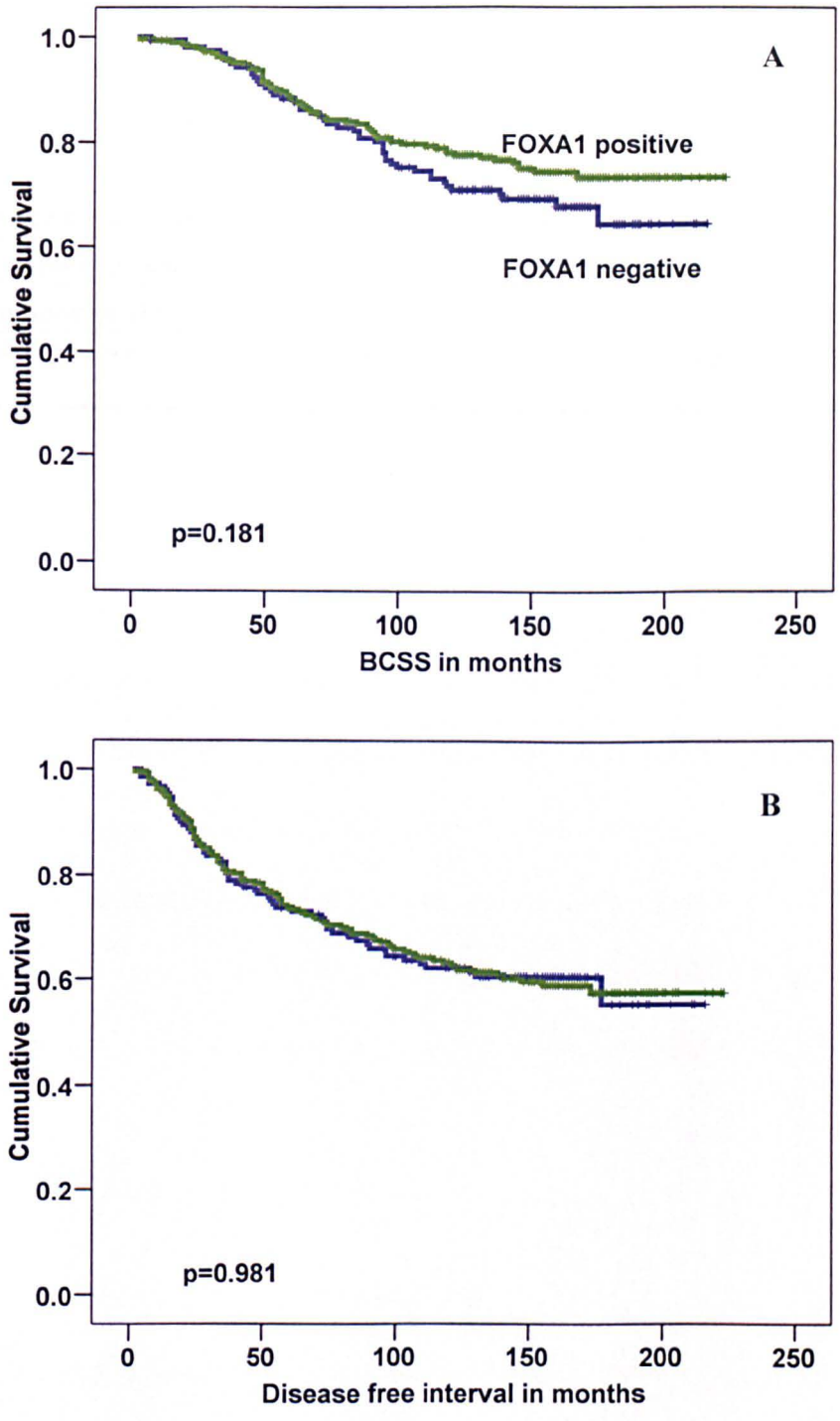


**Figure 3.2:** Kaplan Meier plots of FOXA1 protein expression

(A) Kaplan Meier plot of FOXA1 protein expression and BCSS in the ER positive cohort. (B) Kaplan Meier analysis of FOXA1 protein expression and disease free interval in ER positive cohort.

**Table 3.5**: Cox proportional hazards analysis for predictors of breast cancer specific survival in the whole series

| Variable | P value | HR | 95% CI | |
|---|---|---|---|---|
| | | | Lower | Upper |
| **FOXA1 expression** | 0.418 | 0.891 | 0.674 | 1.178 |
| **Lymph node stage** | <0.001 | 1.802 | 1.505 | 2.159 |
| **Tumour grade** | <0.001 | 2.024 | 1.611 | 2.545 |
| **Tumour size** | 0.038 | 1.481 | 1.022 | 2.146 |

## 3.3 RERG

### 3.3.1 Introduction

The Ras-related, oestrogen-regulated growth inhibitor (RERG) was initially identified as one of the genes that characterise luminal tumours and its expression was decreased in the aggressive ER-negative subtypes (Finlin et al., 2001). RERG is a GTP-binding protein with intrinsic GTPase activity (Finlin et al., 2001). RERG mRNA expression was found to be induced rapidly in MCF-7 cells stimulated by estradiol and repressed by tamoxifen treatment (Finlin et al., 2001).

The suggested heterogeneity of ER-positive tumours has prompted the need to identify candidate biomarkers to refine their subclassification particularly with respect to their behaviour. Subsequently, in this study we have analysed 47,293 gene transcripts in 128 invasive breast carcinomas using different biostatistical models to identify genes that is strongly associated with ER-positive/luminal tumours and that can be used to stratify them into clinically relevant subgroups.

### 3.3.2 Material and Methods

#### 3.3.2.1 Gene expression study

The study population used was derived from the Nottingham Tenovus Primary Breast Carcinoma Series of women aged 70 years or less, who presented with stage I and II primary operable invasive breast carcinomas.

Total RNA was extracted from a series of frozen breast cancers retrieved from Nottingham Hospitals NHS Trust Tumour Bank between 1986 and 1992 as described in the General Material and Methods chapter.

**Bioinformatics analysis (1): Artificial neural network model**

We identified the ER-positive cases (n=84) by immunohistochemistry in our patient cohort (n=128) and applied an artificial neural network (ANN) model for sample classification to the gene expression data comprising 47,293 inputs for each sample. The output node was coded as 0 if a case was low ER expression (the median H-score<140; n = 42), and 1 if high ER expression (H score> 140; n = 42). Each gene was considered singly as an input to the model. More precisely, the data was analysed using multi-layer perceptron architecture with a sigmoidal transfer function, where weights were updated by a back propagation algorithm as previously described in general material and methods chapter. Inputs were ranked in ascending order based on the predictive error.

**Bioinformatics analysis (2): Ensemble classification and cross-validation analysis**

In a second bioinformatics analysis step, we sought to obtain a robust ranking of genes that are differentially expressed between the ER-positive (n=84) cases and the ER negative non-luminal cases (all other cases) and have high predictive power, by applying an ensemble sample classification method (see General Material and Methods chapter).

#### 3.3.2.2 Immunohistochemistry

The RERG specific rabbit polyclonal antibody (Purified rabbit anti-human RERG polyclonal antibody, 10687-1-AP, Proteintech Group, Chicago, IL, USA) was optimized at a working dilution of 1:20 using randomly selected full-face sections of breast cancer tissue to assess the staining distribution. The detailed method is described in the general material and methods chapter. Negative controls were performed by omitting the primary antibody while positive control of known BC sections was used in each run.

### 3.3.3 Results

#### 3.3.3.1 Novel genes associated with ER-positive status using Artificial Neural Network

High ER expression is associated with good prognosis when compared to low ER expression. Our aim was to identify; using a novel prediction method (ANN), a set of genes that can associate with high ER expression and to validated the genes using protein expression. To study this, ER-positive cases (84 tumours) were categorized according to the level of ER expression into high and low expression using the median of the H-score value (H-score 140).

The ranking order of the ANN results was based on predictive error for the unseen cohort in the Monte Carlo Cross validation with the lowest being higher in the ranking order. **Table 3.6** illustrates top transcripts according to ER status ranked by predictive error.

In this study ESR1, the gene for ER, was ranked as the most important gene for ER membership and this was used a proof for the validation of the model.

#### 3.3.3.2 Novel genes associated with ER-positive luminal phenotype using the ensemble cross- validation analysis

The RERG-gene was selected among the significantly differentially expressed genes in every cycle of a leave-one-out external cross-validation analysis. The prediction models obtained from this procedure distinguished the luminal from the non-luminal samples with an average accuracy of 88.3% (sensitivity: 95.2%, specificity: 75.0%). Very similar results were obtained in a 10-fold cross-validation analysis, which was conducted for further verification (average accuracy: 89%, sensitivity: 95.2%, specificity: 77.3%). **Table 3.7** lists the 30 genes which were identified as being differentially expressed in ER-positive luminal and non-luminal samples. These were identified using both in a leave-one-out and a 10-fold cross-validation analysis, i.e. using different subsets of samples, and they were always selected as significantly differentially expressed in each cycle of the analysis

**Figure 3.3** shows a heat map displaying the microarray expression values of 30 genes (rows) in 128 breast cancer samples (columns) using different colour codes (red = high expression, green = low expression). The 30 rows correspond to the 30 top-ranked genes from the cross-validation analysis, grouped according to the results of an average linkage hierarchical clustering using the Euclidean distance metric of the 30 gene expression vectors (see the dendogram on the left in Figure 3.3). The 128 columns in this figure represent

the microarray samples, grouped into non-luminal samples (left) and ER-positive samples (right). **Figure 3.4A** shows a box plot of RERG gene (mRNA) expression (normalised expression value) in ER-positive versus non-luminal samples with higher expression in ER-positive cohort, while **Figure 3.4B** shows a box plot of RERG gene (mRNA) expression (normalised expression value) in different tumour grades which shows that the expression of RERG mRNA is higher in low grade tumours.

RERG was therefore selected for further study using a protein expression assay to assess the biological and prognostic significance of its protein expression in large breast cancer patient cohort as well as in the ER-positive subgroup.

**Table 3.6:** A gene rank of ER expression status

(Summary of step 1 of the ANN approach- 10 genes shown)

| Gene | Selection Error |
|---|---|
| ESR1 | 0.403422327 |
| **RERG** | 0.438626499 |
| AMN1 | 0.441492448 |
| ZNF271 | 0.445580899 |
| PCDHA5 | 0.446326207 |
| PRKAR2B | 0.447933195 |
| TCEAL1 | 0.448787999 |
| CTBP2 | 0.449052205 |
| LDB3 | 0.449750785 |
| DDIT4 | 0.451080472 |

**Table 3.7**: A gene list of 30 genes to differentiate between luminal-like (ER-positive) and non-luminal cases (ER-) using cross-validation analysis ranked by z-score

| Gene identifier | z-score | Gene |
|---|---|---|
| GI_4503602-S | 5.7 | ESR1 |
| GI_14249703-S | 5.7 | **RERG** |
| GI_9951924-S | 5.7 | CA12 |
| GI_37551139-S | 5.7 | C6orf115 |
| GI_34452698-S | 5.7 | ACTR3 |
| GI_22779933-S | 5.7 | WDR19 |
| GI_38455428-S | 5.7 | AGR3 |
| GI_38146007-A | 5.7 | TTC8 |
| GI_40788002-S | 5.7 | PSME4 |
| GI_4503928-S | 5.7 | **GATA3** |
| GI_22748948-S | 5.7 | IGF1R |
| GI_29126237-S | 5.7 | BTF3 |
| GI_37552339-S | 5.7 | KDM4B |
| GI_34304343-A | 5.7 | PSAT1 |
| GI_29728071-S | 5.7 | TBC1D9 |
| GI_34147362-S | 5 | CHAC1 |
| GI_4885496-S | 4.2 | MYB |
| GI_7706686-S | 4.2 | EVL |
| GI_31341936-S | 3.5 | C1orf64 |
| GI_21614543-S | 2.7 | S100A8 |
| GI_40255152-S | 2.7 | KCTD6 |
| GI_21614495-S | 2 | VAV3 |
| GI_4502846-S | 2 | CIRBP |
| GI_30581115-S | 1.2 | SOX11 |
| GI_22035691-A | 1.2 | GFRA1 |
| GI_16507967-S | 0.49 | KCNK15 |
| GI_32698779-S | 0.49 | CMYA5 |
| GI_18152766-S | 0.49 | SYTL4 |
| GI_10835186-S | 0.49 | SOD2 |
| GI_37595559-S | 0.49 | DNALI1 |

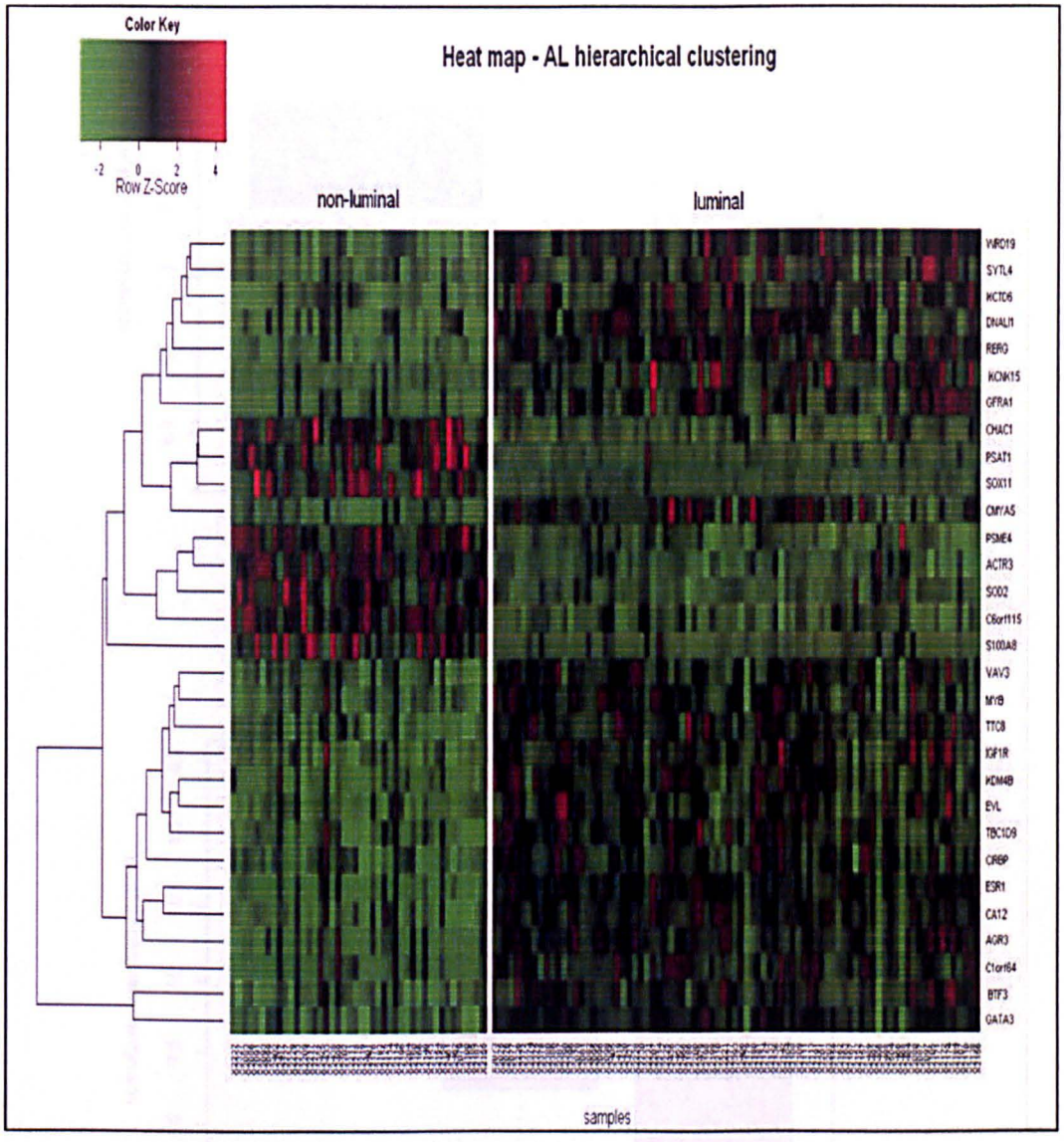


**Figure 3.3:** A heatmap created to visualise the differential expression of the 30 top-ranked genes identified by the cross validation analysis

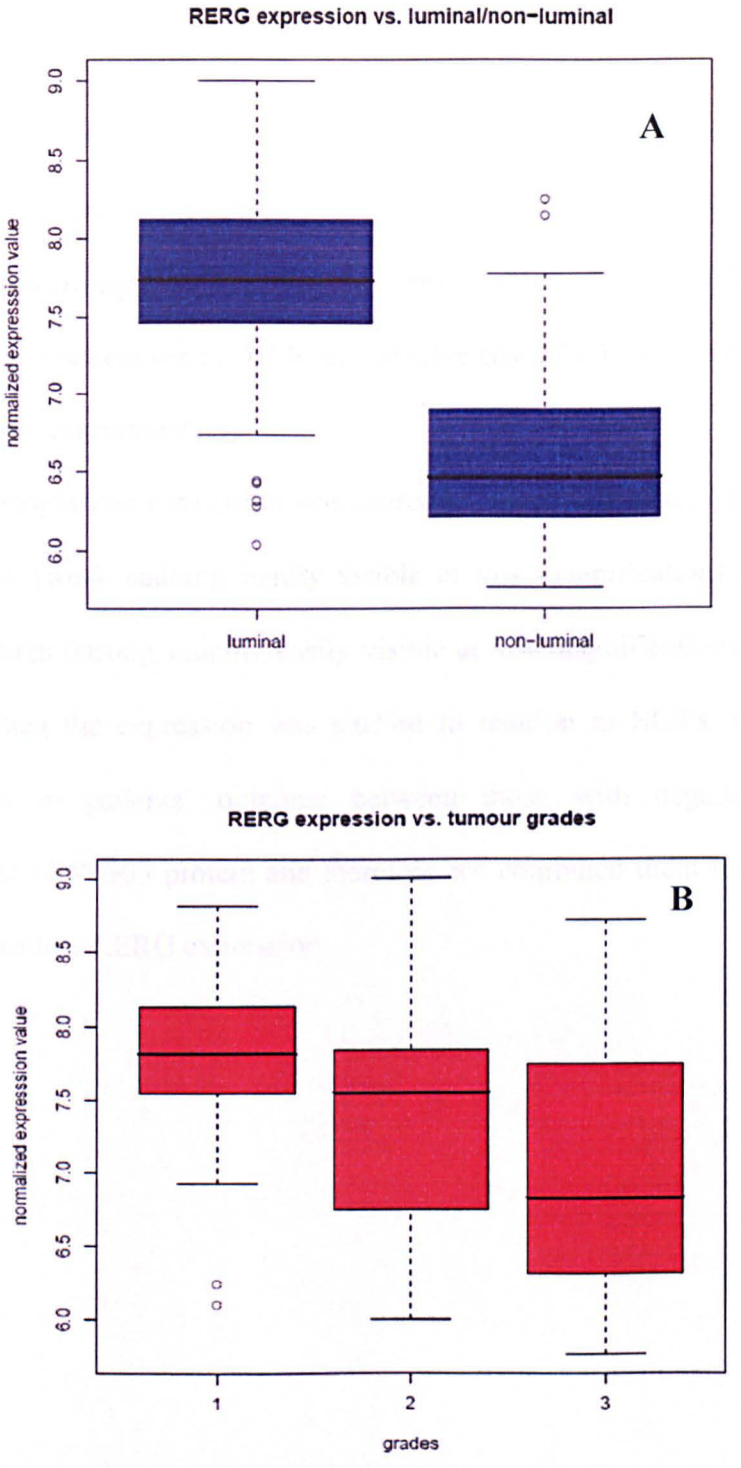


**Figure 3.4:** Box plots of RERG gene expression values

(A) In luminal vs. non-luminal samples. (B) In different tumour grades.

### 3.3.4 Expression of RERG protein in breast cancer using immunohistochemistry

Evaluation of RERG protein expression in breast cancer showed that the immunoreactivity was localised to the cytoplasm of invasive tumour cells **(Fig 3.5)** and was strongly expressed in the luminal cells in the normal breast acini. Of the whole patient series, 1,140 informative cases for RERG expression were available for assessment.

RERG cytoplasmic expression was scored as negative (no staining) in 28% of cases, low (weak staining hardly visible at low magnification) in 45.6% of cases or high (strong staining easily visible at low magnification) in 26.4% of cases. When the expression was studied in relation to BCSS, we found no difference in patients' outcome between those with negative and low expression of RERG protein and therefore we combined them into one group of negative/low RERG expression.

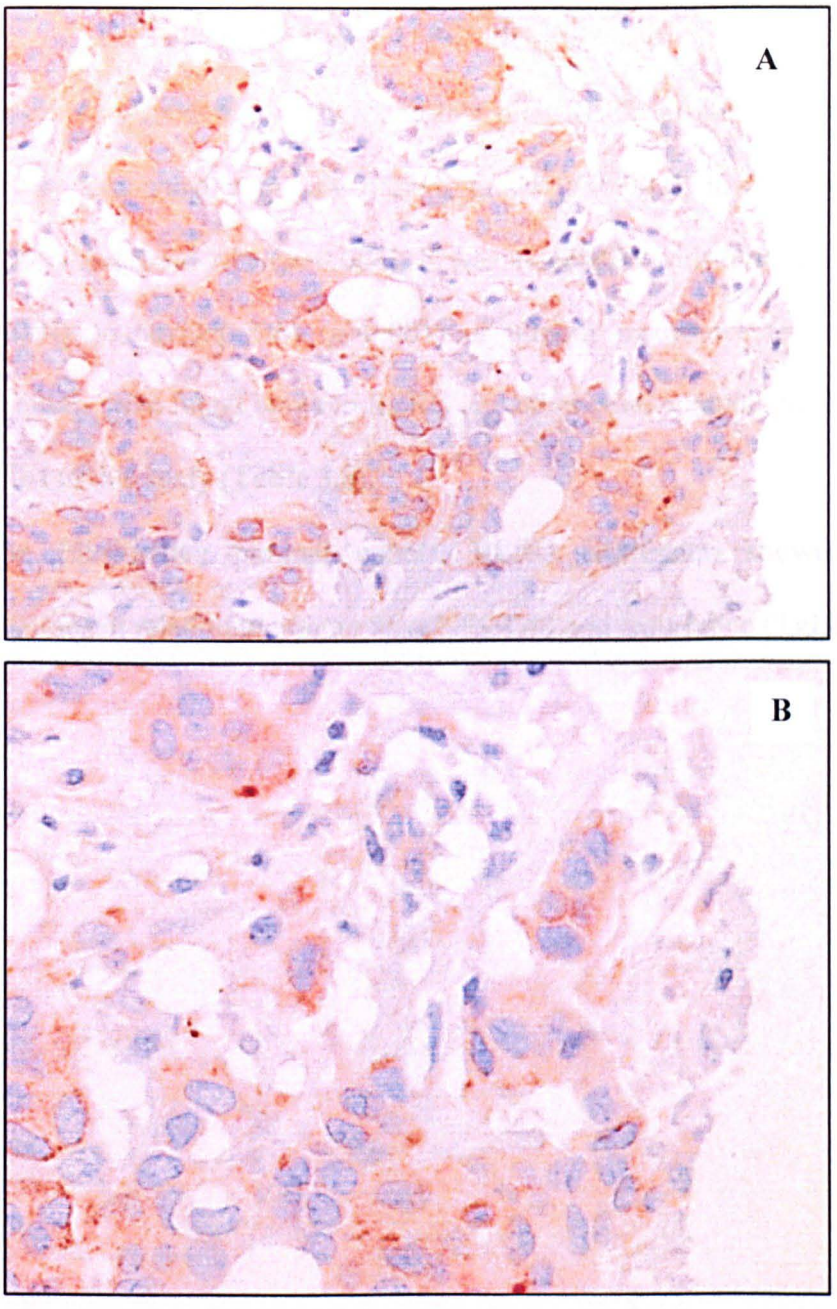


**Figure 3.5:** RERG expression in breast cancer

TMA core of grade 2 invasive breast cancer with strong RERG cytoplasmic expression. (A) Lower magnification (x200) (B) Higher magnification (x400)

#### 3.3.4.1 Correlation between RERG protein expression and other clinicopathological variables

In the whole patient series, we found that high RERG protein expression was positively associated with low tumour grade ($p$=0.002), low mitotic counts ($p$=0.006) and good NPI ($p$=0.006). It was associated with tumours that were less likely to develop DM ($p$=0.001) or tumour recurrence ($p$=0.003). No associations were found between RERG and other clinicopathological variables included in this study **(Table 3.8)**.

In the ER-positive luminal cohort, RERG expression showed similar associations in relation to tumour size, NPI, DM and recurrence **(Table 3.9)**.

**Table 3.8:** Relation of RERG immunostaining to other clinicopathological variables in the whole series

| Variable | Low | High | $\chi^2$ | p value |
|---|---|---|---|---|
| **Age** | | | 4.870 | 0.182 |
| <40 | 70(79.5) | 18(20.5) | | |
| 40-50 | 251(76.1) | 79(23.9) | | |
| 51-60 | 277(73.3) | 101(26.7) | | |
| >60 | 241(70.1) | 103(29.9) | | |
| **Size** | | | 4.604 | 0.032 |
| **≤2 cm** | 406(70.9) | 167(29.1) | | |
| >2 cm | 432(76.5) | 133(23.5) | | |
| **LN Stage** | | | 1.334 | 0.513 |
| 1(Negative) | 497(72.6) | 188(27.4) | | |
| 2(1-3 LN) | 261(74.6) | 89(25.4) | | |
| 3(>3 LN) | 79(77.5) | 23(22.5) | | |
| **Grade** | | | 12.419 | 0.002 |
| 1 | 127(66.8) | 63(33.2) | | |
| 2 | 260(70.3) | 110(29.7) | | |
| 3 | 451(78) | 127(22) | | |
| **NPI** | | | 10.330 | 0.006 |
| Good | 212(67.3) | 103(32.7) | | |
| Moderate | 470(75 | 157(25) | | |
| Poor | 157(79.3) | 41(20.7) | | |
| **DM** | | | 11.948 | 0.001 |
| No | 565(70.9) | 232(29.1) | | |
| Positive | 266(80.9) | 63(19.1) | | |
| **Recurrence** | | | 8.642 | 0.003 |
| No | 474(70.6) | 197(29.4) | | |
| Positive | 348(78.6) | 95(21.4) | | |
| **Tumour type** | | | 4.783 | 0.443 |
| Ductal/NST | 498(75) | 166(25) | | |
| Lobular | 94(72.9) | 35(27.1) | | |
| Tubular and Tubular mixed | 163(70.6) | 68(29.4) | | |
| Medullary | 25(83.3) | 5(16.7) | | |
| Other special types* | 12(75) | 4(25) | | |
| Mixed** | 47(67.1) | 23(32.9) | | |
| **Mitosis** | | | 10.274 | 0.006 |
| 1 | 268(68.7) | 122(31.3) | | |
| 2 | 144(73.5) | 52(26.5) | | |
| 3 | 407(78.1) | 114(21.9) | | |
| **Menopause** | | | 6.116 | 0.016 |
| Premenopausal | 341(77.7) | 98(22.3) | | |
| Postmenopausal | 498(71) | 203(29) | | |

*Includes Mucoid, invasive cribriform and invasive papillary carcinoma, ** Include ductal/NST mixed with lobular or special type

**Table 3.9**: Relation of RERG immunostaining to other clinicopathological variables in the ER-positive cohort

| Variable | Low | High | $\chi^2$ | p value |
|---|---|---|---|---|
| **Age** | | | 1.875 | 0.599 |
| <40 | 31(70.5) | 13(29.5) | | |
| 40-50 | 160(73.4) | 58(26.6) | | |
| 51-60 | 187(72.2) | 72(27.8) | | |
| >60 | 177(68.1) | 83(31.9) | | |
| **Size** | | | 5.335 | 0.021 |
| **≤2 cm** | 288(67.6) | 138(32.4) | | |
| >2 cm | 266(75.1) | 88(24.9) | | |
| **LN Stage** | | | 2.044 | 0.360 |
| 1(Negative) | 325(69.1) | 145(30.5) | | |
| 2(1-3 LN) | 182(73.4) | 66(26.6) | | |
| 3(>3 LN) | 46(75.4) | 15(24.6) | | |
| **Grade** | | | 5.379 | 0.068 |
| 1 | 111(65.7) | 58(34.3) | | |
| 2 | 227(69.8) | 98(30.2) | | |
| 3 | 216(75.5) | 70(24.5) | | |
| **NPI** | | | 7.313 | 0.026 |
| Good | 185(65.8) | 96(34.2) | | |
| Moderate | 285(72.7) | 107(27.3) | | |
| Poor | 85(78.7) | 23(21.3) | | |
| **DM** | | | 9.537 | 0.002 |
| No | 382(68.2) | 178(31.8) | | |
| Positive | 170(79.4) | 44(20.6) | | |
| **Recurrence** | | | 7.189 | 0.007 |
| No | 321(67.9) | 152(32.1) | | |
| Positive | 226(76.9) | 68(23.1) | | |
| **Tumour type** | | | 3.301 | 0.654 |
| Ductal/NST | 272(70.6) | 113(29.4) | | |
| Lobular | 90(76.9) | 27(23.1) | | |
| Tubular and Tubular mixed | 142(70) | 61(30) | | |
| Medullary | 3(75) | 1(25) | | |
| Other special types | 9(75) | 3(25) | | |
| Mixed | 39(65) | 21(35) | | |
| **Mitosis** | | | 3.223 | 0.200 |
| 1 | 238(68.6) | 109(31.4) | | |
| 2 | 116(72) | 45(28) | | |
| 3 | 186(75.3) | 61(24.7) | | |
| **Menopause** | | | 2.984 | 0.084 |
| Premenopausal | 203(74.9) | 68(25.1) | | |
| Postmenopausal | 352(69) | 158(31) | | |

### 3.3.4.2 Correlation between RERG protein expression and other biomarkers

In the whole patient series, RERG protein expression was found to be positively associated with markers of luminal differentiation such as CK19 CK18 ($p$=0.001), CK7/8($p$=0.002), p27 ($p$=0.005), E-cadherin ($p$=0.001), ER ($p$=0.001) and androgen receptor (AR) ($p$<0.001).

In contrast, RERG expression was inversely associated with MIB1 ($p$=0.005) **(Table 3.10)**.

In the ER-positive cohort, RERG expression retained similar associations. No significant associations were found between RERG and other biomarkers included in the study.

a

**Table 3.10:** Relation of RERG immunostaining to other biomarkers in the whole series

| Variable | Negative RERG | Positive RERG | $\chi^2$ | *p*-value |
|---|---|---|---|---|
| **CK5/6** | | | 0.003 | 0.954 |
| Negative | 688(73.7) | 245(26.3) | | |
| Positive | 125(73.5) | 45(26.5) | | |
| **CK14** | | | 0.290 | 0.590 |
| Negative | 711(73.8) | 253(26.2) | | |
| Positive | 92(76) | 29(24) | | |
| **CK18** | | | 10.823 | 0.001 |
| Negative | 118(84.9) | 21(15.1) | | |
| Positive | 647(71.7) | 256(28.3) | | |
| **CK19** | | | 4.829 | 0.028 |
| Negative | 90(82.6) | 19(17.4) | | |
| Positive | 726(72.8) | 271(27.2) | | |
| **CK7/8** | | | 6.128 | 0.013 |
| Negative | 17(100) | 0(0) | | |
| Positive | 800(73.4) | 290(26.6) | | |
| **ER** | | | 10.938 | 0.001 |
| Negative | 249(80.8) | 59(19.2) | | |
| Positive | 555(71.1) | 226(28.9) | | |
| **PgR** | | | 0.026 | 0.872 |
| Negative | 384(74) | 122(26) | | |
| Positive | 449(73.6) | 161(26.4) | | |
| **AR** | | | 23.614 | <0.001 |
| Negative | 300(82.9) | 62(17.1) | | |
| Positive | 469(69) | 211(31) | | |
| **p53** | | | 3.839 | 0.050 |
| Negative | 571(71.7) | 225(28.3) | | |
| Positive | 223(77.7) | 64(22.3) | | |
| **BRCA1** | | | 5.163 | 0.023 |
| Negative | 114(82) | 25(18) | | |
| Positive | 586(72.9) | 218(27.1) | | |
| **Bcl-2** | | | 2.280 | 0.131 |
| Negative | 268(77.5) | 78(22.5) | | |
| Weak | 385(72.9) | 143(27.1) | | |
| **MIB1** | | | 7.915 | 0.005 |
| Low | 172(67.7) | 82(32.3) | | |
| High | 488(76.9) | 147(23.1) | | |
| **P-cadherin** | | | 2.601 | 0.107 |
| Negative | 321(71.7) | 127(28.3) | | |
| Positive | 382(76.2) | 119(23.8) | | |
| **E-cadherin** | | | 11.370 | 0.001 |
| Negative | 332(79.6) | 85(20.4) | | |
| Positive | 473(70.4) | 199(29.6) | | |
| **FOXA1** | | | 8.082 | 0.004 |
| Negative | 344(78.9) | 92(21.1) | | |
| Positive | 272(70.3) | 115(29.7) | | |
| **HER2** | | | 0.243 | 0.622 |
| Negative | 711(73.8) | 253(26.2) | | |
| Positive | 103(75.7) | 33(24.3) | | |
| **EGFR** | | | 5.524 | 0.019 |
| Negative | 596(75.3) | 196(24.7) | | |
| Positive | 132(67) | 65(33) | | |
| **p27** | | | 7.711 | 0.005 |
| Negative | 317(79.1) | 84(20.9) | | |
| Positive | 264(70.4) | 111(29.6) | | |

### 3.3.4.3 Correlation between RERG protein expression and patient outcome

**(A) Univariate analysis**

Breast cancer patients with strong RERG expression showed a significantly longer BCSS (LR=12.267, $p<0.001$) and longer DMFI (LR=7.472, $p=0.006$). The association with longer BCSS was also confirmed in the group of patients that did not receive systemic therapy (n=397) (LR=6.467, $p=0.01$).

In ER-positive group, patients with strong RERG expression also showed a significantly longer BCSS (LR=9.887, $p=0.002$) **(Fig 3.6A)** and longer DMFI (LR=7.205, $p=0.007$) **(Fig 3.6B)**.

In the ER-positive tamoxifen-only treated patients, high RERG expression indicated better response to tamoxifen monotherapy (LR=4.553, $p=0.033$).

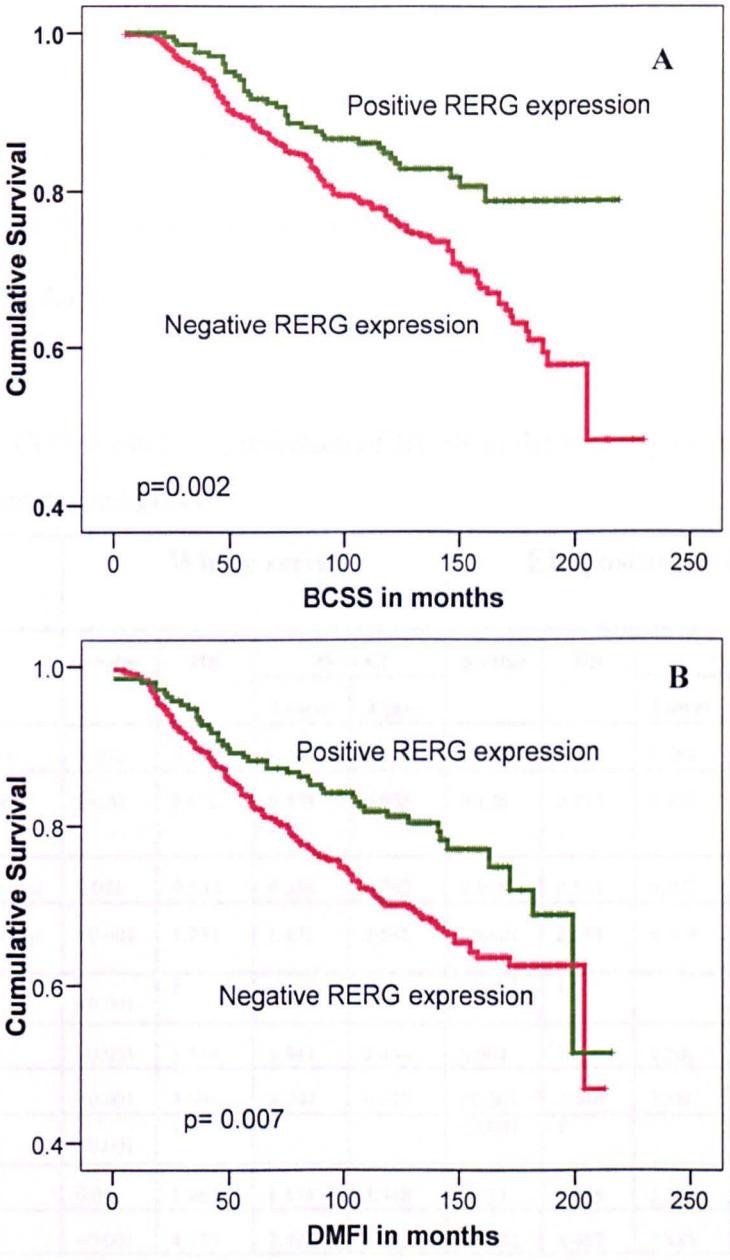


**Figure 3.6:** Kaplan Meier plots of RERG protein expression in the ER-positive luminal-like cohort in relation to (A) BCSS and (B) DMFI

**(B) Multivariate analysis**

Multivariate analyses including well-established prognostic variables showed that RERG expression was an independent prognostic marker for longer BCSS in the whole series (Hazard ratio (HR) =0.573, $p$ =0.001, 95% CI =0.411-0.799) and in ER+ luminal-like cohort (HR =0.555, $p$ =0.006, 95% CI =0.364-0.846) **(Table 3.11)**.

**Table 3.11**: COX model for predictors of BCSS in the whole patient series and in the ER-positive subgroup

| | **Whole series** | | | | **ER-positive cohort** | | | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **p value** | **HR** | **95 % CI** | | **p value** | **HR** | **95 % CI** | |
| | | | **Lower** | **Upper** | | | **Lower** | **Upper** |
| RERG expression | 0.001 | 0.573 | 0.411 | 0.799 | 0.006 | 0.555 | 0.364 | 0.846 |
| Endocrine therapy given | 0.020 | 0.670 | 0.478 | 0.938 | 0.126 | 0.713 | 0.463 | 1.100 |
| Chemotherapy given | 0.001 | 0.508 | 0.338 | 0.762 | 0.028 | 0.521 | 0.292 | 0.931 |
| Tumour size (>2cm) | <0.001 | 1.951 | 1.472 | 2.585 | <0.001 | 2.083 | 1.464 | 2.963 |
| Tumour stage 1 | <0.001 | 1 | | | <0.001 | 1 | | |
| Tumour stage 2 | <0.001 | 1.814 | 1.341 | 2.454 | 0.004 | 1.769 | 1.201 | 2.606 |
| Tumour stage 3 | <0.001 | 4.604 | 3.204 | 6.617 | <0.001 | 3.948 | 2.388 | 6.526 |
| Tumour grade 1 | <0.001 | 1 | | | <0.001 | 1 | | |
| Tumour grade 2 | 0.011 | 1.982 | 1.174 | 3.348 | 0.021 | 1.955 | 1.107 | 3.454 |
| Tumour grade 3 | <0.001 | 4.175 | 2.495 | 6.985 | <0.001 | 4.487 | 2.483 | 8.107 |

## 3.4 GATA3

### 3.4.1 Introduction

Recent gene expression studies identified GATA3 as a marker of Luminal A breast cancer subtype (Sorlie et al., 2001). It has been documented that GATA3 is an essential regulator of mammary morphogenesis and luminal differentiation and normally expressed at high levels in association with ER in luminal epithelial cells (Asselin-Labat et al., 2007). Functionally, GATA3 is an important factor that accompanies the undiffentiated breast cells on their development to form luminal epithelial cells (Asselin-Labat et al., 2007, Kouros-Mehr et al., 2006). Due to its strong relation to ER, GATA3 is involved in growth control and the maintenance of the differentiated state in epithelial cells in ER-positive breast tumours (Usary et al., 2004).

The GATA family consists of six members (GATA1-6) that can be separated into two groups based on their expression patterns and sites. GATA1, GATA2 and GATA3 are expressed in hematopoietic cell lineages and are essential for differentiation, proliferation of hematopoietic stem cells, and the development of T lymphocytes (Ko and Engel, 1993). GATA4, GATA5, and GATA6 are expressed mainly in the cardiovascular system, liver, lung, pancreas, and intestine (Abba et al., 2006).

The importance of GATA3 as a possible candidate luminal marker is due to its involvement in a positive cross-regulatory cycle with the ER gene, where each one is required for the transcription of the other gene (Dydensborg et al., 2009). The role of GATA3 in oestrogen signalling requires this direct positive

regulation of the expression of the ER alpha gene itself by GATA3 which binds to two cis-regulatory elements located within the ER alpha gene, and this is required for RNA polymerase II recruitment to ER alpha promoters (Dydensborg et al., 2009).

GATA3 could contribute to the transcriptional upregulation of MUC1 gene expression in some breast carcinomas with luminal phenotype (Abba et al., 2006). Moreover, GATA3, in addition to ER, is linked to FOXA1 and the three genes form a network that can influence the biology of ER-positive luminal-like breast cancer (Badve and Nakshatri, 2009).

Previous studies have shown an important role of GATA3 in inhibiting the development of metastatic breast cancer by regulating key genes involved in metastatic breast tumour progression to the lung including ID1/-3, KRTHB1, LY6E and RARRES3 (Dydensborg et al., 2009).

In addition to its biological role in ER-positive breast cancer, GATA3 has been previously suggested as a marker of hormone therapy response (Fang et al., 2009). Testing the ER-positive breast cancer for GATA3 using immunohistochemistry might improve the prediction of hormone therapy response (Parikh et al., 2005).

Our analysis of the gene expression data of 128 frozen breast cancer cases has also confirmed its strong relation to the ER-positive luminal phenotype. For this reason, we have studied its protein expression in a cohort of well characterised series of breast cancer to validate our findings and to assess its role in the phenotypic characterisation of ER-positive breast cancer.

### 3.4.2 Material and Methods

#### 3.4.2.1 Gene expression study

The study population used was derived from the Nottingham Tenovus Primary Breast Carcinoma Series of women aged 70 years or less, who presented with stage I and II primary operable invasive breast carcinomas.

Total RNA was extracted from a series of 128 frozen breast cancers retrieved from Nottingham Hospitals NHS Trust Tumour Bank between 1986 and 1992 as described in the General Material and Methods chapter.

**Bioinformatics analysis: Ensemble classification and cross-validation analysis**

GATA3 gene was identified as being differentially expressed between ER-positive and ER negative cases by applying an ensemble sample classification method to the gene microarray data (see General Material and Methods chapter).

#### 3.4.2.2 GATA3 protein expression study

Breast cancer tissue microarrays were prepared and immunohistochemical staining of the sections was performed according to the Streptavidin-Biotin complex using as described in the general material and methods. GATA3 (HG3-31) mouse monoclonal antibody raised against human recombinant GATA3 (Santa Cruz Biotechnology, Inc., CA, USA) was used at an optimised working dilution of 1:80. To unmask the antigens, the sections were microwaved in Tris EDTA buffer pH 8.5 for 20 minutes.

Negative controls were obtained by omitting the primary antibody. Breast cancers sections were used as positive controls.

The xtile program was used to categorise the cases into high and low expression (≥ 60 H-score).

### 3.4.3 GATA3 expression results

#### 3.4.3.1 Identification of GATA3 gene as a candidate luminal marker

**Novel genes associated with ER-positive luminal phenotype using the ensemble cross- validation analysis**

The GATA3 gene was selected among the significantly differentially expressed genes in every cycle of a leave-one-out external cross-validation analysis. The prediction models obtained from this procedure distinguished the luminal from the non-luminal samples with an average accuracy of 88.3% (sensitivity: 95.2%, specificity: 75.0%).

Very similar results were obtained in a 10-fold cross-validation analysis, which was conducted for further verification (average accuracy: 89%, sensitivity: 95.2%, specificity: 77.3%). **Table 3.7** shows the 30 genes identified. GATA3 gene expression was significantly associated with luminal cases ($p<0.001$) **(Fig 3.7).**

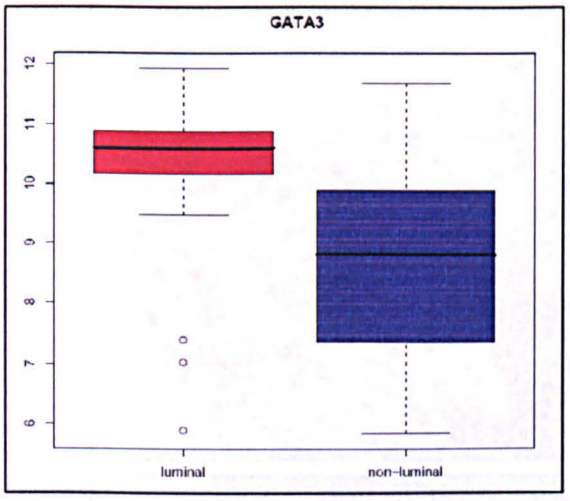


**Figure 3.7:** Boxplot of GATA3 gene normalised expression values in luminal and non-luminal samples

### 3.4.3.2 GATA3 immunohistochemical results

Evaluation of GATA3 protein expression in invasive breast cancer showed that the immunoreactivity was localised to the nuclei of invasive tumour cells with homogenous distribution **(Fig 3.8)** and was strongly expressed in the nuclei of luminal cells of normal breast acini. Of the whole series, 1,045 informative cases for GATA3 expression were available for assessment. In the whole series, 25% of cases were positive for GATA3 protein expression and 33.3% of cases were positive in ER-positive cohort. About 98% of GATA3 positive cases were also ER positive.

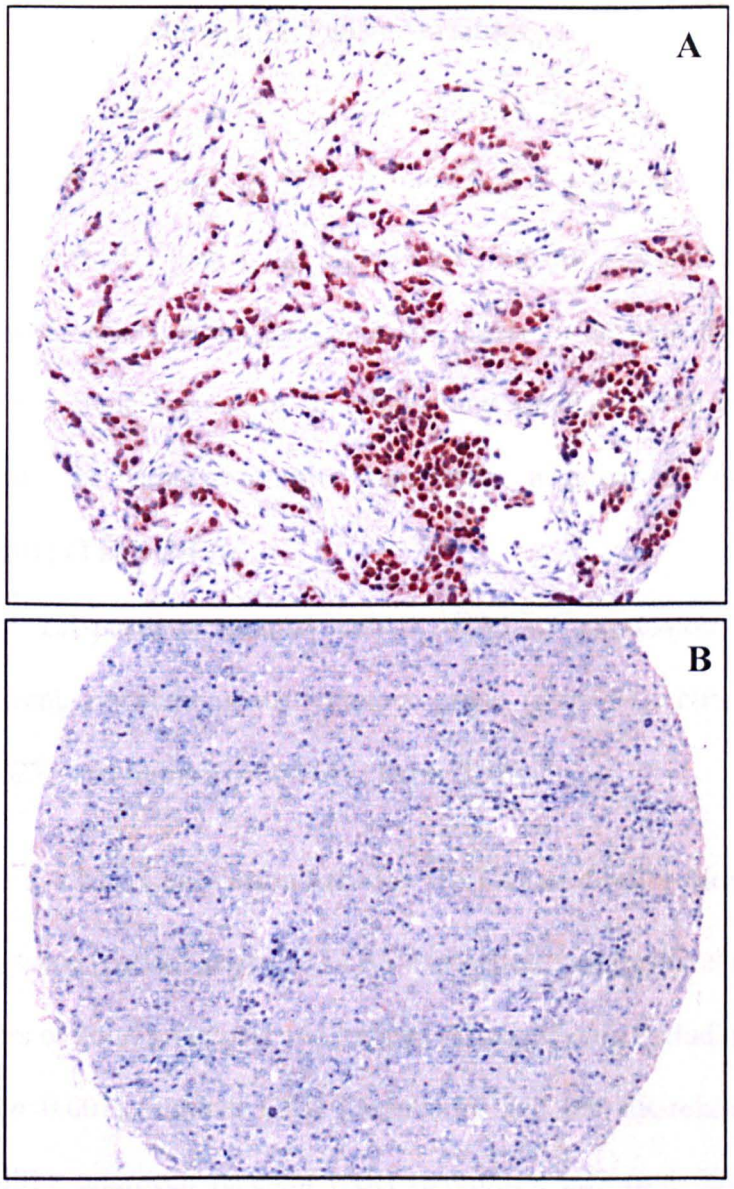


**Figure 3.8:** GATA3 expression in breast cancer

(A) Strong nuclear expression in grade 2 ductal carcinoma (x100)
(B) Negative expression in grade 3 invasive ductal carcinoma (x100)

#### 3.4.3.3 Correlation between GATA3 expression and other clinicopathological variables

In the whole series, high GATA3 expression was positively associated with tumours of small size ($p$=0.002), low tumour grade ($p$<0.001), low mitotic counts ($p$<0.001), and good NPI group ($p$<0.001). GATA3 expression was associated with tumours that were less likely to develop DM ($p$=0.004) and showed a high expression in tubular and tubular mixed tumour types. In contrast, the expression was completely negative in medullary cancer ($p$<0.001) **(Table 3.12)**.

In the ER-positive luminal cohort, GATA3 expression showed similar significant associations with tumour grade ($p$<0.001), NPI ($p$<0.001), DM ($p$=0.023), and mitosis ($p$<0.001) **(Table 3.13)**.

#### 3.4.3.1 Correlation between GATA3 and other biomarkers

In the whole patient series, GATA3 expression was positively associated with markers of good prognosis and luminal differentiation including ER ($p$<0.001), PgR ($p$<0.001), luminal CKs, E-cadherin, and the ER-related gene FOXA1 ($p$<0.001), androgen receptor (AR) ($p$<0.001), p27 ($p$<0.001) and Bcl-2. In contrast, GATA3 expression was inversely associated with the expression of basal CKs, Ki67 (MIB1) ($p$<0.001), p53 ($p$<0.001) and HER2 **(Table 3.14)**.

In the ER-positive luminal-like group, GATA3 expression showed significant positive associations with PgR, p27 and FOXA1. In contrast, it was inversely associated with MIB1 expression **(Table 3.15)**.

**Table 3.12:** Relation of GATA3 expression to other clinicopathological variables in the whole series

| Variable | GATA3 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | High | Total | | |
| **Patients' age** | | | | 2.411 | 0.492 |
| <40 | 62(76.5) | 19(23.5) | 81 | | |
| 40-50 | 220(73.1) | 81(26.9) | 301 | | |
| 51-60 | 266(78.2) | 74(21.8) | 340 | | |
| >60 | 240(75) | 80(25) | 320 | | |
| **Tumour size** | | | | 10.039 | 0.002 |
| **≤1.5 cm** | 361(71.2) | 146(28.8) | 507 | | |
| >1.5 cm | 423(79.7) | 108(20.3) | 531 | | |
| **Lymph node stage** | | | | 3.656 | 0.161 |
| 1(Negative) | 467(73.5) | 168(26.5) | 635 | | |
| 2(1-3 LN) | 240(78.2) | 67(21.8) | 307 | | |
| 3(>3 LN) | 77(80.2) | 19(19.8) | 96 | | |
| **Tumour Grade** | | | | 118.2 | <0.001 |
| 1 | 97(60.2) | 64(39.8) | 161 | | |
| 2 | 194(59.9) | 130(40.1) | 324 | | |
| 3 | 493(89.2) | 60(10.8) | 553 | | |
| **NPI** | | | | 64.783 | <0.001 |
| Good | 160(58.4) | 114(41.6) | 274 | | |
| Moderate | 467(79.7) | 119(20.3) | 586 | | |
| Poor | 158(88.3) | 21(11.7) | 179 | | |
| **DM** | | | | 8.329 | 0.004 |
| No | 517(72.9) | 192(27.1) | 709 | | |
| Positive | 264(81.2) | 61(18.8) | 325 | | |
| **Recurrence** | | | | 2.825 | 0.107 |
| No | 424(73.4) | 154(26.6) | 578 | | |
| Positive | 346(77.9) | 98(22.1) | 444 | | |
| **VI** | | | | 11.673 | 0.003 |
| No | 417(74.1) | 146(25.9) | 563 | | |
| Probable | 85(67.5) | 41(32.5) | 126 | | |
| Definite | 281(81.4) | 64(18.6) | 345 | | |
| **Histologic tumour type** | | | | 61.832 | <0.001 |
| Ductal/NST | 520(82.3) | 112(17.7) | 632 | | |
| Lobular | 53(58.9) | 37(41.1) | 90 | | |
| Tubular and Tubular mixed | 127(64.5) | 70(35.5) | 197 | | |
| Medullary | 28(100) | 0(0) | 28 | | |
| Other special types* | 8(47.1) | 9(52.9) | 17 | | |
| Mixed** | 40(67.8) | 19(32.2) | 59 | | |
| **Mitosis** | | | | 118.3 | <0.001 |
| 1 | 183(57.2) | 137(42.8) | 320 | | |
| 2 | 137(71) | 56(29) | 193 | | |
| 3 | 445(90.1) | 49(9.9) | 494 | | |

*Includes Mucoid, invasive cribriform and invasive papillary carcinoma, ** Include ductal/NST mixed with lobular or special type

**Table 3.13:** Relation of GATA3 expression to other biomarkers in the ER-positive cohort

| Variable | GATA3 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | High | Total | | |
| **Patients' age** | | | | 10.816 | .0130 |
| <40 | 21(52.5) | 19(47.5) | 40 | | |
| 40-50 | 108(60) | 72(40) | 180 | | |
| 51-60 | 150(69.4) | 66(30.6) | 216 | | |
| >60 | 173(71.8) | 68(28.2) | 68 | | |
| **Tumour size** | | | | 2.234 | 0.142 |
| **≤1.5 cm** | 228(64 | 128(36) | 356 | | |
| >1.5 cm | 223(69.5) | 98(30.5) | 321 | | |
| **Lymph node stage** | | | | 5.034 | 0.081 |
| 1(Negative) | 258(63.2) | 150(36.8) | 408 | | |
| 2(1-3 LN) | 153(71.8) | 60(28.2) | 213 | | |
| 3(>3 LN) | 38(70.4) | 16(29.6) | 54 | | |
| **Tumour Grade** | | | | 35.505 | <0.001 |
| 1 | 84(61.3) | 53(38.7) | 137 | | |
| 2 | 158(56.6) | 121(43.4) | 276 | | |
| 3 | 209(80.1) | 52(19.9) | 261 | | |
| **NPI** | | | | 18.393 | <0.001 |
| Good | 134(57.5) | 99(42.5) | 233 | | |
| Moderate | 241(68.9) | 109(31.1) | 350 | | |
| Poor | 77(81.1) | 18(18.9) | 95 | | |
| **DM** | | | | 5.386 | 0.023 |
| No | 309(64.1) | 173(35.9) | 482 | | |
| Positive | 141(73.4) | 51(26.6) | 192 | | |
| **Recurrence** | | | | 1.601 | 0.213 |
| No | 254(64.6) | 139(35.4) | 393 | | |
| Positive | 192(69.3) | 85(30.7) | 277 | | |
| **VI** | | | | 8.274 | 0.016 |
| No | 229(64.1) | 128(35.9) | 357 | | |
| Probable | 56(59.6) | 38(40.4) | 94 | | |
| Definite | 166(73.8) | 59(26.2) | 225 | | |
| **Histologic tumour type** | | | | 12.869 | 0.045 |
| Ductal/NST | 256(71.1) | 104(28.9) | 360 | | |
| Lobular | 48(61.5) | 30(38.5) | 78 | | |
| Tubular and Tubular mixed | 106(62.7) | 63(37.3) | 169 | | |
| Medullary | 4(100) | 0(0) | 4 | | |
| Other special types | 4(36.4) | 7(63.6) | 11 | | |
| Mixed | 31(62) | 19(38) | 50 | | |
| **Mitosis** | | | | 34.955 | <0.001 |
| 1 | 156(55.7) | 124(44.3) | 280 | | |
| 2 | 103(66.5) | 52(33.5) | 155 | | |
| 3 | 180(80.7) | 43(19.3) | 223 | | |

**Table 3.14:** Relation of GATA3 expression to other biomarkers in the whole series

| Variable | GATA3 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | High | Total | | |
| **CK5/6** | | | | 18.776 | <0.001 |
| Negative | 601(72.9) | 223(27.1) | 824 | | |
| Positive | 158(88.3) | 21(11.7) | 179 | | |
| **CK14** | | | | 8.113 | 0.003 |
| Negative | 647(74.6) | 220(25.4) | 867 | | |
| Positive | 107(86.3) | 17(13.7) | 124 | | |
| **CK18** | | | | 37.064 | <0.001 |
| Negative | 140(95.9) | 6(4.1) | 146 | | |
| Positive | 570(72.5) | 216(27.5) | 786 | | |
| **CK19** | | | | 12.205 | <0.001 |
| Negative | 100(89.3) | 12(10.7) | 112 | | |
| Positive | 663(74.3) | 229(25.7) | 892 | | |
| **ER** | | | | 110.5 | <0.001 |
| Negative | 294(97.7) | 7(2.3) | 301 | | |
| Positive | 452(66.7) | 226(33.3) | 678 | | |
| **PgR** | | | | 69.659 | <0.001 |
| Negative | 394(88.3) | 52(11.7) | 446 | | |
| Positive | 348(65.4) | 184(34.6) | 532 | | |
| **AR** | | | | 63.081 | <0.001 |
| Negative | 328(90.1) | 36(9.9) | 364 | | |
| Positive | 383(67.4) | 185(32.6) | 568 | | |
| **p53** | | | | 18.495 | <0.001 |
| Negative | 499(72.4) | 190(27.6) | 689 | | |
| Positive | 248(85.2) | 43(14.8) | 291 | | |
| **MIB1** | | | | 22.539 | <0.001 |
| low | 134(62.3) | 81(37.7) | 215 | | |
| High | 475(78.8) | 128(21.2) | 603 | | |
| **P-cadherin** | | | | 24.698 | <0.001 |
| Negative | 256(68.1) | 124(31.9) | 389 | | |
| Positive | 379(82.8) | 79(17.2) | 458 | | |
| **E-cadherin** | | | | 5.690 | <0.001 |
| Negative | 293(80.7) | 70(19.3) | 363 | | |
| Positive | 459(74) | 161(26) | 620 | | |
| **HER2** | | | | 14.457 | <0.001 |
| Negative | 635(74.4) | 219(25.6) | 845 | | |
| Positive | 128(88.9) | 16(11.1) | 144 | | |
| **p27** | | | | 33.667 | <0.001 |
| Negative | 321(87.7) | 45(12.3) | 366 | | |
| Positive | 243(70) | 104(30) | 347 | | |
| **EGFR** | | | | 4.194 | 0.041 |
| Negative | 527(74.5) | 180(25.5) | 707 | | |
| Positive | 141(82) | 31(18) | 172 | | |
| **Bcl-2** | | | | 47.111 | <0.001 |
| Negative | 276(89) | 34(11) | 310 | | |
| Positive | 315(67.7) | 151(32.4) | 466 | | |
| **FOXA1** | | | | 67.218 | <0.001 |
| Negative | 356(89.9) | 40(10.1) | 396 | | |
| Positive | 218(64.9) | 118(35.1) | 336 | | |

**Table 3.15:** Relation of GATA3 expression to other biomarkers in the ER-positive cohort

| Variable | GATA3 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | High | Total | | |
| **CK5/6** | | | | 0.142 | 0.756 |
| Negative | 411(66.6) | 206(33.4) | 617 | | |
| Positive | 32(64) | 18(36) | 50 | | |
| **CK14** | | | | 0.017 | 1.000 |
| Negative | 409(67) | 201(33) | 610 | | |
| Positive | 36(67.9) | 17(32.1) | 53 | | |
| **CK18** | | | | 3.701 | 0.071 |
| Negative | 25(83.3) | 5(16.7) | 30 | | |
| Positive | 404(66.4) | 204(33.6) | 608 | | |
| **CK19** | | | | 0.843 | 0.398 |
| Negative | 30(73.2) | 11(26.8) | 41 | | |
| Positive | 419(66.2) | 214(33.8) | 633 | | |
| **PgR** | | | | 4.154 | 0.046 |
| Negative | 121(72.9) | 45(27.1) | 166 | | |
| Positive | 322(64.3) | 179(35.7) | 501 | | |
| **AR** | | | | 10.888 | 0.001 |
| Negative | 121(78.1) | 34(21.9) | 155 | | |
| Positive | 308(63.8) | 175(36.2) | 483 | | |
| **p53** | | | | 0.012 | 1.000 |
| Negative | 363(67) | 179(33) | 542 | | |
| Positive | 83(67.5) | 40(32.5) | 123 | | |
| **MIB1** | | | | 9.138 | 0.003 |
| Low | 98(56.3) | 76(43.7) | 174 | | |
| High | 258(69.5) | 113(30.5) | 371 | | |
| **P-cadherin** | | | | 0.241 | 0.654 |
| Negative | 228(65.7) | 119(34.3) | 347 | | |
| Positive | 157(67.7) | 75(32.3) | 323 | | |
| **E-cadherin** | | | | 3.504 | 0.068 |
| Negative | 163(71.8) | 64(28.2) | 227 | | |
| Positive | 283(64.6) | 155(35.4) | 438 | | |
| **HER2** | | | | 4.856 | 0.030 |
| Negative | 396(66) | 204(34) | 600 | | |
| Positive | 48(80) | 12(20) | 60 | | |
| **p27** | | | | 6.720 | 0.011 |
| Negative | 143(78.6) | 39(21.4) | 182 | | |
| Positive | 191(67.5) | 92(32.5) | 283 | | |
| **EGFR** | | | | 0.044 | 0.902 |
| Negative | 344(66.7) | 172(33.3) | 516 | | |
| Positive | 59(67.7) | 28(32.2) | 87 | | |
| **Bcl-2** | | | | 6.373 | 0.014 |
| Negative | 92(76.7) | 28(23.3) | 120 | | |
| Positive | 256(64.3) | 142(35.7) | 398 | | |
| **FOXA1** | | | | 30.612 | <0.001 |
| Negative | 181(82.6) | 38(17.4) | 219 | | |
| Positive | 157(59.5) | 107(40.5) | 264 | | |

### 3.4.3.2 Correlation between GATA3 expression and patient outcome

**(A) Univariate analysis**

Breast cancer patients with strong GATA3 expression showed a significantly longer BCSS (LR=16.329, $p$<0.001) **(Fig 3.9A)** and longer DMFI (LR=13.067, $p$<0.001) **(Fig 3.9B).**

Similar associations were found in the ER-positive luminal group in terms of BCSS (LR=10.149, $p$=0.001; **Fig 3.10A**) and DMFI (LR=7.153, $p$=0.007; **Fig 3.10B**).

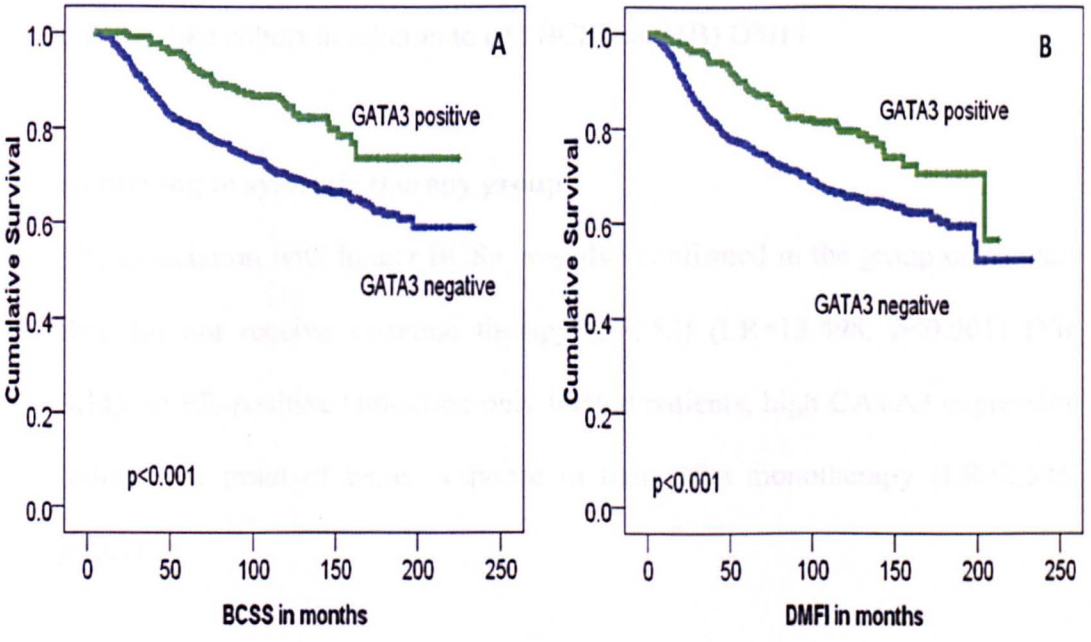


**Figure 3.9:** Kaplan Meier plots of GATA3 expression in the whole series in relation to (A) BCSS and (B) DMFI

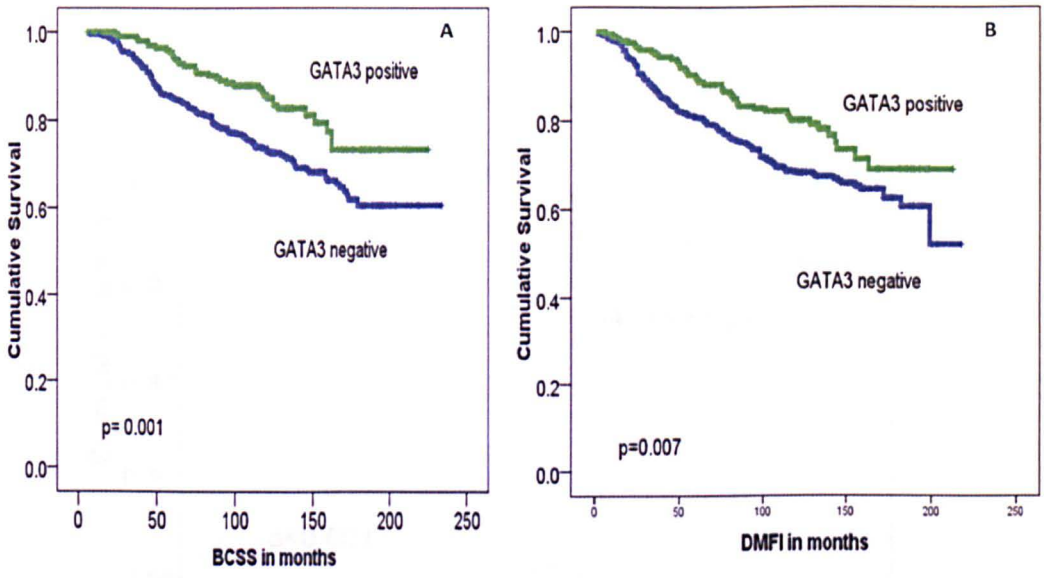


**Figure 3.10:** Kaplan Meier plots of GATA3 expression in ER-positive luminal-like cohort in relation to (A) BCSS and (B) DMFI

**According to systemic therapy groups**

The association with longer BCSS was also confirmed in the group of patients that did not receive systemic therapy (n=352) (LR=13.498, $p$<0.001) **(Fig 3.11)**. In ER-positive tamoxifen-only treated patients, high GATA3 expression indicated a trend of better response to tamoxifen monotherapy (LR=2.546, $p$=0.111).

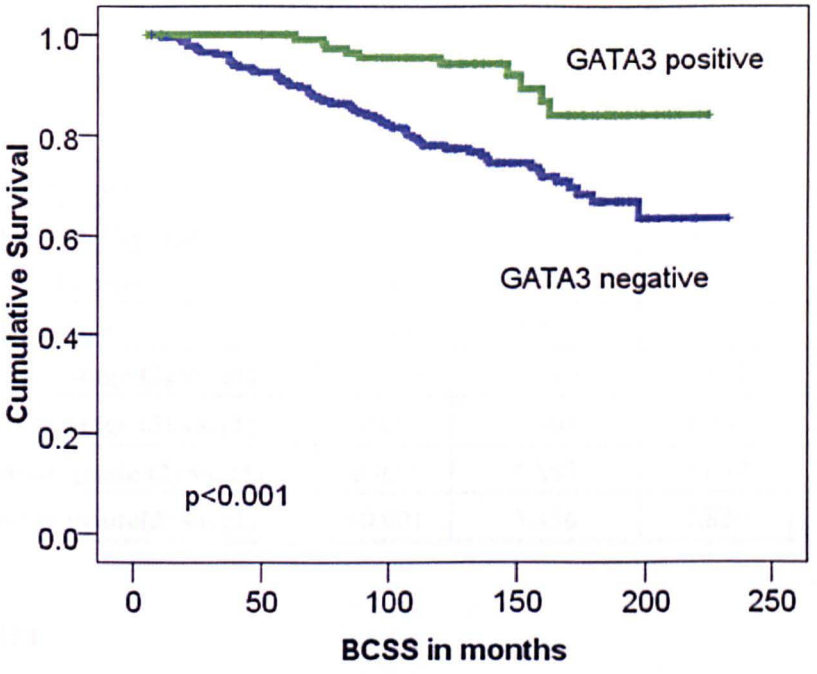


**Figure 3.11:** Kaplan Meier plot of GATA3 expression in untreated patient group in relation to BCSS

**(B) Multivariate analysis**

**BCSS**

Multivariate Cox regression analyses including tumour size, tumour grade, lymph node stage and systemic therapy groups showed that GATA3 expression was an independent prognostic marker for longer BCSS in the whole series (HR=0.665, $p$=0.030, 95% CI =0.459-0.762) and with border-line significance in the ER-positive luminal-like cohort (HR =0.666, $p$=0.056, 95% CI =0.439-1.010) **(Table 3.16)**.

**Table 3.16:** COX analysis model of GATA3 protein expression, tumour grade, LN stage, tumour size and adjuvant therapies in the ER-positive cohort

| Variable | p value | HR | 95 % CI | |
|---|---|---|---|---|
| | | | Lower | Upper |
| **GATA3 expression** | 0.056 | 0.666 | 0.439 | 1.010 |
| **Endocrine Therapy** | 0.120 | 0.697 | 0.443 | 1.099 |
| **Chemotherapy** | 0.213 | 0.695 | 0.392 | 1.233 |
| **Tumour size** | <0.001 | 2.272 | 1.562 | 3.304 |
| **Tumour stage (2) vs. (1)** | <0.001 | 2.246 | 1.484 | 3.400 |
| **Tumour stage (3) vs. (1)** | <0.001 | 3.407 | 1.980 | 5.864 |
| **Tumour grade (2) vs. (1)** | 0.037 | 1.883 | 1.037 | 3.418 |
| **Tumour grade(3) vs. (1)** | <0.001 | 3.436 | 1.820 | 6.485 |

**DMFI**

Multivariate Cox regression analysis including the same well-established prognostic variables as above showed that GATA3 expression was an independent prognostic marker for longer DMFI in the whole series (HR=0.682, $p$=0.028, 95% CI =0.484-0.960) but not in the ER-positive luminal-like cohort (HR =0.715, $p$ =0.085, 95% CI =0.488-1.047).

## 3.5 XBP1

### 3.5.1 Introduction

Human X box-binding protein 1 (XBP-1) was originally identified as a protein binding to the cis-acting X box which presents in the promoter regions of target genes (Lacroix and Leclercq, 2004). Gene expression profiling of breast cancer tissue has previously shown an association between ER and XBP-1 expression because of its association with Luminal A breast cancer (Sorlie et al., 2001).

XBP1 is stimulated by endoplasmic reticulum stress as part of the unfolded protein response (UPR). UPR is a cellular stress response related to the endoplasmic reticulum. If the stress is weak, this pathway acts in a protective manner, while if the stress is strong, it will induce apoptosis. Over-expression of the UPR may also be clinically important because it reduces the effect of certain types of chemotherapy, such as doxorubicin (Scriven et al., 2009).

XBP-1 is a key transcriptional regulator of the UPR that activates genes involved in protein folding, secretion, and degradation to restore endoplasmic reticulum function (Hetz et al., 2008, Yoshida et al., 2001).

Romero-Ramirez and co-workers studied the effect of hypoxia on XBP1 in vitro using mouse embryonic fibroblasts. They showed that hypoxia could increase XBP1 at the transcriptional level and activated splicing of its mRNA, resulting in increased levels of XBP1 protein. After exposure to hypoxia, the XBP1-deficient cells showed increased apoptosis while loss of XBP1 significantly inhibited tumour growth due to a reduced capacity of the tumour cells to survive in a hypoxic microenvironment. They concluded that, XBP1 is

an essential survival factor for hypoxic stress and tumour growth which could be targeted therapeutically to eliminate hypoxia and inhibit tumour proliferation (Romero-Ramirez et al., 2004).

The unfolded protein response as regulated by XBP1 and GRP78 was associated with a more favourable course of the disease of acute myeloid leukaemia (Schardt et al., 2009).

The oestrogenic stimulation was sufficient to induce downstream regulators of UPR activation such as XBP1 (Rzymski and Harris, 2007) and this may explain why XBP1 is identified as a Luminal A marker in the gene expression studies (Sorlie et al., 2001). Previously, oestrogen stimulation has also induced XBP1 overexpression on western blotting analysis study (Scriven et al., 2009). In another study, the XBP1 mRNA expression in ER-positive breast cancers was 2.7 fold as much as that in ER negative breast cancers (Bertucci et al., 2000).

Since the XBP1 mRNA expression pattern is correlated with ER and upregulated in the luminal subset of breast cancers (West et al., 2001, Sorlie et al., 2001), it may play an important role in luminal ER breast cancer growth and represent a new target for therapeutic intervention.

Because of the importance of ER signalling in the regulation of breast cancer development and progression, the potential role of XBP1 in the ER-positive breast cancer biology and subclassification was investigated

### 3.5.2 Material and Methods

Tissue microarrays were prepared and the immunohistochemical staining and optimization was performed using the streptavidin-biotin complex method using DakoCytomation Techmate 500 plus (DakoCytomation, Cambridge, UK) (general material and methods chapter). To unmask the antigens, the sections were microwaved in Tris EDTA buffer pH 8.5 for 23 minutes. Rabbit polyclonal XBP1 antibody (NB100-80861, Novus Biologicals Inc., Littleton, CO, USA) was optimized at a working dilution of 0.5µ/ml using full-face sections and TMAs to assess the staining distribution.

Negative controls were performed by omitting the primary antibody and substitution with diluent. Positive breast cancer cases were used as positive controls. Scoring was performed using a web-based interface (Distiller; Slidepath Ltd, Dublin, Ireland) using the intensity of the cytoplasmic staining. For the intensity, a score of 0, 1, 2 and 3 was used.

### 3.5.3 XBP1 immunohistochemical results

XBP1 was detected in the cytoplasm of the malignant breast cancer cells. After excluding the uninformative TMA cores from the study, 1111 tumours were available for XBP1 assessment. In the whole series, 10.3% of cases were negative for XBP1 protein expression, 38% showed weak expression, 36.9% showed moderate expression and 14.8% had a strong expression. In the ER-positive cohort (n=760), 9.1% of tumours were negative, 39.2% showed weak expression, 36.7% showed moderate expression and 15% were strongly positive **(Fig 3.12A&B)**. Patients with strong XBP1 expression showed a

shorter survival in comparison to other groups in the ER-positive cohort, subsequently, the data was categorised into two groups of strong expression versus the others.

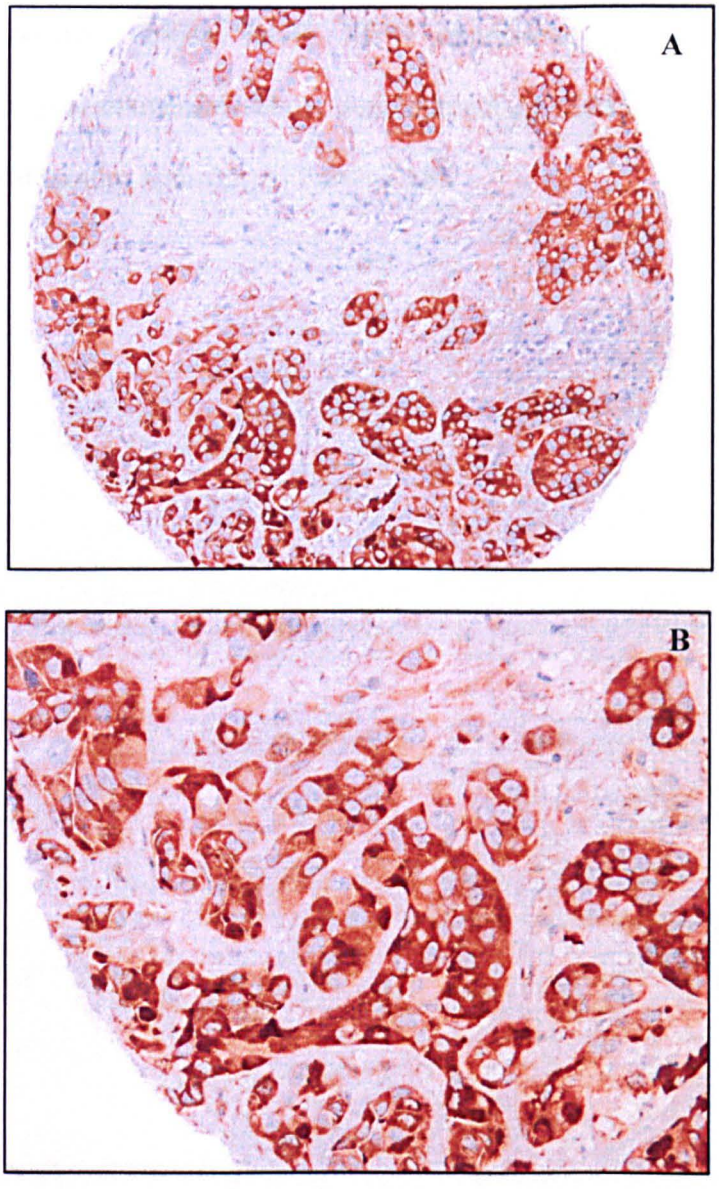


**Figure 3.12:** XBP1 strong cytoplasmic expression in grade 2 ductal cancer

A) Low magnification (x100) B) High magnification (x200)

#### 3.5.3.1 Correlation between XBP1 expression and other clinicopathological variables

In the whole series, XBP1 expression was associated with younger age ($p$=0.002) and premenopausal status ($p$<0.001) as summarised in **(Table 3.17)**.

In the ER-positive cohort, strong XBP1 expression was associated with younger age, premenopausal status, development of distant metastasis (DM) ($p$=0.001) and tumour recurrence **(Table 3.18)**.

**Table 3.17:** Relation of XBP1 expression to other clinicopathological variables in the whole series

| Variable | XBP1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | High | Total | | |
| **Patients' age** | | | | 15.284 | 0.002 |
| <40 | 63(84) | 12(16) | 75 | | |
| 40-50 | 253(79.3) | 66(20.7) | 319 | | |
| 51-60 | 312(86.2) | 50 (13.8) | 362 | | |
| >60 | 319(89.9) | 36(10.1) | 355 | | |
| **Tumour size** | | | | 2.987 | 0.087 |
| **≤1.5 cm** | 319(87.9) | 44(12.1) | 363 | | |
| >1.5 cm | 628(84) | 120(16) | 748 | | |
| **Lymph node stage** | | | | 5.087 | 0.079 |
| 1(Negative) | 584(86.6) | 90(13.4) | 674 | | |
| 2(1-3 LN) | 276(84.4) | 51(15.6) | 327 | | |
| 3(>3 LN) | 84(78.5) | 23(21.5) | 107 | | |
| **Tumour Grade** | | | | 3.055 | 0.217 |
| 1 | 158(85.5) | 27(14.6) | 185 | | |
| 2 | 327(87.7) | 46(12.3) | 373 | | |
| 3 | 461(83.5) | 91(16.5) | 552 | | |
| **NPI** | | | | 5.725 | 0.057 |
| Good | 273(88.1) | 37(11.9) | 310 | | |
| Moderate | 524(85.3) | 90(14.7) | 614 | | |
| Poor | 150(80.2) | 37(19.8) | 187 | | |
| **DM** | | | | 3.629 | 0.063 |
| No | 662(86.9) | 100(13.1) | 762 | | |
| Positive | 278(82.5) | 59(17.5) | 337 | | |
| **Recurrence** | | | | 4.236 | 0.046 |
| No | 546(87.2) | 80(12.8) | 626 | | |
| Positive | 384(82.8) | 80(17.2) | 464 | | |
| **VI** | | | | 5.010 | 0.082 |
| No | 533(86) | 87(14) | 620 | | |
| Probable | 115(89.8) | 13(10.2) | 128 | | |
| Definite | 296(82.2) | 64(17.8) | 360 | | |
| **Histologic tumour type** | | | | 7.768 | 0.169 |
| Ductal/NST | 544(84.1) | 103(15.9) | 647 | | |
| Lobular | 111(92.5) | 9(7.5) | 120 | | |
| Tubular and Tubular mixed | 199(86.5) | 31(13.5) | 130 | | |
| Medullary | 25(80.6) | 6(19.4) | 31 | | |
| Other special types | 13(76.5) | 4(23.5) | 17 | | |
| Mixed | 55(83.3) | 11(16.7) | 66 | | |
| **Mitosis** | | | | 5.196 | 0.074 |
| 1 | 332(88.1) | 45(11.9) | 377 | | |
| 2 | 167(87.4) | 24(12.6) | 191 | | |
| 3 | 419(83) | 86(17) | 505 | | |
| **Menopausal status** | | | | 14.728 | <0.001 |
| Premenopausal | 336(80) | 84(20) | 420 | | |
| Postmenopausal | 611(88.4) | 80(11.6) | 691 | | |

**Table 3.18:** Relation of XBP1expression to other biomarkers in the ER-positive cohort of patient

| Variable | XBP1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | High | Total | | |
| **Patients' age** | | | | 12.976 | 0.005 |
| <40 | 25(75.8) | 8(24.2) | 33 | | |
| 40-50 | 162(78.6) | 44(21.4) | 206 | | |
| 51-60 | 162(87.2) | 32(12.8) | 250 | | |
| >60 | 241(88.9) | 30(11.1) | 271 | | |
| **Tumour size** | | | | 3.147 | 0.089 |
| **≤1.5 cm** | 237(88.1) | 32(11.9) | 269 | | |
| >1.5 cm | 409(83.3) | 82(16.7) | 491 | | |
| **Lymph node stage** | | | | 4.836 | 0.089 |
| 1(Negative) | 394(86.4) | 62(13.6) | 456 | | |
| 2(1-3 LN) | 203(84.6) | 37(15.4) | 240 | | |
| 3(>3 LN) | 47(75.8) | 15(24.2) | 62 | | |
| **Tumour Grade** | | | | 3.078 | 0.215 |
| 1 | 139(85.3) | 24(14.7) | 163 | | |
| 2 | 287(87.2) | 42(12.8) | 329 | | |
| 3 | 220(82.1) | 48(17.9) | 268 | | |
| **NPI** | | | | 3.946 | 0.139 |
| Poor | 239(87.9) | 33(12.1) | 272 | | |
| Moderate | 323(84.3) | 60(15.7) | 383 | | |
| Good | 84(80) | 21(20) | 105 | | |
| **DM** | | | | 11.412 | 0.001 |
| No | 473(87.9) | 65(12.1) | 538 | | |
| Positive | 169 (78.2) | 47(21.8) | 216 | | |
| **Recurrence** | | | | 13.474 | <0.001 |
| No | 393(88.9) | 49(11.1) | 442 | | |
| Positive | 243(79.2) | 64(20.8) | 307 | | |
| **VI** | | | | 3.907 | 0.142 |
| No | 354(86.1) | 57(13.9) | 411 | | |
| Probable | 93(88.6) | 12(11.4) | 105 | | |
| Definite | 197(81.4) | 45(18.6) | 242 | | |
| **Histologic type** | | | | 8.180 | 0.147 |
| Ductal/NST | 311(83.4) | 62(16.6) | 373 | | |
| Lobular | 100(91.7) | 9(8.3) | 109 | | |
| Tubular | 173(86.1) | 28(13.9) | 201 | | |
| Medullary | 4(100) | 0(0) | 4 | | |
| Other special types | 10(71.4) | 4(28.6) | 14 | | |
| Mixed | 48(81.4) | 11 (18.6) | 18.6 | | |
| **Mitosis** | | | | 4.673 | 0.097 |
| 1 | 295(88.1) | 40(11.9) | 335 | | |
| 2 | 134(85.4) | 23(14.6) | 157 | | |
| 3 | 195(81.6) | 44(18.4) | 239 | | |
| **Menopausal status** | | | | 14.860 | <0.001 |
| Premenopausal | 198(78) | 56(22) | 254 | | |
| Postmenopausal | 448(88.5) | 58(11.5) | 506 | | |

### 3.5.3.2 Correlation between XBP1 expression and other biomarkers

In the whole series, XBP1 was associated with the expression of C-MYC, and p53 ($p<0.001$). The tables summarise the correlations. We did not find significant correlation between XBP1 and ER or luminal CKs **(Table 3.19)**.

In the ER-positive luminal-like cohort, XBP1 was associated with the expression of p53 ($p<0.001$) **(Table 3.20)**.

**Table 3.19:** Relation of XBP1 expression to other biomarkers in the whole series of breast cancer patients

| Variable | XBP1 Expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | High | Total | | |
| **CK5/6** | | | | 0.244 | 0.647 |
| Negative | 766(85.5) | 130(14.5) | 896 | | |
| Positive | 153(84.1) | 29(15.9) | 182 | | |
| **CK14** | | | | 0.298 | 0.698 |
| Negative | 780(84.9) | 139(15.1) | 919 | | |
| Positive | 117(86.7) | 18(13.3) | 135 | | |
| **CK18** | | | | 0.919 | 0.355 |
| Negative | 109(82.6) | 23(17.4) | 132 | | |
| Positive | 734(85.7) | 122(14.3) | 856 | | |
| **CK19** | | | | 0.335 | 0.650 |
| Negative | 82(87.2) | 12(12.8) | 94 | | |
| Positive | 834(85) | 147(15) | 981 | | |
| **ER** | | | | 0.001 | 1.000 |
| Negative | 250(85) | 44(15) | 294 | | |
| Positive | 646(85) | 114(15) | 760 | | |
| **PgR** | | | | 0.070 | 0.861 |
| Negative | 399(85.4) | 68(14.6) | 467 | | |
| Positive | 493(84.9) | 88(15.1) | 581 | | |
| **p53** | | | | 14.497 | <0.001 |
| Negative | 678(87.7) | 95(12.3) | 773 | | |
| Positive | 220(78.3) | 61(21.7) | 281 | | |
| **AR** | | | | 1.000 | 0.352 |
| Negative | 309(84) | 59(16) | 368 | | |
| Positive | 541(86.3) | 86(13.7) | 627 | | |
| **MIB1** | | | | 1.162 | 0.299 |
| Low | 212(86.5) | 33(13.5) | 245 | | |
| High | 514(83.6) | 101(16.4) | 615 | | |
| **P-cadherin** | | | | 0.116 | 0.777 |
| Negative | 339(84.5) | 62(15.5) | 401 | | |
| Positive | 414(85.4) | 71(14.6) | 585 | | |
| **E-cadherin** | | | | 1.562 | 0.244 |
| Negative | 359(87.1) | 53(12.9) | 412 | | |
| Positive | 539(84.4) | 100(15.6) | 639 | | |
| **HER2** | | | | 0.049 | 0.797 |
| Negative | 790(85.5) | 134(14.5) | 924 | | |
| Positive | 117(84.8) | 21(15.2) | 138 | | |
| **C-MYC** | | | | 37.951 | <0.001 |
| Negative | 117(92.9) | 9(7.1) | 126 | | |
| Low | 294(89.4) | 35(10.6) | 329 | | |
| Moderate | 287(84.9 | 51(15.1) | 51 | | |
| High | 118(70.7) | 49(29.3) | 167 | | |

**Table 3.20:** Relation of XBP1 expression to other biomarkers in the ER-positive cohort

| Variable | XBP1 Expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | High | Total | | |
| **CK5/6** | | | | 0.235 | 0.844 |
| Negative | 588(84.8) | 105(15.2) | 693 | | |
| Positive | 48(87.3) | 7(12.7) | 55 | | |
| **CK14** | | | | 0.355 | 0.700 |
| Negative | 574(84.8) | 103(15.2) | 677 | | |
| Positive | 50(87.7) | 7(12.3) | 57 | | |
| **CK18** | | | | 0.361 | 0.785 |
| Negative | 25(89.3) | 3(10.7) | 28 | | |
| Positive | 581(85.2) | 101(14.8) | 682 | | |
| **CK19** | | | | 1.284 | 0.341 |
| Negative | 33(91.7) | 3(8.3) | 36 | | |
| Positive | 607(84.8) | 109(15.2) | 716 | | |
| **PgR** | | | | 0.605 | 0.481 |
| Negative | 164(86.8) | 25(13.2) | 189 | | |
| Positive | 472(84.4) | 87(15.6) | 559 | | |
| **AR** | | | | 0.114 | 0.802 |
| Negative | 146(86.4) | 23(13.6) | 169 | | |
| Positive | 460(85.3) | 79(14.7) | 539 | | |
| **p53** | | | | 15.754 | <0.001 |
| Negative | 538(87.6) | 76(12.4) | 614 | | |
| Positive | 101(74.3) | 35(25.7) | 136 | | |
| **MIB1** | | | | 0.416 | 0.552 |
| Low | 175(85.8) | 29(14.2) | 204 | | |
| High | 325(83.8) | 63(16.2) | 388 | | |
| **P-cadherin** | | | | 0.366 | 0.576 |
| Negative | 310(84.5) | 57(15.5) | 367 | | |
| Positive | 237(86.2) | 38(13.8) | 275 | | |
| **E-cadherin** | | | | 1.521 | 0.243 |
| Negative | 249(87.4) | 36(12.6) | 285 | | |
| Positive | 391(84.1) | 74(15.9) | 465 | | |
| **HER2** | | | | 2.989 | 0.119 |
| Negative | 587(85.7) | 98(14.3) | 685 | | |
| Positive | 44(77.2) | 13(22.8) | 57 | | |
| **C-MYC** | | | | 9.960 | 0.019 |
| Negative | 75(91.5) | 7(8.5) | 82 | | |
| Low | 197(87.9) | 27(12.1) | 224 | | |
| Moderate | 206(84.4) | 38(15.6) | 244 | | |
| Strong | 79(76.7) | 96(14.7) | 103 | | |

### 3.5.3.3 Correlation between XBP1 expression and patient outcome

In whole series, XBP1 strong cytoplasmic expression was not associated with patients' survival. In the ER-positive cohort, XBP1 strong cytoplasmic intensity was associated with shorter breast cancer specific survival (BCSS) ($p$=0.012) **(Fig 3.13A)** and shorter distant metastasis free interval (DMFI) ($p$=0.008) **(Fig 3.13B).** In ER-positive cohort, multivariate analysis of XBP1 expression, tumour size, tumour grade and lymph node stage showed that XBP1 was not an independent prognostic factor in relation to BCSS (HR=1.277, $p$=0.186, 95%CI=0.889-1.835) and DMFI (HR=1.261, $p$=0.181, 95%CI=0.898-1.772)

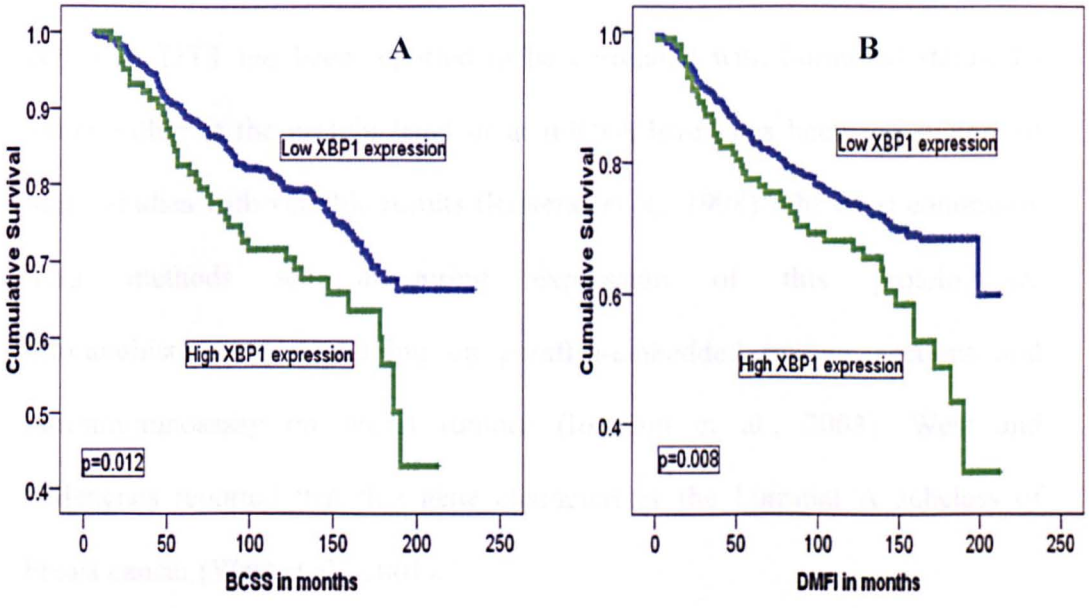


**Figure 3.13:** Kaplan Meier plots of XBP1 protein expression in ER-positive luminal-like cohort in relation to (A) BCSS (B) DMFI

## 3.6 TFF1

### 3.6.1 Introduction

Trefoil factor-1 (TFF1), also known as pS2, belongs to the family of TFFs that share a characteristic three loop structure named trefoil or P domain. The human TFF1 protein is predominantly expressed in the surface epithelial cells of the gastric mucosa and usually increases during mucosal inflammation. Functionally, TFF1 is a secreted protein that stabilizes the mucous gel overlying the gastrointestinal mucosa to provide a physical barrier against various irritating agents (Ioachim et al., 2003).

TFF1 protein expression is induced by oestrogen through oestrogen receptors (ER), and is known to be inhibited by antiestrogen in the MCF-7 breast cancer cell line. TFF1 has been reported to be correlated with hormonal status. Its value, either at the protein level or at mRNA level, has been the subject of many studies with variable results (Ribieras et al., 1998). The most commonly used methods for measuring expression of this protein are immunohistochemical staining on paraffin-embedded tumour sections and radioimmunoassay on breast tumour (Ioachim et al., 2003). West and colleagues reported that this gene characterizes the Luminal A subclass of breast cancer (West et al., 2001).

Although it is associated with ER-positive status, a recent study by has suggested a potential oncogenic role of TFF1 (Amiry et al., 2009).

### 3.6.2 Material and Methods

Tissue microarrays were prepared and the immunohistochemical staining was performed using the streptavidin-biotin complex method using DakoCytomation Techmate 500 plus (DakoCytomation, Cambridge, UK) (general material and methods chapter). To unmask the antigens, the sections were microwaved in citrate buffer pH 6 for 23 minutes.

Mouse monoclonal TFF1 antibody (Ab17829, Abcam, UK) was optimized at a working dilution of 1:2000 using full-face sections and TMAs of breast cancer tissue and normal stomach to assess the heterogeneity and staining distribution. Negative controls were performed by omitting the primary antibody and substitution with diluent. Stomach tissue sections were used as positive controls. The median value of the H-score values (H score $\geq$100) was used to categorize the data.

### 3.6.3 TFF1 immunohistochemical results

Of the whole series, 1056 cores were available for assessment. The pattern of expression was cytoplasmic **(Fig 3.14).**

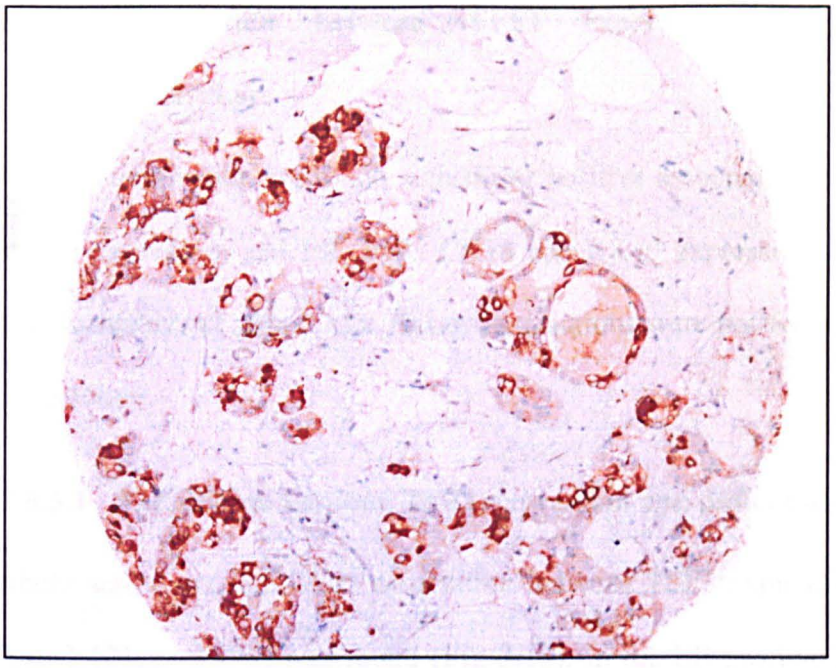

**Figure 3.14:** TFF1 expression in breast cancer

Grade 2 ductal carcinoma with positive TFF1 cytoplasmic expression (x100)

#### 3.6.3.1 Correlation between TFF1 expression and other clinicopathological variables

The relationship with cytoplasmic expression of the TFF1 and the various clinicopathological parameters showed a significant negative correlation with the tumour grade being more positive in low grade tumours ($p$<0.001).

Non-significant correlations were found between TFF1 expression and the other pathological parameters including age, tumour size, lymph node stage, distant metastasis and local recurrence.

No associations were found between TFF1 protein expression and the other clinicopathological variables in the ER-positive cohort **(Table 3.21).**

#### 3.6.3.1 Correlation between TFF1 expression and other biomarkers

In the whole patient series, we found significant positive associations between TFF1 protein expression and ER, PgR, CK18 and CK19 expression, but not with other biomarkers **(Table 3.22).** Theses associations were not found in the ER-positive cohort.

#### 3.6.3.1 Correlation between TFF1 expression and patient outcome

In the whole series, no significant association between TFF1 expression and BCSS (LR=1.101, $p$=0.294) was found **(Fig 3.15).** In the ER-positive patient cohort, we also found no significant association between TFF1 expression and BCSS (LR=0.469, $p$=0.493) **(Fig 3.16)** or DMFI (LR=2.422, $p$=0.120).

**Table 3.21:** Relation of TFF1 immunostaining to other clinicopathological variables in the ER-positive cohort

| Variable | TFF1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Negative | Positive | Total | | |
| **Patients' age** | | | | 4.083 | 0.253 |
| <40 | 17(47.2) | 19(52.8) | 36 | | |
| 40-50 | 79(39.9) | 119(60.1) | 198 | | |
| 51-60 | 109(46.8) | 124(53.2) | 233 | | |
| >60 | 115((49.4) | 118(50.6) | 233 | | |
| **Tumour size** | | | | 0.006 | 0.940 |
| **≤2 cm** | 168(45.8) | 199(54.2) | 367 | | |
| >2 cm | 151(45.5) | 181(54.5) | 332 | | |
| **Lymph node stage** | | | | 0.195 | 0.907 |
| 1(Negative) | 194(46.3) | 225(53.7) | 419 | | |
| 2(1-3 LN) | 98(44.5) | 122(55.5) | 220 | | |
| 3(>3 LN) | 26(44.8) | 32(55.2) | 58 | | |
| **Tumour Grade** | | | | 5.706 | 0.058 |
| 1 | 73(44.2) | 92(55.8) | 165 | | |
| 2 | 115(41.2) | 164(58.8) | 279 | | |
| 3 | 131(51.4) | 124(48.6) | 255 | | |
| **NPI** | | | | 0.497 | 0.780 |
| Poor | 50(48.5) | 53(51.5) | 103 | | |
| Moderate | 158(45.8) | 187(54.2) | 345 | | |
| Good | 112(44.4) | 140(55.6) | 252 | | |
| **DM** | | | | 2.406 | 0.131 |
| No | 235(47.8) | 257(52.2) | 492 | | |
| Positive | 83(41.3) | 118(58.7) | 201 | | |
| **Recurrence** | | | | 2.944 | 0.089 |
| No | 192(48.1) | 207(51.9) | 399 | | |
| Positive | 120(41.5) | 169(58.5) | 289 | | |
| **VI** | | | | 1.880 | 0.391 |
| No | 171)43.6) | 221(56.4) | 392 | | |
| Probable | 42(47.2) | 47(52.8) | 89 | | |
| Definite | 106(49.3) | 109(50.7) | 215 | | |
| **Histologic tumour type** | | | | 5.183 | 0.521 |
| Ductal/NST | 167(48.8) | 175(51.2) | 342 | | |
| Lobular | 37(42.5) | 50(57.5) | 87 | | |
| Tubular and Tubular mixed | 81(42.2) | 111(57.8) | 192 | | |
| Medullary | 1(20) | 4(80) | 5 | | |
| Other special types* | 8(53.3) | 7(46.7) | 15 | | |
| Mixed** | 25(46.3) | 29(53.7) | 54 | | |
| **Mitosis** | | | | 2.716 | 0.257 |
| 1 | 126(42.6) | 170(57.4) | 296 | | |
| 2 | 73(50) | 73(50) | 146 | | |
| 3 | 109(48) | 118(52) | 227 | | |
| **Menopausal status** | | | | 1.022 | 0.341 |
| Premenopausal | 107(43.1) | 141(56.9) | 248 | | |
| Postmenopausal | 213(47.1) | 239(52.9) | 452 | | |

*Includes Mucoid, invasive cribriform and invasive papillary carcinoma, ** Include ductal/NST mixed with lobular or special type

**Table 3.22**: Relation of the TFF1 expression to other biomarkers in the whole series

| Variable | TFF1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Negative | Positive | Total | | |
| **CK5/6** | | | | 0.002 | 1.000 |
| Negative | 409(49.8) | 412(50.2) | 821 | | |
| Positive | 93(50) | 93(50) | 186 | | |
| **CK14** | | | | 1.545 | 0.243 |
| Negative | 429(50.4) | 422(49.6) | 851 | | |
| Positive | 65(44.8) | 80(55.2) | 145 | | |
| **CK18** | | | | 9.790 | 0.002 |
| Negative | 84(64.1) | 47(35.9) | 131 | | |
| Positive | 387(49.4) | 397(50.6) | 784 | | |
| **CK19** | | | | 6.773 | 0.010 |
| Negative | 55(62.5) | 33(37.5) | 88 | | |
| Positive | 439(48) | 476(52) | 915 | | |
| **ER** | | | | 15.749 | <0.001 |
| Negative | 164(59.9) | 110(40.1) | 274 | | |
| Positive | 320(45.7) | 380(54.3) | 700 | | |
| **PgR** | | | | 7.287 | 0.008 |
| Negative | 240(54.5) | 200(45.5) | 440 | | |
| Positive | 242(45.8) | 286(54.2) | 528 | | |
| **AR** | | | | 4.360 | 0.043 |
| Negative | 195(54.8) | 161(45.2) | 356 | | |
| Positive | 273(47.7) | 299(52.3) | 572 | | |
| **p53** | | | | 1.944 | 0.170 |
| Negative | 346(48.6) | 366(51.4) | 712 | | |
| Positive | 140(53.6) | 121(46.4) | 261 | | |
| **BRCA1** | | | | 3.264 | 0.072 |
| Negative | 79(59.4) | 54(40.6) | 133 | | |
| Weak | 338(50.8) | 327(49.2) | 665 | | |
| **MIB1** | | | | 0.163 | 0.696 |
| Low | 117(50.9) | 113(49.1) | 230 | | |
| High | 277(49.3) | 285(50.7) | 562 | | |
| **P-cadherin** | | | | 4.913 | 0.058 |
| Negative | 202(56.7) | 154(43.3) | 356 | | |
| Positive | 220(48.9) | 230(51.1) | 450 | | |
| **E-cadherin** | | | | 1.206 | 0.295 |
| Negative | 205(52.3) | 187(47.7) | 392 | | |
| Positive | 282(48.7) | 297(51.3) | 579 | | |
| **HER2** | | | | 0.383 | 0.581 |
| Negative | 428(50.3) | 423(49.7) | 851 | | |
| Positive | 65(47.4) | (52.6) | 137 | | |
| **EGFR** | | | | 0.509 | 0.485 |
| Negative | 357(52.5) | 323(47.5) | 680 | | |
| Positive | 80(49.4) | 82(50.6) | 162 | | |

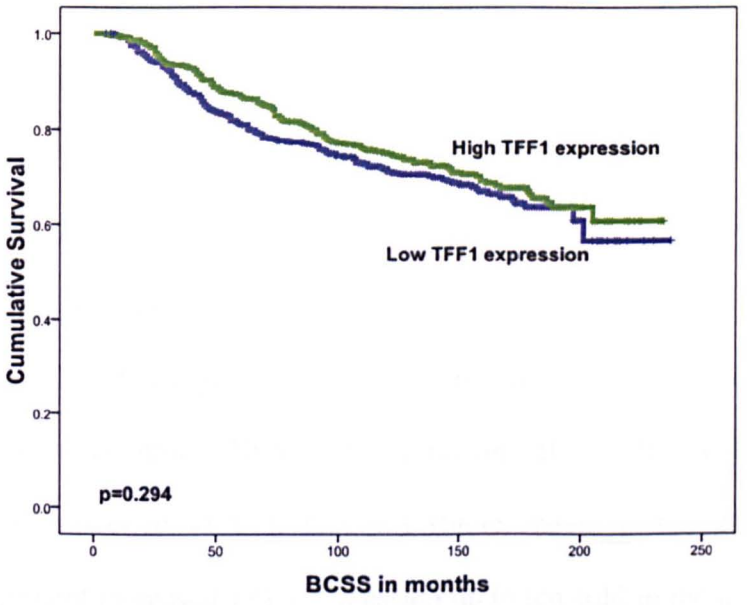


**Figure 3.15:** Kaplan Meier plot of TFF1 expression in relation to BCSS in the whole series

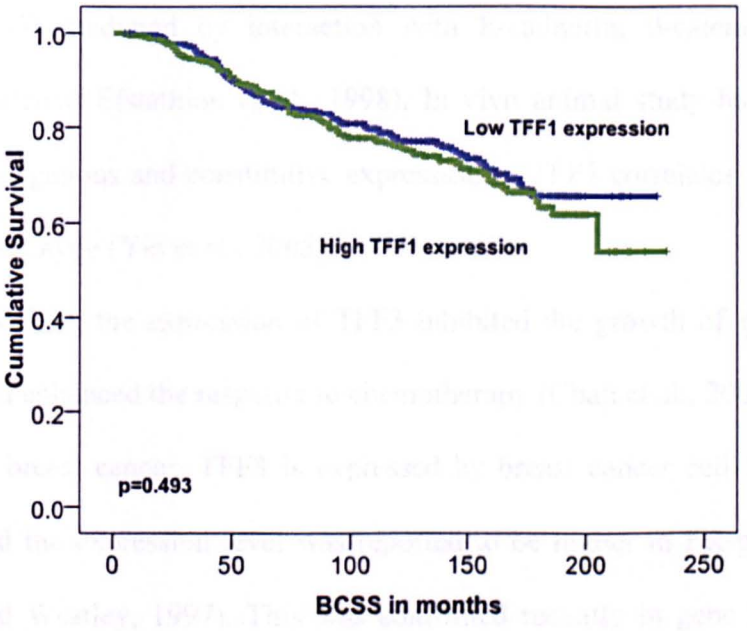


**Figure 3.16:** Kaplan Meier plot of TFF1 expression in relation to BCSS in the ER-positive cohort

**TFF3**

### 3.6.4 Introduction

Trefoil factors (TFF1/pS2, TFF2/SP and TFF3/ITF) are soluble peptides with trefoil domain(s) and C-terminal dimerization domain involved in protection and healing of the human gastrointestinal tract (May and Westley, 1997). In particular, TFF3 plays a key role in mucosal protection, and also in mucosal repair after injury. They have effects on cell motility and spreading in vitro (Poulsom et al., 1997). Previous studies have demonstrated that oestradiol treatment increased TFF3 expression up to ten-fold in the oestrogen-responsive breast cancer cell lines, confirming that TFF3 is regulated by oestrogen in breast cancer cells (May and Westley, 1997).

TFF3 may affect the metastatic potential of tumour cells (May and Westley, 1997) mediated by interaction with E-cadherin, ß-catenin, and associated proteins (Efstathiou et al., 1998). In vivo animal study has shown that both endogenous and constitutive expression of TFF3 correlates with an aggressive phenotype (Yio et al., 2005).

Blocking the expression of TFF3 inhibited the growth of gastric cancer cells and enhanced the response to chemotherapy (Chan et al., 2005).

In breast cancer, TFF3 is expressed by breast cancer cell lines and tumours, and the expression level was reported to be higher in ER-positive cells (May and Westley, 1997). This was confirmed recently in gene expression studies which have repeatedly reported TFF3 as a gene that characterises the Luminal A subtype. Our analysis of the Cambridge gene expression data has demonstrated that TFF3 gene shows a wide variation in the normalised

expression intensity values within the ER positive group implying an important role in the phenotypic characterisation of the ER-positive breast cancer.

The value of TFF3 protein as a prognostic biomarker in defining breast cancer phenotypes remains undetermined especially in ER-positive subtype.

### 3.6.5 Material and Methods

Tissue microarrays were prepared and the immunohistochemical staining was performed using the streptavidin-biotin complex method using DakoCytomation Techmate 500 plus (DakoCytomation, Cambridge, UK) (general material and methods chapter). To unmask the antigens, the sections were microwaved in citrate buffer pH 6 for 23 minutes. Mouse monoclonal TFF3 antibody (Ab57752, Abcam, UK) was optimised at a working dilution of 3μg/ml. Negative controls were performed by omitting the primary antibody and substitution with diluent. The H-score (histochemical score) was used for assessment. The cutoff point was assigned by using the median of H-score values (H-score ≥90).

### 3.6.6 TFF3 immunohistochemical results

The expression of TFF3 was detected in the cytoplasm of the malignant cells **(Fig 3.17A&B)** with decreased expression in the normal acini. After excluding the uninformative TMA cores from the study, 1020 tumours were available for assessment. In the whole series, 52% of cases were TFF3 positive while in the ER-positive cohort, 62% of cases were TFF3 positive.

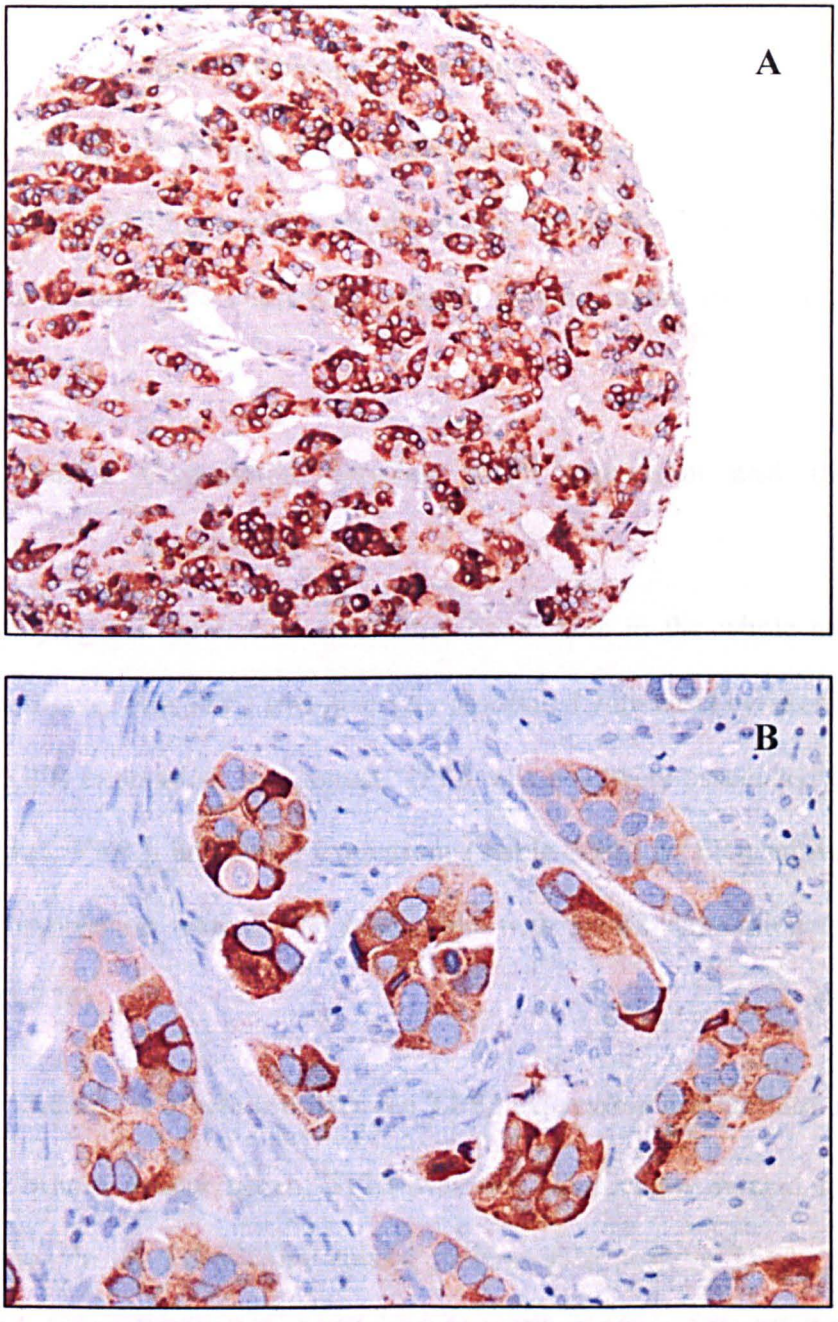


**Figure 3.17:** TFF3 expression in breast cancer

(A) TMA core of grade 2 ductal carcinoma with strong TFF3 expression (x100). (B) TMA core of grade 2 ductal carcinoma with strong TFF3 expression (x200).

#### 3.6.6.1 Correlation between TFF3 expression and the other clinicopathological variables

In the whole series, high TFF3 expression was associated with low grade tumours, good NPI group, low mitotic counts, DM and VI **(Table 3.23)**. In ER-positive cohort, TFF3 expression retained its association with DM ($p$=0.007) **(Table 3.25)**.

#### 3.6.6.1 Correlation between TFF3 expression and the other biomarkers

On studying the correlation with other biomarkers in the whole series, our results showed that TFF3 was inversely associated with basal cytokeratins, p53 and EGFR expression. In contrast, TFF3 was positively associated with ER, PgR, AR, HER2, and Bcl-2 expression **(Table 3.24)**. In ER-positive cohort, TFF3 expression retained its association with HER2 expression ($p$=0.001) **(Table 3.26)**.

#### 3.6.6.1 Correlation between TFF3 expression and patient outcome

In the whole patient series, TFF3 was not significantly related to patient outcome. In the ER-positive cohort, patients with positive TFF3 expression showed shorter BCSS (LR=5.895, $p$=0.015) **(Fig 3.18)** and DMFI (LR=6.174, $p$=0.013) **(Fig 3.19)**. Multivariate Cox regression analysis including tumour size, tumour grade and lymph node stage revealed that TFF3 was not an independent prognostic marker of BCSS (HR=1.452, $p$=0.061, 95%CI=0.986-1.854) or DMFI (HR=1.340, $p$=0.053, 95%CI=0.996-1.804).

Table 3.23: Relation of TFF3 expression to other clinicopathological variables in the whole series

| Variable | TFF3 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Negative | Positive | Total | | |
| **Patients' age** | | | | 4.528 | 0.210 |
| <40 | 44(55.7) | 35(44.3) | 79 | | |
| 40-50 | 139(47.3) | 155(52.7) | 294 | | |
| 51-60 | 159(49.8) | 160(50.2) | 319 | | |
| >60 | 144(43.9) | 184(56.1) | 328 | | |
| **Tumour size** | | | | 1.898 | 0.175 |
| **≤1.5 cm** | 139(44.4) | 174(55.6) | 313 | | |
| >1.5 cm | 347(49.1) | 360(50.9) | 707 | | |
| **Lymph node stage** | | | | 2.826 | 0.243 |
| 1(Negative) | 297(48.3) | 318(51.7) | 615 | | |
| 2(1-3 LN) | 150(48.7) | 158(51.3) | 308 | | |
| 3(>3 LN) | 37(39.4) | 57(60.6) | 94 | | |
| **Tumour Grade** | | | | 27.492 | <0.001 |
| 1 | 70(42.4) | 95(57.6) | 165 | | |
| 2 | 125(37.9) | 205(62.1) | 330 | | |
| 3 | 291(55.5) | 233(44.5) | 524 | | |
| **NPI** | | | | 12.577 | 0.002 |
| Good | 104(38.7) | 165(61.3) | 269 | | |
| Moderate | 299(51.7) | 279(48.3) | 578 | | |
| Poor | 83(48) | 90(52) | 173 | | |
| **DM** | | | | 7.042 | 0.008 |
| No | 388(50.5) | 331(49.5) | 669 | | |
| Positive | 144(41.7) | 201(58.3) | 345 | | |
| **Recurrence** | | | | 6.702 | 0.011 |
| No | 282(51) | 271(49) | 553 | | |
| Positive | 193(42.8) | 258(57.2) | 451 | | |
| **VI** | | | | 9.560 | 0.008 |
| No | 294(52.1) | 270(47.9) | 564 | | |
| Probable | 52(42.3) | 71(57.7) | 123 | | |
| Definite | 140(42.4) | 190(57.6) | 330 | | |
| **Histologic tumour type** | | | | 46.120 | <0.001 |
| Ductal/NST | 311(50.8) | 301(49.2) | 612 | | |
| Lobular | 31(32.3) | 65(67.7) | 96 | | |
| Tubular and Tubular mixed | 93(44.7) | 115(55.3) | 208 | | |
| Medullary | 28(90.3) | 3(9.7) | 31 | | |
| Other special types | 8(53.3) | 7(46.7) | 15 | | |
| Mixed | 15(25.9) | 43(74.1) | 58 | | |
| **Mitosis** | | | | 28.587 | <0.001 |
| 1 | 133(41) | 191(59) | 324 | | |
| 2 | 68(38.2) | 110(61.8) | 178 | | |
| 3 | 273(57) | 206(43) | 479 | | |
| **Menopausal status** | | | | 1.425 | 0.247 |
| Premenopausal | 197(50) | 197(50) | 394 | | |
| Postmenopausal | 289(46.2) | 337(53.8) | 626 | | |

*

**Table 3.24:** Relation of TFF3 expression to other biomarkers in the whole series

| Variable | TFF3 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Negative | Positive | Total | | |
| **CK5/6** | | | | 36.441 | <0.001 |
| Negative | 351(43.1) | 463(56.9) | 814 | | |
| Positive | 120(68.2) | 56(31.8) | 176 | | |
| **CK14** | | | | 9.473 | 0.002 |
| Negative | 380(45.5) | 455(54.5) | 835 | | |
| Positive | 78(60) | 52(40) | 130 | | |
| **CK18** | | | | 80.452 | <0.001 |
| Negative | 106(84.1) | 20(15.9) | 126 | | |
| Positive | 320(41.1) | 458(58.9) | 778 | | |
| **CK19** | | | | 23.612 | <0.001 |
| Negative | 63(72.4) | 24(27.6) | 87 | | |
| Positive | 409(45.2) | 493(54.8) | 899 | | |
| **ER** | | | | 86.762 | <0.001 |
| Negative | 197(71.1) | 80(28.9) | 277 | | |
| Positive | 262(38) | 427(62) | 689 | | |
| **PgR** | | | | 37.947 | <0.001 |
| Negative | 252(58.5) | 179(41.5) | 431 | | |
| Positive | 205(38.5) | 327(61.5) | 532 | | |
| **AR** | | | | 32.956 | <0.001 |
| Negative | 211(59.6) | 143(40.4) | 354 | | |
| Positive | 223(40.1) | 333(59.9) | 556 | | |
| **p53** | | | | 9.752 | 0.002 |
| Negative | 311(44.6) | 387(55.4) | 698 | | |
| Positive | 151(55.7) | 120(44.3) | 271 | | |
| **Bcl-2** | | | | 21.299 | <0.001 |
| Negative | 185(58) | 134(42) | 319 | | |
| Positive | 202(41.4) | 286(58. | 488 | | |
| **MIB1** | | | | 1.876 | 0.195 |
| Low | 89(42.2) | 121(57.6) | 210 | | |
| High | 273(47.9) | 297(52.1) | 570 | | |
| **P-cadherin** | | | | 25.561 | <0.001 |
| Negative | 136(36.9) | 233(63.1) | 369 | | |
| Positive | 241(54.6) | 200(45.4) | 441 | | |
| **E-cadherin** | | | | 2.807 | 0.099 |
| Negative | 192(50.9) | 185(49.1) | 377 | | |
| Positive | 267(45.4) | 231(54.6) | 588 | | |
| **HER2** | | | | 21.783 | <0.001 |
| Negative | 428(50.4) | 421(49.6) | 849 | | |
| Positive | 37(28.5) | 93(71.5) | 130 | | |
| **EGFR** | | | | 15.871 | <0.001 |
| Negative | 292(43.3) | 383(56.7) | 675 | | |
| Positive | 102(60.4) | 67(39.6) | 169 | | |

**Table 3.25:** Relation of TFF3 expression to other clinicopathological variables in the ER-positive cohort

| Variable | TFF3 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Negative | Positive | Total | | |
| **Patients' age** | | | | 3.016 | 0.389 |
| <40 | 12(33.3) | 24(66.7) | 36 | | |
| 40-50 | 64(433.7) | 126(66.3) | 190 | | |
| 51-60 | 89(41.4) | 126(58.6) | 215 | | |
| >60 | 97(39.1) | 151(60.9) | 248 | | |
| **Tumour size** | | | | 0.020 | 0.934 |
| **≤1.5 cm** | 87(37.7) | 144(62.3) | 231 | | |
| >1.5 cm | 175(38.2) | 283(61.8) | 458 | | |
| **Lymph node stage** | | | | 3.844 | 0.146 |
| 1(Negative) | 154(37.7) | 254(62.3) | 408 | | |
| 2(1-3 LN) | 91(41) | 131(59) | 222 | | |
| 3(>3 LN) | 15(26.8) | 41(73.2) | 56 | | |
| **Tumour Grade** | | | | 0.533 | 0.766 |
| 1 | 59(40.7) | 86(59.3) | 145 | | |
| 2 | 107(37.3) | 180(62.7) | 287 | | |
| 3 | 96(37.5) | 160(62.5) | 265 | | |
| **NPI** | | | | 0.282 | 0.868 |
| Good | 35(35.7) | 63(64.3) | 98 | | |
| Moderate | 328(38.7) | 219(48.3) | 357 | | |
| Poor | 89(48) | 145(52) | 234 | | |
| **DM** | | | | 7.289 | 0.007 |
| No | 192(41.5) | 271(58.5) | 463 | | |
| Positive | 69 (30.8) | 155(69.2) | 224 | | |
| **Recurrence** | | | | 4.144 | 0.042 |
| No | 156(41.4) | 221(58.6) | 377 | | |
| Positive | 103(33.8) | 202(66.2) | 305 | | |
| **VI** | | | | 4.685 | 0.096 |
| No | 154(41.4) | 218(58.6) | 372 | | |
| Probable | 38(38.4) | 61(61.6) | 99 | | |
| Definite | 70(32.4) | 146(67.6) | 216 | | |
| **Histologic tumour type** | | | | 8.593 | 0.126 |
| Ductal/NST | 136(38.3) | 219(61.7) | 355 | | |
| Lobular | 29(32.6) | 60(67.4) | 89 | | |
| Tubular and Tubular mixed | 93(42.8) | 103(57.2) | 180 | | |
| Medullary | 3(75) | 1(25) | 4 | | |
| Other special types* | 4(40) | 6(60) | 10 | | |
| Mixed** | 13(25.5) | 38 (74.5) | 51 | | |
| **Mitosis** | | | | 1.468 | 0.480 |
| 1 | 115(40.1) | 172(59.9) | 287 | | |
| 2 | 49(34.3) | 94(65.7) | 143 | | |
| 3 | 90(39.5) | 138(60.5) | 228 | | |
| **Menopausal status** | | | | 1.800 | 0.187 |
| Premenopausal | 82(34.6) | 155(65.4) | 237 | | |
| Postmenopausal | 180(39.8) | 272(60.2) | 452 | | |

**Table 3.26:** Relation of TFF3 expression to other biomarkers in ER-positive cohort

| Variable | TFF3 Expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Negative | Positive | Total | | |
| **CK5/6** | | | | 0.178 | 0.765 |
| Negative | 240(38.3) | 387(61.7) | 627 | | |
| Positive | 18(35.3) | 33(64.7) | 51 | | |
| **CK14** | | | | 1.180 | 0.311 |
| Negative | 233(38.3) | 357(61.7) | 608 | | |
| Positive | 17(30.9) | 38(69.1) | 55 | | |
| **CK18** | | | | 0.023 | 1.000 |
| Negative | 10(35.7) | 18(64.3) | 28 | | |
| Positive | 228(37.1) | 386(62.9) | 614 | | |
| **CK19** | | | | 0.156 | 0.719 |
| Negative | 14(41.2) | 20(58.8) | 34 | | |
| Positive | 245(37.8) | 403(62.2) | 648 | | |
| **PgR** | | | | 0.184 | 0.668 |
| Negative | 61(36.3) | 107(63.7) | 168 | | |
| Positive | 195(38.2) | 316(61.8) | 511 | | |
| **AR** | | | | 0.397 | 0.572 |
| Negative | 57(35.6) | 103(64.4) | 160 | | |
| Positive | 184(38.4) | 295(61.6) | 479 | | |
| **p53** | | | | 0.022 | 0.919 |
| Negative | 213(38.3) | 343(61.7) | 556 | | |
| Positive | 47(37.6) | 78(62.4) | 125 | | |
| **Bcl-2** | | | | 1.874 | 0.181 |
| Negative | 43(32.8) | 188(67.2) | 131 | | |
| Weak | 165(39.5) | 253(60.5) | 418 | | |
| **MIB1** | | | | 0.922 | 0.343 |
| Low | 71(40.7) | 105(59.7) | 176 | | |
| High | 128(36.1) | 227(63.9) | 355 | | |
| **P-cadherin** | | | | 0.303 | 0.600 |
| Negative | 117(34.9) | 218(65.1) | 335 | | |
| Positive | 91(37.1) | 154(62.9) | 245 | | |
| **E-cadherin** | | | | 0.001 | 1.000 |
| Negative | 98(38.1) | 159(61.9) | 257 | | |
| Positive | 162(38.2) | 262(61.8) | 424 | | |
| **HER2** | | | | 10.770 | 0.001 |
| Negative | 247(39.8) | 374(60.2) | 621 | | |
| Positive | 9(17) | 44(83) | 53 | | |
| **EGFR** | | | | 0.660 | 0.464 |
| Negative | 188(35.9) | 336(64.1) | 524 | | |
| Positive | 34(40.5) | 50(59.5) | 84 | | |

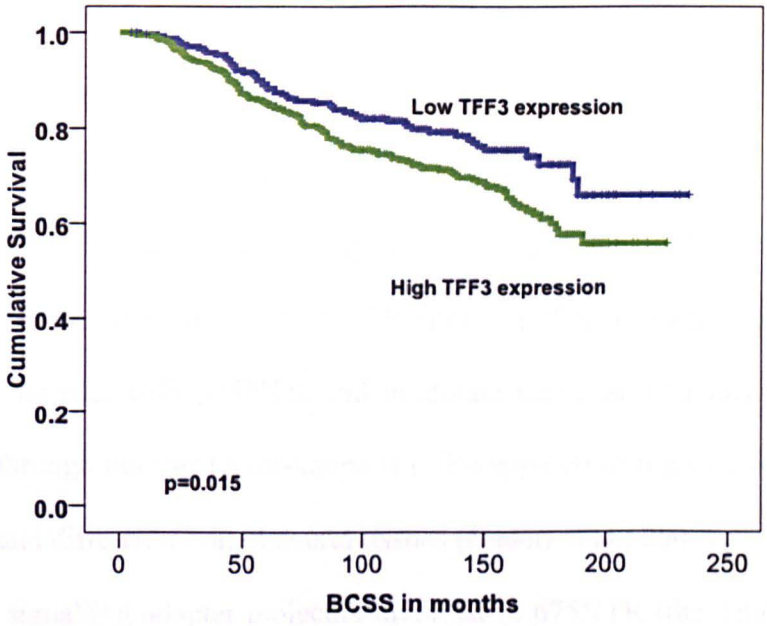


**Figure 3.18:** Kaplan Meier plot of TFF3 expression in relation to BCSS in the ER-positive cohort

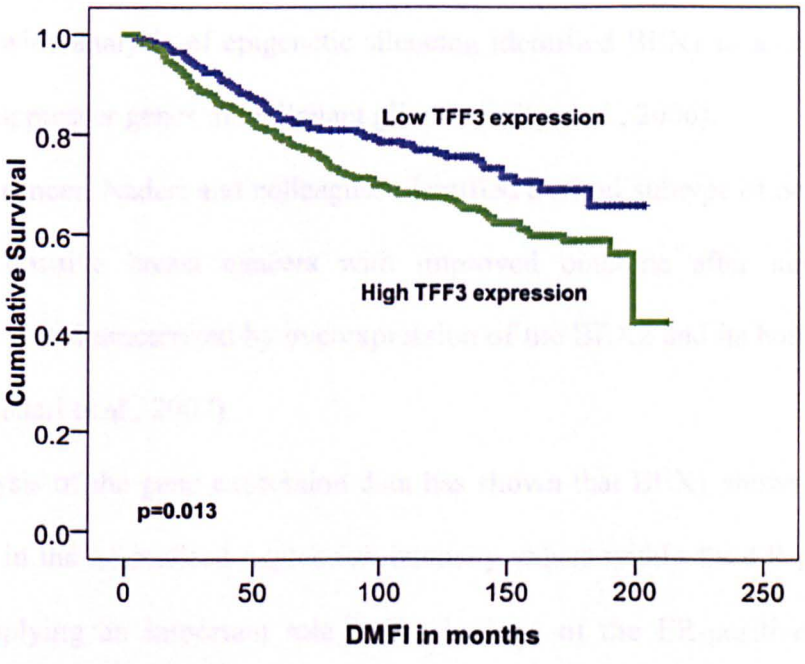


**Figure 3.19:** Kaplan Meier plot of TFF3 expression in relation to DMFI in the ER-positive cohort

## 3.7 BEX1

### 3.7.1 Introduction

BEX1 (brain expressed X-linked gene) maps on the Xq22 in humans (Brown and Kay, 1999). It belongs to a family of genes, including BEX1, NGFRAP1 (BEX3), BEXL1 (BEX4), and, NGFRAP1L1 (BEX5). Both BEX1 and NGFRAP1 interact with p75NTR and modulate nerve growth factor (NGF) signalling through nuclear factor-kappa B (NF-kappa B) to regulate cell cycle, apoptosis, and differentiation in neural tissues (Naderi et al., 2007).

BEX1 is a signalling adapter molecule involved in p75NTR (the neurotrophin receptor) /NGFR signalling. In the central nervous system, it has been suggested that it inhibits the neuronal differentiation in response to nerve growth factor (NGF) (Vilar et al., 2006, Naderi et al., 2007, Foltz et al., 2006). Genome-wide analysis of epigenetic silencing identified BEX1 as a candidate tumour suppressor genes in malignant glioma (Foltz et al., 2006).

In breast cancer, Naderi and colleagues identified a novel subtype of oestrogen receptor positive breast cancers with improved outcome after tamoxifen treatment and characterized by overexpression of the BEX2 and its homologue BEX1 (Naderi et al., 2007).

Our analysis of the gene expression data has shown that BEX1 shows a wide variation in the normalised expression intensity values within the ER positive group implying an important role in the biology of the ER-positive breast cancer. Available studies in the literature regarding BEX1 are minimal and largely unexplored in breast cancer.

### 3.7.2 Material and Methods

Tissue microarrays were prepared and the immunohistochemical staining was performed using the streptavidin-biotin complex method using DakoCytomation Techmate 500 plus (DakoCytomation, Cambridge, UK) (general material and methods chapter). To unmask the antigens, the sections were microwaved in citrate buffer pH 6 for 23 minutes. Rabbit polyclonal BEX1 antibody (Ab69032, Abcam, UK) was optimised at a working dilution of 1:3500. Negative controls were performed by omitting the primary antibody and substitution with diluent. Breast carcinoma sections were used as positive controls. The H-score (histochemical score) was used for assessment. Using x-tile program, H-score of 100 was selected as the optimal cutoff point for data categorisation.

### 3.7.3 BEX1 expression results

The pattern of staining in breast carcinoma was cytoplasmic **(Fig 3.20)**, with increased expression in the normal breast acini. After excluding the uninformative TMA cores from the study, 1,106 tumours were available for assessment.

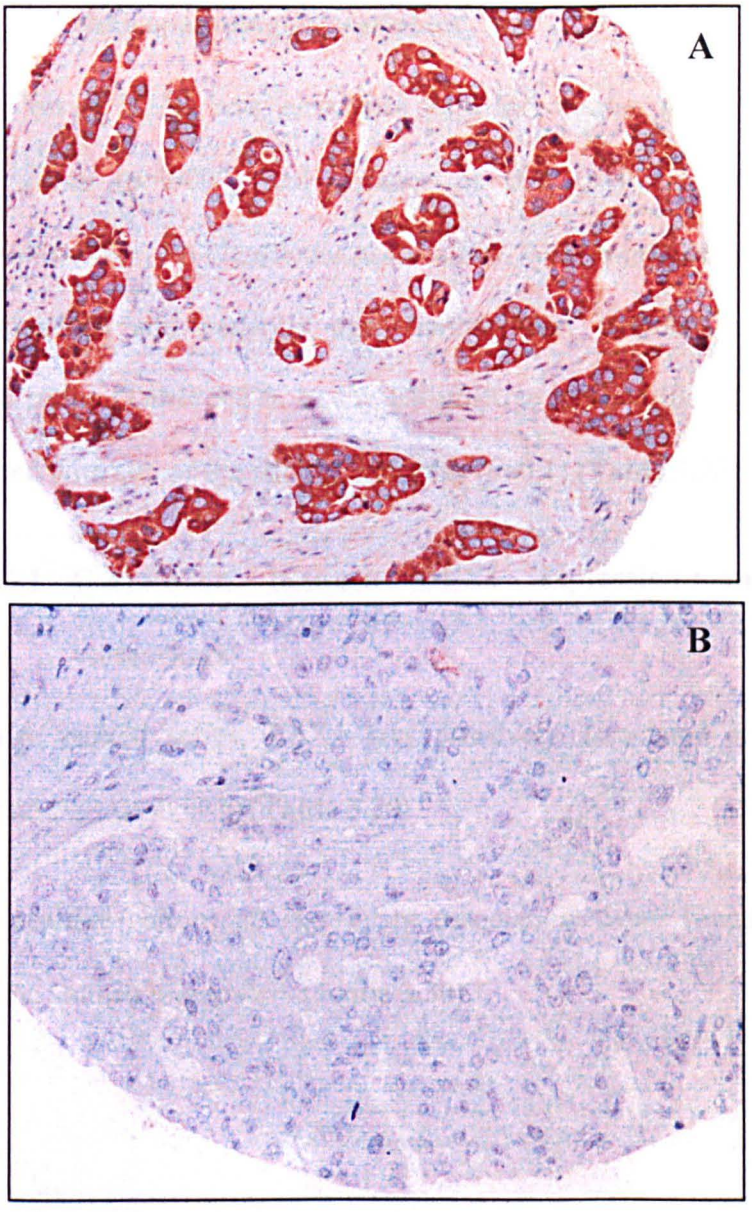


**Figure 3.20:** BEX1 expression in breast cancer

(A) Grade 2 ductal cancer NST with strong BEX1 expression (x100)

(B) Grade 3 ductal cancer NST with negative BEX1 expression (x200)

#### 3.7.3.1 Correlation between BEX1 expression and other clinicopathological variables

The expression of BEX1 was associated with low tumour grade ($p$=0.008), good NPI group ($p$=0.002) and absence of DM ($p$=0.008) and tumour recurrence ($p$=0.007). No significant associations were found between BEX1 protein expression and patient's age, tumour size, vascular invasion or menopausal status **(Table 3.27)**. In the ER-positive luminal-like subtype was not associated with any of the studied variables ($p$>0.1) **(Table 3.28)**.

#### 3.7.3.1 Correlation between BEX1 expression and other biomarkers

In the whole patient series, BEX1 was positively associated with CK18 ($p$=0.002) and PgR ($p$=0.001) **(Table 3.29)**.

In the ER-positive cohort, no significant associations were found between BEX1 and the studied biomarkers **(Table 3.30)**.

**Table 3.27:** Relation of BEX1 expression to other clinicopathological variables in the whole series

| Variable | BEX1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | High | Total | | |
| **Patients' age** | | | | 7.535 | 0.057 |
| <40 | 31(41.3) | 44(58.7) | 75 | | |
| 40-50 | 83(25.8) | 239(74.2) | 322 | | |
| 51-60 | 112(31) | 249(69) | 361 | | |
| >60 | 106(30.5) | 242(69.5) | 348 | | |
| **Tumour size** | | | | | |
| **≤1.5 cm** | 101(27.4) | 267(72.6) | 368 | | |
| >1.5 cm | 231(31.3) | 507(68.7) | | | |
| **Lymph node stage** | | | | 2.742 | 0.254 |
| 1(Negative) | 194(28.2) | 493(71.8) | 687 | | |
| 2(1-3 LN) | 106(33.1) | 214(66.9) | 320 | | |
| 3(>3 LN) | 31(32.3) | 65(67.7) | 96 | | |
| **Tumour Grade** | | | | 9.674 | 0.008 |
| 1 | 43(21.9) | 153(78.1) | 196 | | |
| 2 | 105(29) | 257(71) | 362 | | |
| 3 | 184(33.6) | 363(66.4) | 547 | | |
| **NPI** | | | | 12.946 | 0.002 |
| Good | 71(22.3) | 248(77.7) | 319 | | |
| Moderate | 200(32.9) | 408(67.1) | 608 | | |
| Poor | 61(34.1) | 118(65.9) | 179 | | |
| **DM** | | | | 7.266 | 0.008 |
| No | 209(27.4) | 553(72.6) | 762 | | |
| Positive | 118(35.5) | 214(64.5) | 332 | | |
| **Recurrence** | | | | 7.534 | 0.007 |
| No | 164(26.2) | 463(73.8) | 627 | | |
| Positive | 155(33.8) | 303(66.2) | 458 | | |
| **VI** | | | | 0.191 | 0.909 |
| No | 185(29.8) | 436(70.2) | 621 | | |
| Probable | 37(28.9) | 91(71.1) | 128 | | |
| Definite | 109(30.8) | 245(69.2) | 354 | | |
| **Histologic tumour type** | | | | 14.750 | 0.011 |
| Ductal/NST | 208(32) | 441(68) | 649 | | |
| Lobular | 40(35.4) | 73(64.6) | 113 | | |
| Tubular and Tubular mixed | 47(20.6) | 181(79.4) | 228 | | |
| Medullary | 8(24.2) | 25(75.8) | 33 | | |
| Other special types* | 9(42.9) | 12(57.1) | 21 | | |
| Mixed** | 20(32.3) | 42(67.7) | 62 | | |
| **Mitosis** | | | | 8.292 | 0.016 |
| 1 | 100(26.4) | 279(73.6) | 379 | | |
| 2 | 51(26.7) | 140(73.3) | 191 | | |
| 3 | 172(34.6) | 325(65.4) | 497 | | |
| **Menopausal status** | | | | 0.699 | 0.417 |
| Premenopausal | 119(28.5) | 298(71.5) | 417 | | |
| Postmenopausal | 213(30.9) | 476(69.1) | 689 | | |

*Includes Mucoid, invasive cribriform and invasive papillary carcinoma, ** Include ductal/NST mixed with lobular or special type

**Table 3.28:** Relation of BEX1 expression to other clinicopathological variables in the ER-positive cohort

| Variable | BEX1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | High | Total | | |
| **Patients' age** | | | | 3.395 | 0.335 |
| <40 | 7(22.6) | 24(77.4) | 31 | | |
| 40-50 | 50(24) | 158(76) | 208 | | |
| 51-60 | 72(28.9) | 177(71.1) | 249 | | |
| >60 | 82(31.1) | 182(68.9) | 264 | | |
| **Tumour size** | | | | 0.575 | 0.498 |
| **≤1.5 cm** | 71(26.4) | 198(73.6) | 269 | | |
| >1.5 cm | 140(29) | 343(71) | 483 | | |
| **Lymph node stage** | | | | 4.552 | 0.103 |
| 1(Negative) | 117(25.3) | 345(74.7) | 462 | | |
| 2(1-3 LN) | 74(31.9) | 158(68.1) | 232 | | |
| 3(>3 LN) | 19(34.5) | 36(65.5) | 55 | | |
| **Tumour Grade** | | | | 3.430 | 0.180 |
| 1 | 38(22.5) | 131(77.5) | 169 | | |
| 2 | 94(30) | 219(70) | 313 | | |
| 3 | 79(29.4) | 190(70.6) | 269 | | |
| **NPI** | | | | 5.877 | 0.053 |
| Good | 62(22.8) | 210(77.2) | 272 | | |
| Moderate | 117(30.9) | 262(69.1) | 379 | | |
| Poor | 32(31.7) | 69(68.3) | 101 | | |
| **DM** | | | | 6.569 | 0.012 |
| No | 135(25.4) | 396(74.6) | 531 | | |
| Positive | 75(34.7) | 141(65.3) | 216 | | |
| **Recurrence** | | | | 4.124 | 0.046 |
| No | 108(24.8) | 327(75.2) | 435 | | |
| Positive | 97(31.6) | 210(68.4) | 307 | | |
| **VI** | | | | 0.401 | 0.818 |
| No | 114(27.8) | 296(72.2) | 410 | | |
| Probable | 27(26) | 77(74) | 104 | | |
| Definite | 69(29.2) | 167(70.8) | 236 | | |
| **Histologic type** | | | | 9.887 | 0.079 |
| Ductal/NST | 112(29.6) | 266(70.4) | 378 | | |
| Lobular | 36(36) | 64(64) | 100 | | |
| Tubular and Tubular mixed | 40(20.2) | 158(79.8) | 198 | | |
| Medullary | 1(25) | 3(75) | 4 | | |
| Other special types* | 5(31.2) | 11(68.8) | 16 | | |
| Mixed** | 17(30.4) | 39(69.6) | 56 | | |
| **Mitosis** | | | | 0.631 | 0.730 |
| 1 | 91(27.5) | 240(72.5) | 331 | | |
| 2 | 41(27) | 111(73) | 152 | | |
| 3 | 72(30.1) | 167(69.9) | 239 | | |
| **Menopausal status** | | | | 6.573 | 0.012 |
| Premenopausal | 55(22.1) | 194(77.9) | 249 | | |
| Postmenopausal | 156(31) | 347(69) | 503 | | |

**Table 3.29:** Relation of BEX1 expression to other biomarkers in the whole series

| Variable | BEX1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | High | Total | | |
| **CK5/6** | | | | 0.109 | 0.789 |
| Negative | 265(29.9) | 622(70.1) | 887 | | |
| Positive | 56(31.1) | 124(68.9) | 180 | | |
| **CK14** | | | | 0.278 | 0.687 |
| Negative | 280(30.6) | 635(69.4) | 915 | | |
| Positive | 38(28.4) | 96(71.6) | 134 | | |
| **CK18** | | | | 9.842 | 0.002 |
| Negative | 55(42) | 76(58) | 131 | | |
| Positive | 241(28.5) | 606(71.5) | 847 | | |
| **CK19** | | | | 3.234 | 0.078 |
| Negative | 36(38.3) | 58(61.7) | 94 | | |
| Positive | 285(29.4) | 685(70.6) | 970 | | |
| **ER** | | | | 6.760 | 0.010 |
| Negative | 105(36.3) | 184(63.7) | 289 | | |
| Positive | 211(28.1) | 541(71.9) | 752 | | |
| **PgR** | | | | 11.515 | 0.001 |
| Negative | 162(35.8) | 291(64.2) | 453 | | |
| Positive | 151(26) | 430(74) | 581 | | |
| **AR** | | | | 4.156 | 0.045 |
| Negative | 127(34.7) | 239(65.3) | 366 | | |
| Positive | 174(28.5) | 437(71.5) | 611 | | |
| **p53** | | | | 2.391 | 0.128 |
| Negative | 242(31.6) | 524(68.4) | 766 | | |
| Positive | 74(26.6) | 204(73.4) | 278 | | |
| **MIB1** | | | | 1.200 | 0.315 |
| Low | 64(26.2) | 180(73.8) | 244 | | |
| High | 180(30) | 420(70) | 600 | | |
| **P-cadherin** | | | | 0.498 | 0.507 |
| Negative | 128(31.6) | 277(68.4) | 405 | | |
| Positive | 137(29.4) | 329(70.6) | 406 | | |
| **E-cadherin** | | | | 5.444 | 0.022 |
| Negative | 138(34.8) | 259(65.2) | 397 | | |
| Positive | 180(27.9) | 465(72.1) | 645 | | |
| **HER2** | | | | 1.580 | 0.235 |
| Negative | 289(31.4) | 632(68.6) | 921 | | |
| Positive | 36(26.1) | 102(73.9) | 138 | | |
| **EGFR** | | | | 0.001 | 1.000 |
| Negative | 222(30.1) | 515(69.9) | 737 | | |
| Positive | 52(30.2) | 120(69.8) | 172 | | |
| **Bcl-2** | | | | 4.201 | 0.045 |
| Negative | 111(32.4) | 232(67.6) | 343 | | |
| Positive | 134(25.9) | 383(74.1) | 517 | | |

**Table 3.30:** Relation of BEX1 expression to other biomarkers in the ER-positive cohort

| Variable | BEX1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | High | Total | | |
| **CK5/6** | | | | 3.138 | 0.080 |
| Negative | 198(28.7) | 491(71.3) | 689 | | |
| Positive | 9(17.3) | 43(82.7) | 52 | | |
| **CK14** | | | | 1.696 | 0.223 |
| Negative | 196(29.2) | 476(70.8) | 672 | | |
| Positive | 12(21.1) | 45(78.9) | 57 | | |
| **CK18** | | | | 4.848 | 0.033 |
| Negative | 13(46.4) | 15(53.6) | 28 | | |
| Positive | 184(27.3) | 489(72.7) | 673 | | |
| **CK19** | | | | 0.599 | 0.464 |
| Negative | 13(33.3) | 26(66.7) | 39 | | |
| Positive | 195(27.6) | 511(72.4) | 706 | | |
| **PgR** | | | | 4.984 | 0.028 |
| Negative | 62(34.1) | 120(65.9) | 182 | | |
| Positive | 142(25.5) | 414(74.5) | 556 | | |
| **AR** | | | | 2.596 | 0.116 |
| Negative | 56(33.3) | 112(66.7) | 168 | | |
| Positive | 142(26.9) | 386(73.1) | 528 | | |
| **p53** | | | | 6.538 | 0.070 |
| Negative | 184(30.2) | 426(69.8) | 610 | | |
| Positive | 25(19.1) | 106(80.6) | 131 | | |
| **MIB1** | | | | 0.001 | 1.000 |
| low | 54(27.3) | 144(72.7) | 198 | | |
| High | 103(27.2) | 275(72.8) | 378 | | |
| **E-cadherin** | | | | 3.803 | 0.053 |
| Negative | 89(32.4) | 186(67.6) | 275 | | |
| Positive | 120(25.7) | 347(74.3) | 467 | | |
| **P-cadherin** | | | | 3.803 | 0.058 |
| Negative | 112(30.5) | 255(69.5) | 367 | | |
| Positive | 62(23.5) | 202(76.5) | 264 | | |
| **HER2** | | | | 1.440 | 0.295 |
| Negative | 196(29) | 481(71) | 677 | | |
| Positive | 13(21.7) | 47(78.3) | 60 | | |
| **EGFR** | | | | 2.276 | 0.163 |
| Negative | 164(28.8) | 406(71.2) | 570 | | |
| Positive | 19(21.1) | 71(78.9) | 90 | | |

### 3.7.3.1 Correlation between BEX1 expression and patient outcome

In the whole series, BEX1 was associated with longer BCSS (LR=8.807& $p$=0.003) **(Fig 3.21A)** and longer DMFI (LR=7.926& $p$=0.005) **(Fig 3.21B)**. This association with longer survival was also demonstrated in the ER-positive luminal-like patient cohort regarding BCSS (LR=8.040& $p$=0.005) **(Fig 3.21C)** and DMFI (LR=6.497& $p$=0.011) **(Fig 3.21D).**

Multivariate Cox regression analyses including tumour size, tumour grade, lymph node stage and systemic therapy groups showed that BEX1 expression was an independent prognostic marker for longer BCSS in the whole series (HR=0.725, $p$=0.015, 95% CI=0.559-0.940) and in the ER-positive luminal-like cohort (HR =0.694, $p$=0.033, 95% CI=0.496-0.971) **(Table 3.31)**.

BEX1 expression was also an independent prognostic marker for longer DMFI in the whole series (HR=0.740, $p$=0.017, 95% CI=0.577-0.948) and in the ER-positive luminal-like cohort (HR =0.748, $p$=0.033, 95% CI=0.545-1.027).

**Table 3.31:** COX analysis model of BEX1 protein expression, tumour grade, LN stage, tumour size and adjuvant therapies in the ER-positive cohort in relation to BCSS

| Variable | p value | HR | 95% CI | |
|---|---|---|---|---|
| | | | Lower | Upper |
| **BEX1 expression** | 0.033 | 0.694 | 0.496 | 0.971 |
| **Endocrine Therapy** | 0.060 | 0.668 | 0.439 | 1.017 |
| **Chemotherapy** | 0.022 | 0.502 | 0.278 | 0.905 |
| **Tumour size** | <0.001 | 2.178 | 1.528 | 3.105 |
| **Tumour stage (2) vs. (1)** | 0.001 | 1.998 | 1.350 | 2.958 |
| **Tumour stage (3) vs. (1)** | <0.001 | 3.617 | 2.118 | 6.178 |
| **Tumour grade (2) vs. (1)** | 0.016 | 1.958 | 1.134 | 3.381 |
| **Tumour grade(3) vs. (1)** | <0.001 | 4.822 | 2.720 | 8.550 |

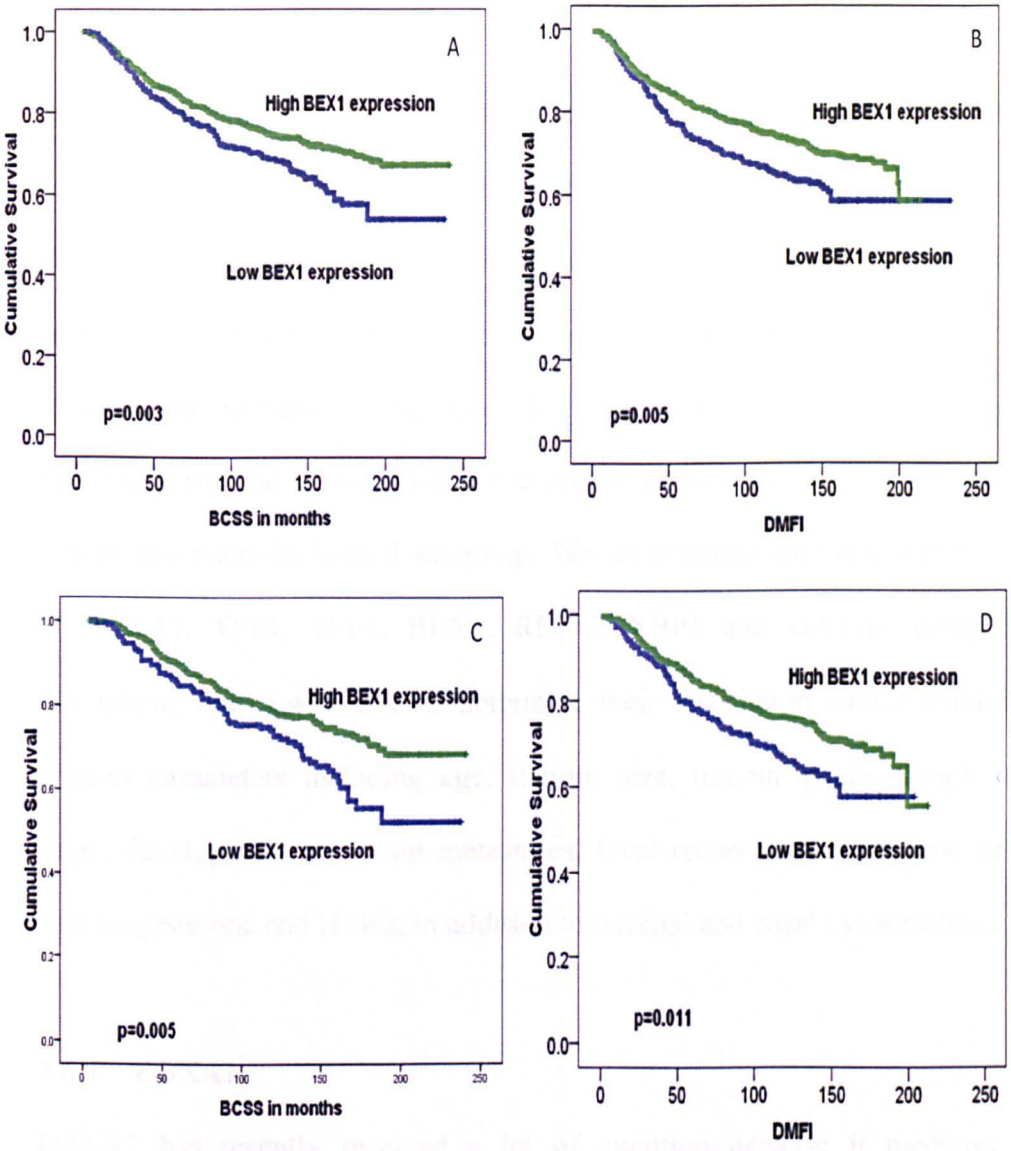


**Figure 3.21:** Kaplan Meier plots of BEX1 expression

(A)Kaplan Meier plot of BEX1 protein expression in relation to BCSS in the whole series. (B) Kaplan Meier plot of BEX1 protein expression in relation to DMFI in the whole series. (C) Kaplan Meier plot of BEX1 protein expression in relation to BCSS in the ER-positive cohort. (D) Kaplan Meier plot of BEX1 protein expression in relation to DMFI in the ER-positive cohort.

## 3.8 Discussion

Oestrogen receptor positive breast cancers form a large proportion of breast cancer patients and within this group much effort is being applied to discover reliable markers that can be used in patient prognosis and in determining response to various methods of postoperative therapies. In this chapter, we reported on biomarkers that have been previously identified using gene expression studies; to study if that was able to consistently classify the luminal cancer into patho-biological subgroup. We investigated the protein expression of FOXA1, TFF1, TFF3, BEX1, RERG, XBP1 and GATA3 using high throughput TMAs with IHC to determine their association with a number of clinical parameters including age, tumour size, tumour grade, lymph node stage, development of distant metastases, local recurrence, expression of ER and progesterone and HER2, in addition to luminal and basal cytokeratins.

### 3.8.1 FOXA1

FOXA1 has recently received a lot of attention because it mediates the expression of 50% of ER-regulated genes (Holmqvist et al., 2005, Laganiere et al., 2005) through its important functional activity on chromatin and the histones H3 and H4 (Cirillo and Zaret, 2007). By opening chromatin, FOXA1 is thought to facilitate the expression of ERα associated genes (Carroll et al., 2005) and it has been speculated that survival and proliferation of breast cancer epithelial cells may be under the control of a pathway that involves ER and

FOXA1. Previous studies of FOXA1 in breast cancer have shown conflicting roles with both growth repression and stimulation effects.

In the present study, we investigated the expression of FOXA1 protein, using immunohistochemistry in a large and well characterised cohort of breast cancer cases using TMAs, to evaluate its biologic and prognostic role in unselected and ER-defined breast cancer subsets. In particular, we investigated whether FOXA1 could be used to subclassify luminal tumours. In unselected breast cancer patients, we found that FOXA1 expression was associated with lower histologic grade, lower mitotic counts, smaller tumour size, and positive expression of hormone receptor positivity (ERα, PgR and AR) and other luminal markers. In addition, FOXA1 expression was positively associated with BRCA1, consistent with previous reports proposing that FOXA1 can block metastatic progression via influencing the BRCA1 associated cell cycle inhibitor p27 expression and regulating E-cadherin expression (Williamson et al., 2006, Liu et al., 2005). In support of this hypothesis, although we found that FOXA1 expression did not show a significant association with the development of distant metastasis there was an inverse relationship trend. Furthermore, FOXA1 expression showed a lack of association with other markers of aggressive tumour phenotype including epidermal growth factor receptors and p53. Moreover, FOXA1 protein expression showed a significant inverse association with the basal phenotype (CK5/6 & CK14 positivity) which is reported to have high proliferative activity and poor prognosis (Rakha et al., 2006).

Studies in MCF-7 cells suggested downregulation of FOXA1 mRNA levels following oestrogen stimulation (Frasor et al., 2003). Interestingly we have observed that positive FOXA1 expression is associated with good survival in patients who did not receive hormonal treatment. These patients are mostly ER negative where the ER-dependent downregulation involving FOXA1 is absent and this might lead to activation of FOXA1 growth inhibitory function. Collectively, these results support a tumour suppressor function for FOXA1 achieved through a growth inhibitory effect. Our findings are in agreement with previous studies showing association of FOXA1 with less-aggressive tumour characteristics (Badve et al., 2007, Wolf et al., 2007).

An important finding of this study is the lack of prognostic significance of FOXA1 expression as assessed by immunohistochemistry in ER-positive tumours, thus contrasting previous results global gene expression profiling studies, which showed that FOXA1 expression is a feature of Luminal A tumours. Our results support the growth inhibitory role of FOXA1 in breast cancer and emphasized its potentially important biological role and the strong association between FOXA1 and ER.

### 3.8.2 RERG

It is thought that novel approaches may be more appropriate for biomarkers discovery in breast cancer. Among the machine learning based methods, Artificial Neural Networks (ANNs) are emerging as valuable tools for this purpose. In this study, we used a transcript expression profiling of 128 frozen breast cancer cases and analysed the normalised expression values using

ANNs. Additionally, a cross-validation analysis in combination with a majority-vote ensemble sample classification was applied in order to obtain a more robust selection and ranking of genes to characterise the ER-positive breast cancer samples. We identified Ras-related, oestrogen-regulated growth inhibitor (RERG) as a candidate marker for differentiating between luminal and non-luminal classes among other genes including GATA3, CA12 and ESR1 which have been reported previously for characterising luminal class membership.

RERG is a GTP-binding protein with intrinsic GTPase activity (Finlin et al., 2001) and was initially identified as one of the genes that characterise Luminal A tumours using gene expression arrays. The expression of RERG has been reported to be decreased in the aggressive ER negative subtypes (Sorlie et al., 2001).

In our analysis of BC gene expression data, RERG correlated with high ER expression status using an ANN model and clearly belonged to the best-ranked genes for differentiating between luminal and non-luminal cases, being selected in each cycle a leave-one-out external cross-validation analysis that provided an average classification accuracy of 88.3%. Although RERG gene expression has been reported to be associated with ER-positive breast cancer in our analysis and previous studies, its protein expression has not been studied in breast cancer. To validate the gene expression findings, we studied RERG protein expression in invasive breast cancer using TMAs and immunohistochemistry. We found good agreement between protein and gene expression results, highlighting the importance of RERG as a candidate

luminal marker. RERG protein expression showed significant associations with luminal CKs (CK18), FOXA1, E-cadherin, steroid receptors and p27 which are all markers associated with good prognosis and luminal phenotype. *In vitro* studies also lend support to its association with good-prognostic phenotypes because RERG mRNA expression is induced rapidly in ER-responsive MCF-7 cells stimulated by oestradiol and repressed by ER-antagonist tamoxifen treatment (Finlin et al., 2001).

In contrast, we found an inverse relation between RERG protein expression and indicators of cell proliferation such as tumour grade, mitosis and MIB1 expression and this observation of altered cellular proliferation has been proposed as an explanation for the difference in prognosis seen within luminal tumours. In agreement with this observation, our protein expression results confirmed that RERG expression is associated with tumours displaying low MIB1 expression supporting the growth inhibitory function of RERG. The association of RERG with low tumour grade was also seen on mRNA level. For these reasons, we propose that within the luminal classes, expression of ER, RERG and MIB1 could be used to define biological subgroups with different prognoses. ER+ RERG+ MIB $^{low}$ could represent a luminal subgroup with good prognosis while those with an ER+ RERG- MIB1$^{high}$ phenotype could define a luminal subclass with poor prognosis.

A key aim of this study was to assess the prognostic ability of RERG protein expression in ER-positive luminal-like BC patients. In this important group of patients, we found that RERG expression was significantly associated with

longer BCSS and longer DMFI which implies its role in subclassification of ER-positive groups into prognostic subgroups.

### 3.8.3 GATA3

In breast cancer, GATA3 has emerged as a strong predictor of tumour differentiation, oestrogen-receptor status, and clinical outcome. Supporting the important prognostic role of GATA3, Dolled-Filhart and colleagues have shown in their clustering study that the minimal discovered set of tissue biomarkers with maximal prognostic or predictive value applied to conventional formalin-fixed, paraffin-embedded tissue sections was GATA3, NAT1, and oestrogen receptor (Dolled-Filhart et al., 2006).

Gene expression studies have shown that Luminal A breast cancers show the highest expression of GATA3 in comparison to Luminal B. In our gene expression study, GATA3 was significantly associated with the luminal-like ER-positive breast cancer. To validate these findings, we have performed protein expression analysis in 1,045 breast tissue samples using high throughput immunohistochemistry on TMAs. The results of this study showed a statistical positive correlation between GATA3 and markers of good prognosis including steroid receptor positivity, luminal CKs, BRCA1, Bcl-2 and FOXA1 in agreement with others (Mehra et al., 2005). These associations provide further evidence that GATA3 could be used as a marker of the ER-positive luminal-like breast cancer. Confirming the association between GATA3 and ER, we found that 97.3% of GATA3 positive tumours were also ER positive. In the ER-positive group, 33.3% of cases were GATA3 positive.

This number is close to the results of Voduc et al. who found that 39% of ER positive casa were also GATA3 positive (Voduc et al., 2008).

Tumour grade is a good indicator of breast cancer progression and differentiation status. It has been shown that high expression of GATA3 correlates with low tumour grade by cDNA microarray studies. Similarly, Mehra and co-workers showed that loss of GATA3 expression is associated with higher histological grade (Mehra et al., 2005). Supporting this, we found a strong correlation between GATA3 protein expression and low tumour grade with the more differentiated breast carcinomas expressing lower levels of GATA3 protein.

Previous studies have shown an important role of GATA3 in inhibiting tumour metastasis through its association with E-cadherin expression in which GATA3 acts as a predominant factor to induce E-cadherin and have presented an evidence of the association of GATA3 with the reversal of EMT in the inhibition of tumour metastasis (Yan et al.). Recently, Kouros-Mehr and colleagues have found that restoration of GATA3 in advanced mammary carcinoma of transgenic animals triggered cancer cell differentiation and subsequently suppressed cancer metastasis (Kouros-Mehr et al., 2008). Supporting these studies, we found a highly significant association between GATA3 and E-cadherin expression with a significant reduction in DM formation in GATA3 expressing tumours.

A key aim of this study was to assess the prognostic role of GATA3 protein expression in ER-positive luminal-like BC patients. In this important group of patients, we found that GATA3 expression was significantly associated with

longer BCSS and longer DMFI which implies its role in subclassification of ER-positive groups into prognostic subgroups.

### 3.8.4 Trefoil factors

TFF1 has been reported to be correlated with hormonal status but its value, either at the protein or mRNA level, has shown contradictory results. TFF1 protein expression is induced by oestrogen through oestrogen receptors (ER), and is known to be inhibited by antiestrogen in the breast cancer (Ribieras et al., 1998).

We found that TFF1 is significantly and positively related to ER and progesterone protein expression, in agreement with the findings of other investigators (Henry et al., 1991). Contrasting with our results Amiry and colleagues demonstrated an oncogenic role for TFF1 and showed that forced expression of TFF1 in mammary carcinoma cells promotes mammary tumour progression in vitro and in vivo and functional inhibition of TFF1 by RNA interference decreased the oncogenic properties of breast carcinoma cells (Amiry et al., 2009).

In this study, there was a non-significant correlation between TFF1 expression and overall survival and the disease free interval. On the other hand, TFF1 was reported as an independent prognostic factor in primary breast cancer and lymph node-negative patients (Foekens et al., 1993).

In our study, we showed that TFF1 was more expressed in low grade tumours, in accordance with other investigators (Ioachim et al., 2003, Speiser et al., 1994).

An important finding of this study was the lack of prognostic significance of TFF1 expression as assessed immunohistochemistry in ER-positive tumours, thus contrasting with previous results from global gene expression profiling studies, which showed that TFF1 expression is a feature of Luminal A tumours.

Our data have shown that TFF3 expression was associated with markers of luminal differentiation including luminal CKs, steroid receptors, Bcl-2 confirming its association with luminal phenotype. Although TFF3 was previously reported to be associated with the good prognosis Luminal A molecular class, our data showed that TFF3 expression is associated with poor outcome in luminal-like cancer and was not related to patients' survival in the whole series. The strong association between TFF3 and HER2 may explain the poor prognosis of ER+TFF3+ phenotype although TFF3 was reported to be a characterising marker of Luminal A subtype. TFF3 could characterise a subgroup of ER-positive with increased HER2 expression. Supporting our findings, Wilson and co-workers reported that TFF3 was overexpressed in HER2- positive breast cancer cells in vitro and was not expressed in HER2-negative cell lines (Wilson et al., 2002). These finding are in agreement with ours that showed a strong association with HER2 protein expression in both ER-positive luminal-like cohort and in the whole series.

Previously, TFF3 expression has been linked to metastatic potential in an animal model of colon cancer and contributed to the malignant behaviour of colon cancer cells (Babyatsky et al., 2009). An in vivo animal study has shown that both endogenous and constitutive expression of TFF3 correlates with an

aggressive phenotype (Yio et al., 2005). The expression of TFF3 was associated with metastasis in ER-positive patient group despite its association with good prognostic markers. This could be explained by its association with the HER2 oncogene.

### 3.8.5 XBP1

X-box-binding protein-1 (XBP-1) is a key transcriptional factor of the UPR that activates genes required for protein folding and degradation to restore endoplasmic reticulum function (Hetz et al., 2008).

XBP1 is stimulated by endoplasmic reticulum stress as part of the unfolded protein response (UPR), which can promote apoptosis or cell survival. Gene expression profiling of breast cancer tissue has previously shown an association between ER and XBP-1 expression because of its association with Luminal A breast cancer (Rzymski and Harris, 2007). The XBP-1 mRNA expression in ER-positive breast cancers was 2.7-fold as much as that in ER-negative breast cancers (Bertucci et al., 2000). Contrary to this, our results demonstrated a lack of significant association between ER and XBP1.

Scriven and co-workers showed for the first time that oestrogenic stimulation is also sufficient to induce downstream effectors of UPR activation such as XBP1 (Scriven et al., 2009).

In a previous study XBP1 was found to be expressed in 90% of breast cancer (Scriven et al., 2009). In this study, about 15 percent showed complete loss of XBP1 protein. Our results showed that strong XBP1 expression was associated with shorter survival in ER-positive luminal-like breast cancer which could be

explained by a possible ER coactivator function of XBP1 as previously shown (Ding et al., 2003) or by its role in the regulation of chromatin unfolding which may be responsible for the enhancement of ER transcriptional function and promoting the growth of tumour cells (Fang et al., 2004).

Furthermore, our results showed important associations between increased C-MYC expression, mutant p53 expression and increased XBP1 expression which further explain the poor prognosis seen in ER+XBP1+ phenotype.

### 3.8.6 BEX1

Our gene expression data has shown that BEX1 shows a great variation in the normalised expression value within the ER-positive cohort indicating an important role in the biology of luminal-like breast cancer. The results of this study showed for the first time the association of BEX1 with markers of good prognosis including lower tumour grade, PgR and luminal CK18, supporting other studies that BEX1 has a tumour suppressor function in other cancers (Foltz et al., 2006).

Furthermore, we found increased expression BEX1 in tubular and tubular mixed histologic tumour type which forms with the lobular cancer the low nuclear grade neoplasia family that mainly fall in the good prognosis luminal-like ER-positive breast cancer (Abdel-Fatah et al., 2008) .

In the whole series and ER-positive luminal-like breast cancer, BEX1 expression was significantly associated with longer survival which implies a potential role in subclassification of ER-positive groups into prognostic

subgroups. BEX1 could be used as a characterising marker of ER-positive breast cancer.

Our data regarding BEX1 are novel and we are the first to report on this marker and its prognostic and biological role in breast cancer using a large well characterised series of patients with long term follow-up.

In conclusion, using novel bioinformatics approaches to analyse high dimension datasets is of value to identify candidate genes to characterise the ER-positive/luminal like breast cancer. Subsequently, these can be used to subclassify these cancers in terms of biology and prognosis.

GATA3, BEX1 and RERG were able to differentiate between luminal-like tumours associated with poor and good prognosis and as such they could be useful markers for the definition of the luminal phenotype.

Although TFF3 and XBP1 are associated with good prognosis Luminal A subtype in the published gene expression studies, they showed associations with shorter BCSS while TFF1 and FOXA1 was not associated with survival in the ER-positive subtype. This could be attributed to the difference in the downstream technique used (RNA in expression profiling as opposed to protein in immunohistochemistry studies) or a post translational modification of the protein product of the gene.

These results may support the view that translation of gene expression profiling studies into clinical practice should be interpreted with care and individual markers may not show the same significance when studied in isolation and not as part of the expression signature.

# 4 Role of some ER coregulators in the biology and outcome of ER-positive breast cancer

## 4.1 Introduction

It is known that oestrogen plays an important role in the development and progression of breast cancer mediated through the oestrogens receptor (ER) in ER positive breast cancer.

Ligand binding to ER produces a specific change in the receptor structure, which releases it from the inhibitory effect of several chaperone proteins and produces receptor dimerization to initiate transcription (Klinge et al., 2004).

Apart from ligand binding to ER, the biologic functions of nuclear receptors, including the ER, are also regulated by a group of proteins known as transcriptional coactivators, as well as by another group of proteins known as transcriptional corepressors (Nair and Vadlamudi, 2007).

Coactivators are recruited to the target gene promoters through protein-protein interactions with the ER rather than by DNA binding and function as linker molecules between DNA binding proteins and DNA protein-modifying enzymes, which facilitate local structural alterations (Ma et al., 1999). It is important to examine the status of the steroid receptor co-regulators to identify their biological and clinical significance in breast cancer.

In this chapter we studied the biological and prognostic role of two ER coactivators including CARM1 and PELP1 with respect to patient outcome and biological associations.

## 4.2 CARM1

### 4.2.1 Introduction

Initially CARM1 (coactivator-associated arginine methyltransferase 1 ) was described as a factor that interacts with and further stimulates the transcription enhancing function of the p160 family of nuclear receptor coactivators that comprise other members such as SRC-1/NCoA1, GRIP1/TIF2/SRC-2/NCoA2, and RAC3/ACTR/AIB1/SRC-3/NCoA3 (Leo and Chen, 2000, Onate et al., 1998). CARM1 can enhance the transcriptional activation by nuclear receptors through methylation of histone H3 at arginine 17 (Miao et al., 2006, Schurter et al., 2001).

Confirming its important role in chromatin remodelling, Lee and co-workers reported on a transient-transfection assay under which the activity of various nuclear receptors is highly dependent on the synergistic action between CARM1, and other protein acetyltransferases, p300, CBP, or p/CAF. This synergy was observed when low levels of nuclear receptors were expressed and was highly dependent on the methyltransferase activity of CARM1 and the acetyltransferase activity of p/CAF, but not the acetyltransferase activity of p300 (Lee et al., 2002). This also suggests that the activation of gene transcription involves chromatin remodelling action of CARM1 which could be recruited by DNA-bound transcription factors.

More recently, CARM1 was also found to associate with p53, suggesting that this enzyme plays important roles in cell proliferation and survival (An et al., 2004). CARM1 knocked out cells showed impaired expression of a subset of

NF-kappaB- dependent genes (Covic et al., 2005) which implies that CARM1 can act as a coactivator of transcription factors other than nuclear receptors, like NF-kappaB (Covic et al., 2005, Miao et al., 2006).

CARM1 is a critical factor in the pathway of oestrogen-stimulated breast cancer growth downstream of ER alpha and upstream of the cell cycle regulatory transcription factor E2F1 and its target genes which include CDC25A, CCNA1, CCNE1, and CCNE2 (Stallcup et al., 2003, Frietze et al., 2008). Thus, CARM1 plays a critical role in breast cancer cell proliferation through the positive regulation of E2F1 expression.

The expression of CARM1 has been linked to the development of other human malignancy especially prostate cancer (Hong et al., 2004).

The value of CARM1 as a prognostic biomarker in the context of defining adverse, proliferative breast cancer phenotypes remains largely unexplored. This particularly applies to the ER-positive / luminal-like breast cancer where there is a pressing and important need to identify prognostic biomarkers for determining clinical outcome.

### 4.2.2 Material and Methods

Tissue microarrays were prepared and the immunohistochemical staining was performed using the streptavidin-biotin complex method using DakoCytomation Techmate 500 plus (DakoCytomation, Cambridge, UK) (see General Material and Methods Chapter). Rabbit polyclonal antibody to CARM1 (NB100-1817; Novus Biologicals Inc., Littleton, CO, USA) was optimized at a working dilution of 1:300 using full-face sections of breast

cancer excision tissue and TMAs to assess the heterogeneity and staining distribution. Negative controls were performed by omitting the primary antibody and substitution with diluent. Positive breast cancer cases were used as positive controls. To unmask the antigens, the sections were microwaved in citrate buffer pH 6 for 23 minutes.

X-tile bioinformatics software was used to define optimal cut off points of the H-score values (<30= negative/low, ≥30 and <150= moderate and ≥150= strong expression). The X-tile program randomly divides the total patient cohort into two separate training and validation sets ranked by patient follow up time. Statistical significance was tested by validating the obtained cut points to the validation set.

### 4.2.3 CARM1 expression results

After excluding the uninformative TMA cores from the study, 1130 tumours were available for assessment. The median age of the patients was 54 years (range 27-70 ±ST=9.897). Fifty one percent of patients had large tumours greater than or equal to 2 cm in size. Fifty eight percent of the tumours were ductal with no special type, 17% of the tumours were grade 1 and 26.7% showed good NPI. Thirty one percent of the patients developed metastatic disease during the period of follow-up and 41.7% developed tumour recurrence. The CARM1 staining pattern was mainly nuclear with homogenous distribution **(Fig 4.1)** being detected in the nuclei of the malignant cells and showed decreased expression in normal acini.

In the whole series, 30.5% of the tumours showed negative or low expression, 51.3% showed moderate expression while 18.2% showed strong expression. In

ER-positive group, 34% of case showed negative and low expression, 51.4% showed moderate expression and 13.4% showed strong expression.

#### 4.2.3.1 Correlation between CARM1 expression and other clinicopathological variables

In the whole series of unselected breast cancer patients, increased CARM1 expression was associated with young age, premenopausal status, high grade tumours, raised mitotic counts ($p<0.001$) and poor NPI group **(Table 4.1)**. No association was found between CARM1 and lymph node stage or vascular invasion.

In the ER-positive group, CARM1 expression was positively associated with higher tumour grade ($p=0.004$), DM ($p=0.001$), tumour recurrence ($p<0.001$), higher mitotic counts ($p=0.001$) and menopausal status ($p=0.001$). We found no associations between CARM1 and LN stage, NPI groups, vascular invasion or histologic tumour types **(Table 4.2)**.

#### 4.2.3.2 Correlation between CARM1 expression and other biomarkers

An inverse association was found between CARM1 expression and ER, PgR, AR, and luminal CK18 expression. Our results showed a positive association between CARM1 expression and basal CKs, MIB1 ($p<0.001$) expression, p53, HER2 and EGFR. No association was found between CARM1 and CK19, BRCA1 or E-cadherin **(Table 4.3)**.

In the ER-positive group, CARM1 expression was positively associated with p53 ($p<0.001$) and P-cadherin ($p<0.001$) **(Table 4.4)**.

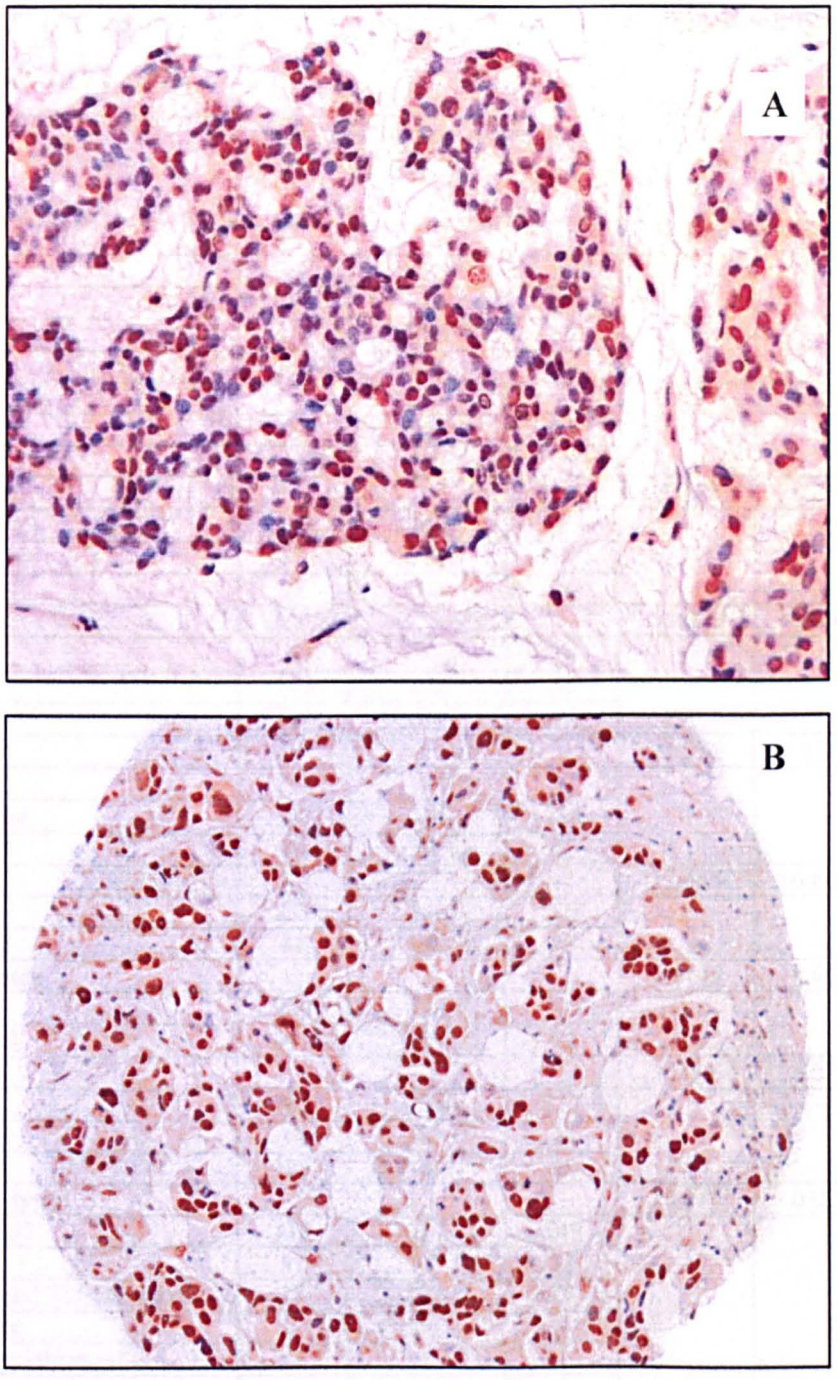


**Figure 4.1:** CARM1 protein expression in breast cancer
(A) Positive expression (full section) (x200) (B) Positive expression in a grade 2 ductal cancer (TMA core) (x100)

**Table 4.1:** Relation of CARM1 expression to other clinicopathological variables in whole series

| Variable | CARM1 nuclear expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | low | Moderate | Strong | | |
| **Age** | | | | 24.606 | <0.001 |
| <40 | 19 | 42 | 23 | | |
| 40-50 | 90 | 156 | 81 | | |
| 51-60 | 117 | 206 | 56 | | |
| >60 | 119 | 175 | 46 | | |
| **Size** | | | | 7.027 | 0.030 |
| **≤2 cm** | 187 | 286 | 88 | | |
| >2 cm | 1157 | 293 | 118 | | |
| **LN Stage** | | | | 5.537 | 0.236 |
| 1(Negative) | 220 | 340 | 123 | | |
| 2(1-3 LN) | 95 | 185 | 57 | | |
| 3(>3 LN) | 28 | 54 | 26 | | |
| **Grade** | | | | 51.608 | <0.001 |
| 1 | 88 | 91 | 14 | | |
| 2 | 114 | 194 | 50 | | |
| 3 | 142 | 294 | 142 | | |
| **NPI** | | | | 31.235 | <0.001 |
| Good | 122 | 149 | 31 | | |
| Moderate | 167 | 341 | 128 | | |
| Poor | 56 | 89 | 47 | | |
| **DM** | | | | 12.648 | 0.002 |
| No | 261 | 386 | 128 | | |
| Positive | 81 | 187 | 75 | | |
| **Recurrence** | | | | 10.145 | 0.006 |
| No | 220 | 318 | 105 | | |
| Positive | 117 | 251 | 92 | | |
| **VI** | | | | 3.181 | 0.528 |
| No | 187 | 335 | 117 | | |
| Probable | 32 | 60 | 24 | | |
| Definite | 126 | 181 | 65 | | |
| **Tumour type** | | | | 19.987 | 0.029 |
| Ductal/NST | 170 | 337 | 149 | | |
| Lobular | 51 | 57 | 13 | | |
| Tubular and Tubular mixed | 77 | 133 | 28 | | |
| Medullary | 7 | 20 | 9 | | |
| Other special types* | 11 | 8 | 1 | | |
| Mixed** | 29 | 24 | 6 | | |
| **Mitosis** | | | | 55.033 | <0.001 |
| 1 | 154 | 188 | 36 | | |
| 2 | 55 | 103 | 30 | | |
| 3 | 123 | 269 | 136 | | |
| **Menopause** | | | | 24.800 | <0.001 |
| Premenopausal | 108 | 216 | 108 | | |
| Postmenopausal | 237 | 363 | 98 | | |

*Includes Mucoid, invasive cribriform and invasive papillary carcinoma,
** Include ductal/NST mixed with lobular or special types

**Table 4.2:** Relation of CARM1 expression to other clinicopathological variables in the ER-positive cohort

| Variable | CARM1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | low | Moderate | Strong | | |
| **Age** | | | | 14.343 | 0.026 |
| <40 | 11 | 24 | 6 | | |
| 40-50 | 64 | 98 | 41 | | |
| 51-60 | 93 | 139 | 29 | | |
| >60 | 103 | 133 | 26 | | |
| **Size** | | | | 1.220 | 0.543 |
| **≤1.5 cm** | 151 | 208 | 51 | | |
| >1.5 cm | 119 | 186 | 51 | | |
| **LN Stage** | | | | 3.688 | 0.450 |
| 1(Negative) | 173 | 225 | 63 | | |
| 2(1-3 LN) | 75 | 135 | 31 | | |
| 3(>3 LN) | 21 | 34 | 8 | | |
| **Grade** | | | | 15.357 | 0.004 |
| 1 | 77 | 77 | 12 | | |
| 2 | 100 | 169 | 43 | | |
| 3 | 93 | 148 | 47 | | |
| **NPI** | | | | 8.478 | 0.076 |
| Good | 108 | 131 | 26 | | |
| Moderate | 123 | 210 | 60 | | |
| Poor | 40 | 53 | 16 | | |
| **DM** | | | | 14.200 | 0.001 |
| No | 212 | 262 | 64 | | |
| Positive | 57 | 129 | 38 | | |
| **Recurrence** | | | | 16.583 | <0.001 |
| No | 183 | 212 | 51 | | |
| Positive | 82 | 175 | 49 | | |
| **VI** | | | | 4.285 | 0.369 |
| No | 150 | 221 | 55 | | |
| Probable | 28 | 46 | 18 | | |
| Definite | 93 | 125 | 29 | | |
| **Tumour type** | | | | 15.287 | 0.122 |
| Ductal/NST | 127 | 195 | 60 | | |
| Lobular | 46 | 52 | 11 | | |
| Tubular and Tubular mixed | 68 | 115 | 25 | | |
| Medullary | 0 | 5 | 0 | | |
| Other special types | 7 | 5 | 1 | | |
| Mixed | 23 | 22 | 5 | | |
| **Mitosis** | | | | 17.677 | 0.001 |
| 1 | 137 | 163 | 29 | | |
| 2 | 45 | 83 | 27 | | |
| 3 | 78 | 132 | 45 | | |
| **Menopause** | | | | 15.189 | 0.001 |
| Premenopausal | 75 | 131 | 50 | | |
| Postmenopausal | 196 | 263 | 52 | | |

**Table 4.3:** Relation of CARM1 expression to other biomarkers in the whole series

| Variable | CARM1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | Moderate | Strong | | |
| **CK5/6** | | | | 33.175 | <0.001 |
| Negative | 294 | 470 | 141 | | |
| Positive | 33 | 94 | 59 | | |
| **CK14** | | | | 21.190 | <0.001 |
| Negative | 303 | 472 | 156 | | |
| Positive | 22 | 77 | 40 | | |
| **CK18** | | | | 11.519 | 0.003 |
| Negative | 32 | 73 | 39 | | |
| Positive | 278 | 439 | 143 | | |
| **CK19** | | | | 3.916 | 0.141 |
| Negative | 30 | 49 | 27 | | |
| Positive | 299 | 508 | 173 | | |
| **ER** | | | | 46.195 | <0.001 |
| Negative | 67 | 164 | 95 | | |
| Positive | 258 | 383 | 99 | | |
| **PgR** | | | | 19.182 | <0.001 |
| Negative | 125 | 226 | 112 | | |
| Positive | 194 | 318 | 82 | | |
| **AR** | | | | 21.951 | <0.001 |
| Negative | 104 | 174 | 94 | | |
| Positive | 209 | 345 | 87 | | |
| **p53** | | | | 52.109 | <0.001 |
| Negative | 271 | 398 | 106 | | |
| Positive | 51 | 153 | 87 | | |
| **BRCA1** | | | | 4.400 | 0.111 |
| Negative | 45 | 60 | 32 | | |
| Positive | 237 | 390 | 126 | | |
| **MIB1** | | | | 20.554 | <0.001 |
| low | 91 | 125 | 21 | | |
| High | 81 | 162 | 68 | | |
| **P-cadherin** | | | | 40.815 | <0.001 |
| Negative | 169 | 193 | 51 | | |
| Positive | 110 | 265 | 112 | | |
| **E-cadherin** | | | | 0.777 | 0.678 |
| Negative | 132 | 222 | 72 | | |
| Positive | 193 | 322 | 121 | | |
| **HER2** | | | | 18.803 | <0.001 |
| Negative | 305 | 481 | 154 | | |
| Positive | 26 | 75 | 41 | | |
| **EGFR** | | | | 16.106 | <0.001 |
| Negative | 254 | 381 | 120 | | |
| Positive | 39 | 98 | 48 | | |
| **Cyclin E** | | | | 9.304 | 0.010 |
| Negative | 97 | 159 | 50 | | |
| Positive | 4 | 15 | 11 | | |

**Table 4.4:** Relation of CARM1 expression to other biomarkers in the ER-positive cohort

| Variable | CARM1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | Moderate | Strong | | |
| **CK5/6** | | | | 9.383 | 0.015 |
| Negative | 251 | 360 | 87 | | |
| Positive | 13 | 30 | 14 | | |
| **CK14** | | | | 4.660 | 0.097 |
| Negative | 252 | 349 | 88 | | |
| Positive | 12 | 31 | 10 | | |
| **CK18** | | | | 0.341 | 0.843 |
| Negative | 12 | 16 | 3 | | |
| Positive | 243 | 353 | 89 | | |
| **CK19** | | | | 0.972 | 0.615 |
| Negative | 17 | 18 | 6 | | |
| Positive | 250 | 370 | 96 | | |
| **PgR** | | | | 3.612 | 0.164 |
| Negative | 74 | 85 | 25 | | |
| Positive | 186 | 303 | 77 | | |
| **AR** | | | | 3.401 | 0.183 |
| Negative | 66 | 72 | 22 | | |
| Positive | 192 | 297 | 70 | | |
| **p53** | | | | 21.084 | <0.001 |
| Negative | 232 | 315 | 68 | | |
| Positive | 30 | 72 | 32 | | |
| **BRCA1** | | | | 3.295 | 0.193 |
| Negative | 29 | 27 | 6 | | |
| Positive | 205 | 390 | 76 | | |
| **MIB1** | | | | 6.902 | 0.032 |
| low | 82 | 107 | 16 | | |
| High | 60 | 90 | 29 | | |
| **P-cadherin** | | | | 15.516 | <0.001 |
| Negative | 157 | 175 | 41 | | |
| Positive | 75 | 155 | 44 | | |
| **E-cadherin** | | | | 1.054 | 0.590 |
| Negative | 101 | 152 | 34 | | |
| Positive | 164 | 232 | 66 | | |
| **HER2** | | | | 5.447 | 0.066 |
| Negative | 247 | 350 | 85 | | |
| Positive | 17 | 33 | 14 | | |
| **EGFR** | | | | 7.163 | 0.028 |
| Negative | 219 | 289 | 71 | | |
| Positive | 24 | 58 | 17 | | |
| **Cyclin E** | | | | 3.535 | 0.171 |
| Negative | 82 | 116 | 27 | | |
| Positive | 1 | 2 | 2 | | |

#### 4.2.3.3 Correlation between CARM1 expresion and patient outcome

**Breast cancer specific survival (BCSS)**

In the whole patient series, an association between CARM1 expression and shorter BCSS was found (Log Rank (LR)=28.786, $p<0.001$) **(Fig 4.2a)**. Multivariate Cox hazard analysis including tumour size, histologic grade, lymph node stage, vascular invasion, systemic therapy groups and CARM1 expression showed that CARM1 expression was an independent predictor of shorter BCSS (Hazard ratio (HR)=2.179, $p<0.001$, 95%CI=1.465-3.242) **(Table 4.5)**.

In the ER-positive cohort, CARM1 expression also showed an association with shorter BCSS (LR=17.994, $p<0.001$) in univariate analysis **(Fig 4.2C)**. In multivariate analysis of ER-positive cohort, strong CARM1 expression was an independent predictor of shorter BCSS (HR=3.084, $p<0.001$, 95% CI=1.768-5.381) **(Table 4.6)**

**Disease free interval (DFI)**

In the whole patient series, an association between CARM1 expression and shorter DFI was found (LR=12.919, $p=0.002$) **(Fig 4.2B)**. Multivariate Cox hazard analysis showed that CARM1 expression was an independent predictor of shorter DFI (HR=1.6, $p=0.004$, 95% CI=1.116-2.205) **(Table 4.5)**. In the ER-positive cohort, CARM1 expression showed an association with shorter DFI (LR=15.004, $p=0.001$) in univariate analysis **(Fig 4.2D)** as well as in multivariate analysis (HR=2.267, $p<0.001$, 95% CI=1.491-3.447) **(Table 4.6)**.

#### 4.2.3.4 Outcome according to systemic therapy groups

Significant associations with shorter BCSS and DFI were maintained when we analysed our data according to the systemic therapy groups.

In the group of patient that did not receive adjuvant therapy (n=387), CARM1 expression showed an association with shorter BCSS (LR=21.200, $p<0.001$) **(Fig 4.3A)** and DFI (LR=11.697, $p=0.003$) **(Fig 4.3B)**.

In the group of ER-positive hormonal therapy treated patients (n=367), CARM1 expression showed an association with shorter BCSS (LR=11.266, $p=0.004$) **(Fig 4.3C)**.

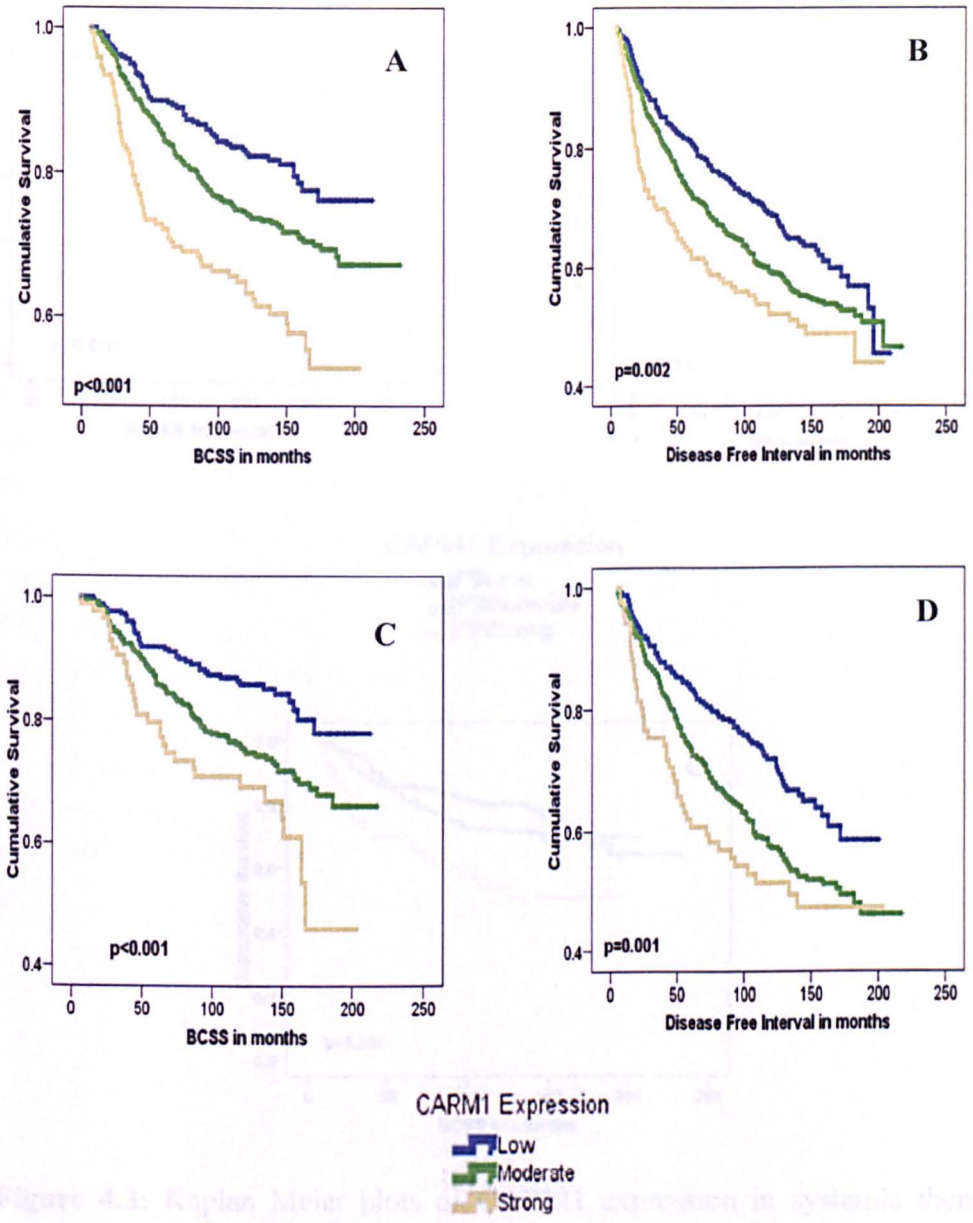


**Figure 4.2:** Kaplan Meier plots of CARM1 expression

(A) Kaplan Meier plot of CARM1 expression in relation to BCSS in the whole series. (B) Kaplan Meier plot of CARM1 expression in relation to DFI in the whole series. (C) Kaplan Meier plot of CARM1 expression in relation to BCSS in the ER-positive patient cohort. (D) Kaplan Meier plot of CARM1 expression in relation to DFI the ER-positive patient cohort.

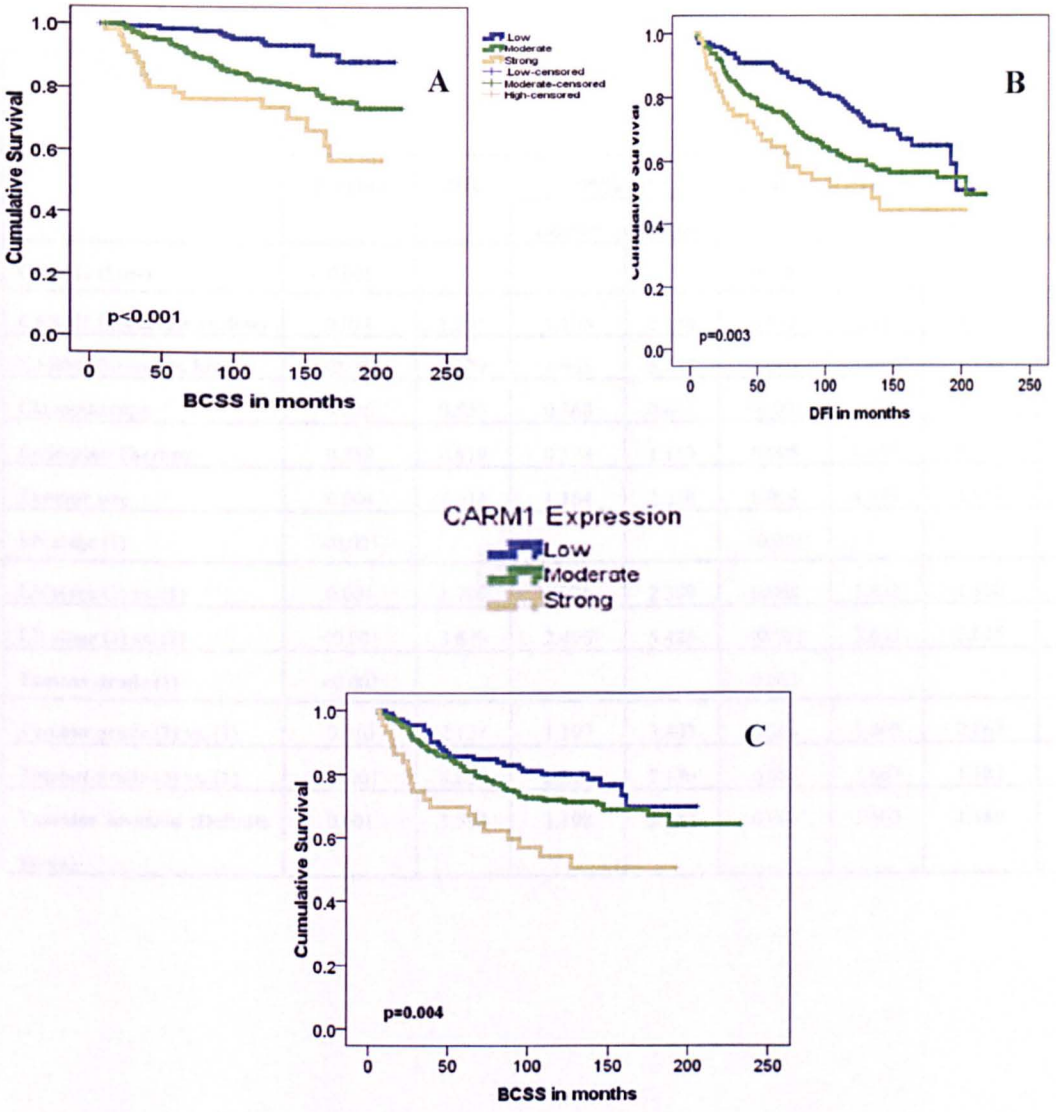


**Figure 4.3:** Kaplan Meier plots of CARM1 expression in systemic therapy groups

(A) Kaplan Meier plot of CARM1 expression in relation to BCSS in the untreated patient group. (B) Kaplan Meier plot of CARM1 expression in relation to DFI in the untreated patient group. (C) Kaplan Meier plot of CARM1 expression in relation to BCSS in the ER-positive tamoxifen only treated patient group

**Table 4.5:** Multivariate COX regression model for predictors of BCSS and DFI in the whole patient series

| Variable | BCSS | | | | DFI | | | |
|---|---|---|---|---|---|---|---|---|
| | P value | HR | 95% CI | | P value | HR | 95% CI | |
| | | | Lower | Upper | | | Lower | Upper |
| CARM1 (Low) | 0.001 | | | | 0.009 | | | |
| CARM1 (Moderate vs. low) | 0.011 | 1.537 | 1.105 | 2.136 | 0.015 | 1.348 | 1.059 | 1.717 |
| CARM1 Strong vs. low) | <0.001 | 2.179 | 1.465 | 3.242 | 0.004 | 1.600 | 1.160 | 2.205 |
| Chemotherapy | 0.006 | 0.556 | 0.368 | 0.842 | <0.001 | 0.544 | 0.387 | 0.766 |
| Endocrine Therapy | 0.259 | 0.819 | 0.578 | 1.159 | 0.005 | 0.675 | 0.513 | 0.889 |
| Tumour size | 0.004 | 1.614 | 1.164 | 2.238 | 0.014 | 1.344 | 1.061 | 1.702 |
| LN stage (1) | <0.001 | | | | <0.001 | | | |
| LN stage (2) vs. (1) | 0.001 | 1.700 | 1.226 | 2.359 | 0.008 | 1.432 | 1.100 | 1.863 |
| LN stage (3) vs. (1) | <0.001 | 3.679 | 2.496 | 5.424 | <0.001 | 3.643 | 2.625 | 5.055 |
| Tumour grade (1) | <0.001 | | | | 0.005 | | | |
| Tumour grade (2) vs. (1) | 0.010 | 2.134 | 1.197 | 3.807 | 0.264 | 1.208 | 0.867 | 1.683 |
| Tumour grade (3) vs. (1) | <0.001 | 4.035 | 2.271 | 7.170 | 0.004 | 1.667 | 1.182 | 2.351 |
| Vascular invasion (Definite vs. no) | 0.001 | 1.598 | 1.198 | 2.133 | 0.001 | 1.502 | 1.189 | 1.897 |

**Table 4.6:** Multivariate COX regression model for predictors of BCSS and DFI in the ER-positive /luminal like subgroup

| Variable | BCSS | | | | DFI | | | |
|---|---|---|---|---|---|---|---|---|
| | P value | HR | 95% CI | | P value | HR | 95% CI | |
| | | | Lower | Upper | | | Lower | Upper |
| **CARM1 (Low)** | <0.001 | | | | <0.001 | | | |
| **CARM1 (Moderate vs. low)** | 0.001 | 2.021 | 1.330 | 3.073 | <0.001 | 1.700 | 1.270 | 2.276 |
| **CARM1 Strong vs. low)** | <0.001 | 3.084 | 1.768 | 5.381 | <0.001 | 2.267 | 1.491 | 3.447 |
| **Chemotherapy** | 0.018 | 0.467 | 0.248 | 0.880 | 0.004 | 0.491 | 0.301 | 0.799 |
| **Endocrine Therapy** | 0.338 | 0.793 | 0.494 | 1.274 | 0.006 | 0.609 | 0.427 | 0.868 |
| **Tumour size** | 0.003 | 1.923 | 1.248 | 2.962 | 0.014 | 1.437 | 1.076 | 1.917 |
| **LN stage (1)** | <0.001 | | | | <0.001 | | | |
| **LN stage (2) vs. (1)** | 0.006 | 1.805 | 1.184 | 2.753 | 0.009 | 1.541 | 1.112 | 2.135 |
| **LN stage (3) vs. (1)** | <0.001 | 4.931 | 2.915 | 8.340 | <0.001 | 4.455 | 2.897 | 6.850 |
| **Tumour grade (1)** | <0.001 | | | | 0.004 | | | |
| **Tumour grade (2) vs. (1)** | 0.028 | 2.034 | 1.080 | 3.831 | 0.138 | 1.320 | 0.915 | 1.905 |
| **Tumour grade (3) vs. (1)** | <0.001 | 4.255 | 2.190 | 8.267 | 0.001 | 1.960 | 1.296 | 2.965 |
| **Vascular invasion (Definite vs. no)** | 0.008 | 1.645 | 1.138 | 2.378 | 0.014 | 1.430 | 1.076 | 1.902 |

## 4.3 PELP1

### 4.3.1 Introduction

PELP1 (proline, glutamate and leucine rich protein 1) consists of 1,282 amino acids and is located on chromosome 17 (Vadlamudi et al., 2001). It improves 17$\beta$-oestradiol (E2) dependent transcriptional activation from the oestrogen response element in a dose-dependent fashion and shows high expression in various tissues especially in the testes, breast, and brain. Importantly, PELP1 may add to the oncogenic properties of cancer cells by acting as a scaffolding protein that relates many signalling processes with ER through its interaction with other oncogenes including SRC, PI3K, STAT3 and EGFR (Vadlamudi et al., 2001).

Previous gene knock down studies of PELP1 have shown reduced E2 activation of Akt signalling pathway significantly, and inhibited E2 genomic transcriptional effects on gene expression in breast cancer cells (Brann et al., 2008). Regulation of aromatase by PELP1 represents a novel mechanism for autocrine oestrogen synthesis which may lead to tumour proliferation (Rajhans et al., 2008). These findings suggest an important tumourigenic role of PELP1 and may open a new targeted therapeutic approach by its inhibition (Nagpal et al., 2008).

Other studies suggest a different mechanism for the oncogenic properties of PELP1 through its involvement in histone remodelling. PELP1 maintains the balanced hypoacetylated state of histones, while ER binding reverses its role through hyperacetylation of histones through an unknown mechanism (Choi et

al., 2004). In addition, it has been suggested that PELP1 contributes to chromatin remodelling by affecting certain types of histone in cancer cells (Nair et al., 2004). In a small previous breast cancer study, PELP1 expression was reported to be up-regulated in higher grade lymph node positive invasive tumours (Rajhans et al., 2008, Rajhans et al., 2007) but the study did not specifically focus on PELP1 expression in ER-positive/luminal cancers.

The value of PELP1 as a prognostic biomarker in defining breast cancer phenotypes remains undetermined. Therefore, our aim was to investigate the clinical relevance and biological relations of PELP1 protein expression in a large series of consecutive patients with invasive breast cancers using high-throughput tissue microarrays (TMAs) and immunohistochemistry and to test its association with other clinically and biologically relevant biomarkers. In addition, PELP1 protein expression was investigated in the ER-positve patients' cohort.

### 4.3.2 Material and Methods

Tissue microarrays were prepared and the immunohistochemical staining was performed using the streptavidin-biotin complex method using DakoCytomation Techmate 500 plus (DakoCytomation, Cambridge, UK) (General Material and Methods Chapter).

Rabbit polyclonal antibody to PELP1 (NB100-1749; Novus Biologicals Inc., Littleton, CO, USA) was optimized at a working dilution of 1:100 using full-face sections of breast cancer tissue to assess the staining distribution. Negative controls were performed by omitting the primary antibody and substitution

with diluent. Peptide blocking with PELP1 antigen (Novus Biologicals, NB100-1749PEP) was performed to verify the antibody specificity. Positive breast cancer cases were used as positive controls.

The H-score (histochemical score) was used for assessment. The X-tile (Camp et al., 2004) program was used to define optimal cut off points of PELP1 H-score values (<5=negative/low, ≥5and<170=moderate and ≥170=strong expression).

### 4.3.3 PELP1 expression results

After excluding the uninformative TMA cores from the study, 1,162 tumours were available for assessment. The median age of the patients was 55 years (range 27-70). Sixty eight percent of patients had tumours greater than or equal to 1.5 cm in size. Fifty nine percent of the tumours were ductal of no special type, 17% of the tumours were grade 1 and 27.8% showed good NPI. Thirty percent of the patients developed metastatic disease during the period of follow-up and 41.7% developed tumour recurrence.

PELP1 staining was detected in the nuclei of the malignant cells as well as in some luminal ductal epithelial cells of associated normal tissues in the cores. Applying the peptide blocking successfully abrogated staining **(Fig 4.4B)**. The In the whole series, 17.2% of the tumours showed negative or low expression, 69.3% showed moderate expression **(Fig 4.4C/D)** while 13.5% showed strong expression **(Fig 4.4A)**. No cytoplasmic staining was observed.

#### 4.3.3.1 Correlation between PELP1 expression and other clinicopathological variables

In the whole series of unselected breast cancer patients, increased PELP1 expression was associated with markers of poor prognosis such as large primary tumour size, raised mitotic counts ($p$=0.004), recurrence and poor NPI group.

No associations were found between PELP1 and patients' age, lymph node stage, vascular invasion and menopausal status **(Table 4.7).** When the analysis was repeated on ER-positive/luminal-like group of tumours (n=768), PELP1 expression showed border line associations with large tumour size and development of tumour recurrence ($p$=0.027) **(Table 4.8).**

#### 4.3.3.1 Correlation between PELP1 expression and other biomarkers

In the whole series, we found significant positive associations between PELP1 expression and biomarkers of poor prognosis including P-cadherin, p53, and CARM1 ($p$<0.001). An inverse association was found between PELP1 expression and ERα, PgR, AR, and CK18 expression. No associations were found between PELP1 and other biomarkers included in the study **(Table 4.9).** In the ER-positive group of tumours, PELP1 expression was associated with CARM1 expression ($p$<0.001).

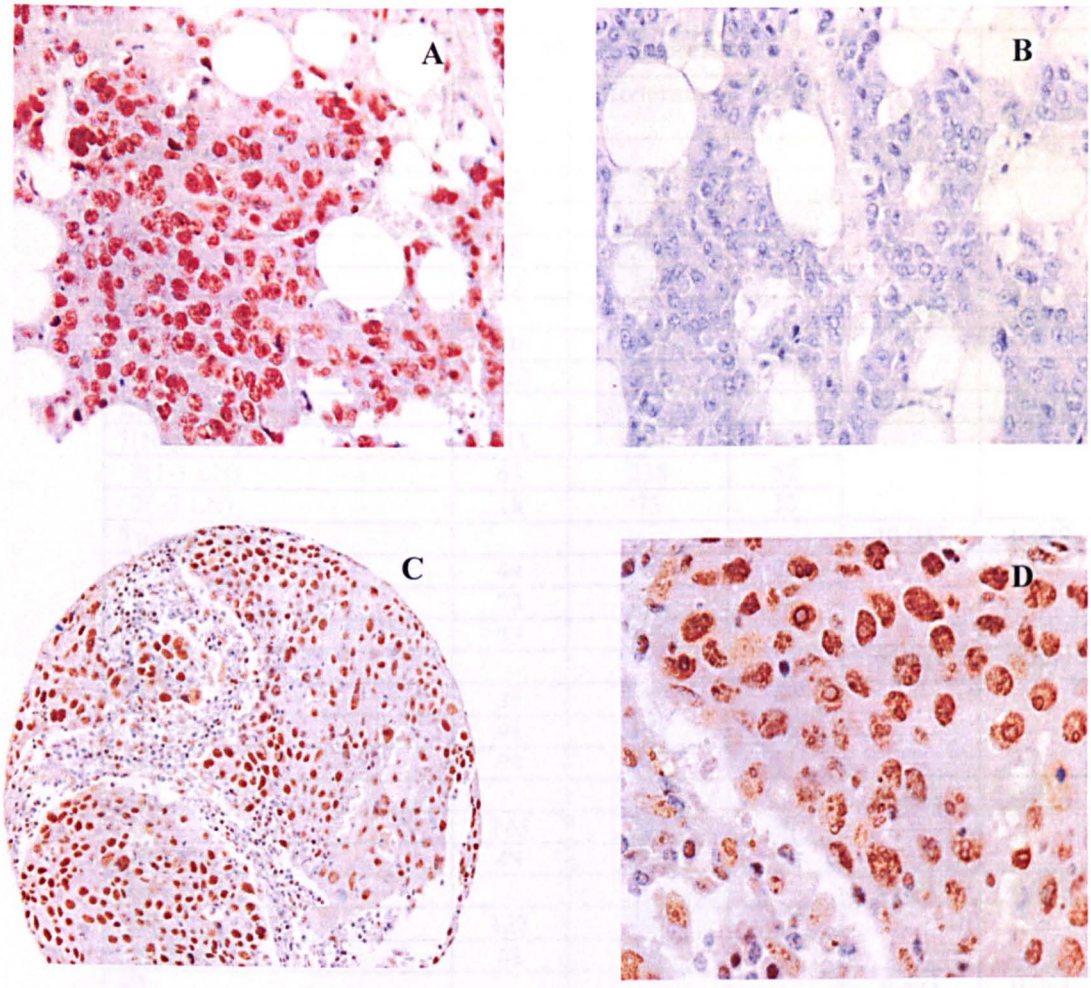


**Figure 4.4:** PELP1 expression in breast cancer

(A&B) PELP1 nuclear staining was lost with application of the peptide blocking, used as a negative control.(Ax200 & Bx200). (C&D) TMA core of a grade 3 ductal carcinoma showing strong positive PELP1 nuclear expression (C x100 & D x400).

**Table 4.7 :** Relation of PELP1 expression to other clinicopathological parameters in the whole series

| Variable | PELP1 Expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | low | Moderate | Strong | | |
| **Patients' age** | | | | 3.069 | 0.8 |
| <40 | 14 | 61 | 12 | | |
| 40-50 | 55 | 231 | 45 | | |
| 51-60 | 78 | 264 | 52 | | |
| >60 | 53 | 249 | 48 | | |
| **Tumour size** | | | | 11.098 | 0.004 |
| **≤1.5 cm** | 80 | 254 | 37 | | |
| >1.5 cm | 120 | 551 | 120 | | |
| **Lymph node stage** | | | | 0.930 | 0.920 |
| 1(Negative) | 118 | 491 | 92 | | |
| 2(1-3 LN) | 63 | 238 | 52 | | |
| 3(>3 LN) | 18 | 73 | 13 | | |
| **Tumour grade** | | | | 10.045 | 0.040 |
| 1 | 44 | 128 | 26 | | |
| 2 | 73 | 247 | 46 | | |
| 3 | 83 | 428 | 85 | | |
| **NPI** | | | | 14.045 | 0.007 |
| Poor | 35 | 137 | 30 | | |
| Moderate | 91 | 451 | 95 | | |
| Good | 74 | 217 | 32 | | |
| **DM** | | | | 6.873 | 0.032 |
| No | 145 | 554 | 97 | | |
| Positive | 49 | 244 | 60 | | |
| **Recurrence** | | | | 11.895 | 0.003 |
| No | 133 | 451 | 78 | | |
| Positive | 62 | 336 | 76 | | |
| **VI** | | | | 0.593 | 0.964 |
| No | 111 | 450 | 83 | | |
| Probable | 23 | 85 | 17 | | |
| Definite | 66 | 268 | 56 | | |
| **Histologic tumour type** | | | | 19.987 | 0.029 |
| Ductal/NST | 109 | 482 | 97 | | |
| Lobular | 33 | 89 | 13 | | |
| Tubular and Tubular mixed | 42 | 156 | 32 | | |
| Medullary | 1 | 22 | 7 | | |
| Other special types* | 7 | 10 | 1 | | |
| Mixed** | 8 | 46 | 7 | | |
| **Mitosis** | | | | 15.465 | 0.004 |
| 1 | 88 | 257 | 48 | | |
| 2 | 37 | 137 | 27 | | |
| 3 | 68 | 388 | 79 | | |
| **Menopausal status** | | | | 1.598 | 0.450 |
| Premenopausal | 67 | 308 | 60 | | |
| Postmenopausal | 133 | 497 | 97 | | |

*Includes mucoid, invasive cribriform and invasive papillary carcinoma, ** Includes ductal/NST mixed with lobular or special types

**Table 4.8:** Relation of PELP1 expression to other clinicopathological variables in the ER-positive cohort

| Variable | PELP1 Expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | low | Moderate | Strong | | |
| **Patients' age** | | | | 8.354 | 0.213 |
| <40 | 5 | 31 | 3 | | |
| 40-50 | 34 | 139 | 24 | | |
| 51-60 | 63 | 175 | 27 | | |
| >60 | 45 | 184 | 38 | | |
| **Tumour size** | | | | 6.945 | 0.031 |
| **≤1.5 cm** | 63 | 189 | 24 | | |
| >1.5 cm | 84 | 340 | 68 | | |
| **Lymph node stage** | | | | 1.109 | 0.893 |
| 1(Negative) | 89 | 322 | 51 | | |
| 2(1-3 LN) | 46 | 167 | 33 | | |
| 3(>3 LN) | 11 | 38 | 8 | | |
| **Tumour Grade** | | | | 4.362 | 0.359 |
| 1 | 37 | 111 | 22 | | |
| 2 | 67 | 217 | 35 | | |
| 3 | 43 | 200 | 35 | | |
| **NPI** | | | | 6.379 | 0.173 |
| Poor | 19 | 69 | 16 | | |
| Moderate | 64 | 267 | 50 | | |
| Good | 64 | 193 | 26 | | |
| **DM** | | | | 2.624 | 0.269 |
| No | 108 | 375 | 60 | | |
| Positive | 36 | 151 | 32 | | |
| **Recurrence** | | | | 7.222 | 0.027 |
| No | 100 | 305 | 47 | | |
| Positive | 45 | 216 | 42 | | |
| **VI** | | | | 4.109 | 0.392 |
| No | 82 | 298 | 43 | | |
| Probable | 19 | 60 | 16 | | |
| Definite | 46 | 169 | 33 | | |
| **Histologic tumour type** | | | | 10.585 | 0.391 |
| Ductal/NST | 69 | 269 | 49 | | |
| Lobular | 29 | 82 | 11 | | |
| Tubular | 38 | 129 | 26 | | |
| Medullary | 0 | 3 | 0 | | |
| Other special types | 5 | 8 | 0 | | |
| Mixed | 6 | 38 | 6 | | |
| **Mitosis** | | | | 8.124 | 0.087 |
| 1 | 80 | 229 | 38 | | |
| 2 | 29 | 108 | 19 | | |
| 3 | 33 | 175 | 32 | | |
| **Menopausal status** | | | | 3.488 | 0.175 |
| Premenopausal | 38 | 178 | 27 | | |
| Postmenopausal | 109 | 351 | 65 | | |

**Table 4.9:** Relation of PELP1 expression to other biomarkers in the whole series

| Variable | PELP1 Expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | Moderate | Strong | | |
| **CK5/6** | | | | 7.090 | 0.029 |
| Negative | 168 | 653 | 119 | | |
| Positive | 23 | 128 | 35 | | |
| **CK14** | | | | 8.090 | 0.018 |
| Negative | 174 | 669 | 121 | | |
| Positive | 16 | 97 | 58 | | |
| **CK18** | | | | 6.262 | 0.044 |
| Negative | 15 | 109 | 25 | | |
| Positive | 163 | 619 | 121 | | |
| **CK19** | | | | 2.393 | 0.302 |
| Negative | 16 | 76 | 20 | | |
| Positive | 175 | 705 | 131 | | |
| **ER** | | | | 12.108 | 0.002 |
| Negative | 40 | 236 | 58 | | |
| Positive | 147 | 529 | 92 | | |
| **PgR** | | | | 11.009 | 0.004 |
| Negative | 58 | 343 | 70 | | |
| Positive | 123 | 420 | 79 | | |
| **AR** | | | | 16.078 | <0.001 |
| Negative | 42 | 279 | 60 | | |
| Positive | 136 | 449 | 81 | | |
| **p53** | | | | 9.372 | 0.009 |
| Negative | 150 | 545 | 106 | | |
| Positive | 33 | 217 | 47 | | |
| **BRCA1** | | | | 4.882 | 0.087 |
| Negative | 21 | 110 | 12 | | |
| Positive | 142 | 552 | 114 | | |
| **Bcl-2** | | | | 10.961 | 0.090 |
| Negative | 28 | 134 | 26 | | |
| Weak | 18 | 113 | 20 | | |
| Moderate | 53 | 162 | 20 | | |
| Strong | 13 | 46 | 6 | | |
| **MIB1** | | | | 8.033 | 0.018 |
| low | 53 | 163 | 20 | | |
| High | 47 | 218 | 45 | | |
| **P-cadherin** | | | | 12.588 | 0.002 |
| Negative | 96 | 295 | 53 | | |
| Positive | 68 | 362 | 80 | | |
| **E-cadherin** | | | | 1.240 | 0.538 |
| Negative | 69 | 312 | 57 | | |
| Positive | 120 | 455 | 92 | | |
| Negative | 88 | 425 | 71 | | |
| Positive | 29 | 161 | 38 | | |
| **CARM1** | | | | 60.987 | <0.001 |
| Low | 75 | 200 | 21 | | |
| Moderate | 49 | 366 | 70 | | |
| Strong | 20 | 105 | 45 | | |

#### 4.3.3.2 Correlation between PELP1 expression and patient outcome

**(A) Breast cancer specific survival (BCSS)**

In the whole patient series, an association between PELP1 expression and shorter BCSS was found (Log Rank (LR) =12.168, $p$=0.002) **(Fig 4.5A)**. Multivariate Cox hazard analysis including tumour size, histologic grade, lymph node stage, vascular invasion, ER expression showed that PELP1 expression was an independent predictor of shorter BCSS (Hazard ratio (HR) =1.349, $p$=0.006, 95%CI=1.091-1.668).

In the ER-positive cohort, PELP1 expression also showed an association with shorter BCSS (LR=7.029, $p$=0.030) in univariate analysis **(Fig 4.5C)**. However, in multivariate Cox analysis of ER-positive cohort, PELP1 was not an independent predictor of BCSS (HR=1.302, $p$ =0.061, 95% CI=0.987-1.717) **(Table 4.10).**

**(A) Disease free interval (DFI)**

In the whole patient series, an association between PELP1 expression and shorter DFI was found (LR=10.336, $p$=0.006) **(Fig 4.5B)**. Multivariate Cox hazard analysis showed that PELP1 expression was an independent predictor of shorter DFI (HR=1.255, $p$=0.011, 95% CI=1.053-1.495).

In the ER-positive cohort, PELP1 expression showed an association with shorter DFI (LR=6.805, $p$=0.033) in univariate analysis **(Fig 4.5D)** as well as in multivariate analysis (HR=1.256, $p$=0.036, 95% CI=1.015-1.553).

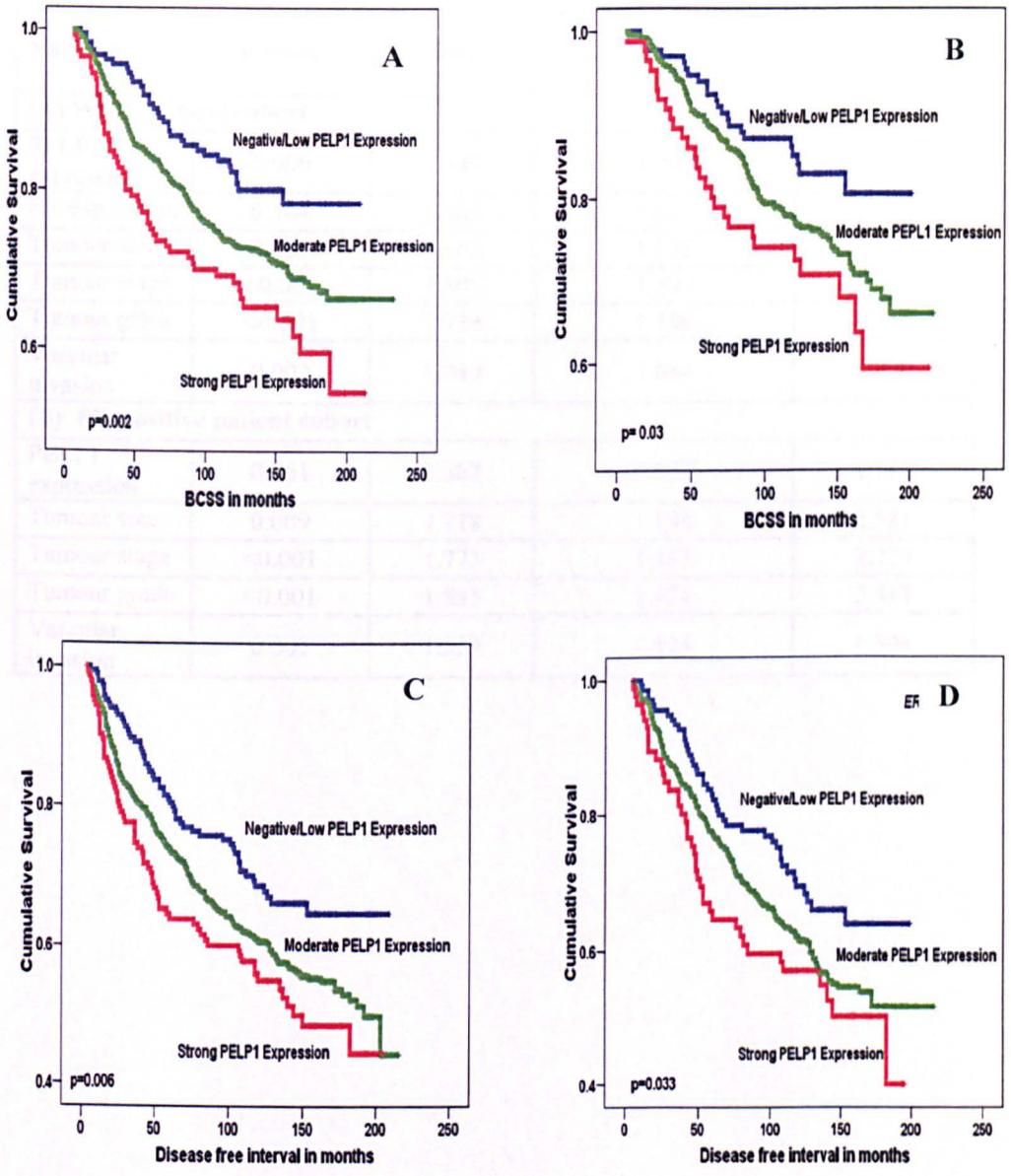


**Figure 4.5:** Kaplan Meier plots of PELP1 expression

(A) Kaplan Meier plot of PELP1 expression in the whole series in relation to BCSS. (B) Kaplan Meier plot of PELP1 expression in the whole series in relation to DFI. (C) Kaplan Meier plot of PELP1 expression in the ER-positive cohort in relation to BCSS. (D) Kaplan Meier plot of PELP1 expression in the ER-positive cohort in relation to DFI.

**Table 4.10:** Multivariate COX regression model for predictors of BCSS (A) The whole patient cohort and (B) ER-positive patient cohort

| Variable | p value | HR | 95 % CI | |
|---|---|---|---|---|
| | | | Lower | Upper |
| **(A) Whole patient cohort** | | | | |
| PELP1 expression | 0.006 | 1.349 | 1.091 | 1.668 |
| ER expression | 0.104 | 0.808 | 0.625 | 1.045 |
| Tumour size | 0.005 | 1.602 | 1.155 | 2.223 |
| Tumour stage | <0.001 | 1.893 | 1.592 | 2.251 |
| Tumour grade | <0.001 | 1.724 | 1.386 | 2.145 |
| Vascular invasion | 0.002 | 1.240 | 1.084 | 1.420 |
| **(B) ER-positive patient cohort** | | | | |
| PELP1 expression | 0.061 | 1.302 | 0.987 | 1.717 |
| Tumour size | 0.009 | 1.718 | 1.144 | 2.581 |
| Tumour stage | <0.001 | 1.775 | 1.413 | 2.229 |
| Tumour grade | <0.001 | 1.885 | 1.474 | 2.412 |
| Vascular invasion | 0.001 | 1.339 | 1.125 | 1.594 |

## 4.4 The effect of combined expression of PELP1 and CARM1 on patient survival

The combined expression of CARM1 and PELP1 was investigated. The group of patients showing combined positive expression of both (Moderate and strong vs. low and negative groups) showed a significant shorter breast cancer specific survival (LR=14.428 and $p$=0.002) **(Fig 4.6)** and this effect was maintained in the ER-positive luminal-like subgroup (LR=13.797 and $p$=0.003).

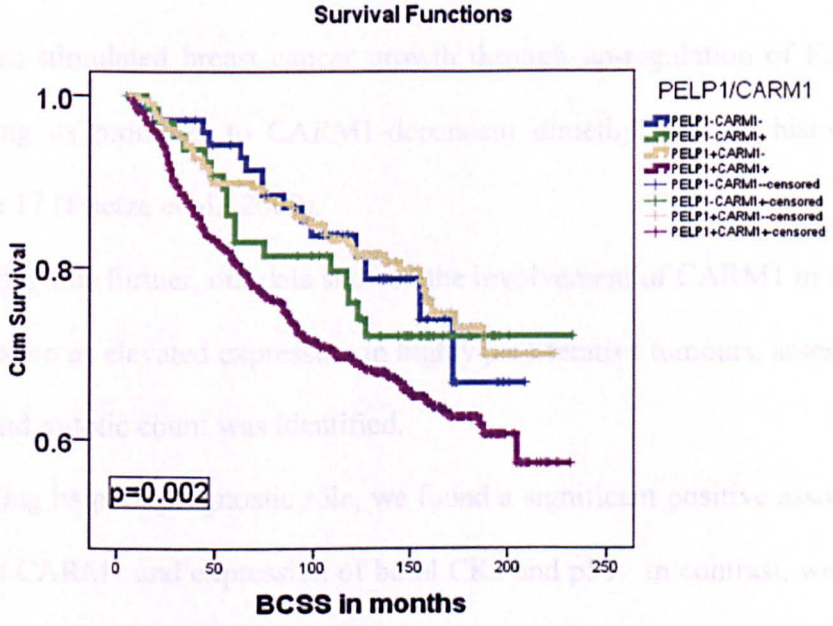


**Figure 4.6:** Kaplan Meier plots of combined CARM1/PELP1 expression in the whole series in relation to BCSS

## 4.5 Discussion

### 4.5.1 CARM1

CARM1 was originally identified because of its ability to interact with p160 family of nuclear receptor coactivators to methylate histone H3 and activates transcription via chromatin remodelling.

We found significant positive associations between CARM1 and known features of poor prognosis including large tumour size, high tumour grade and frequent development of distant metastasis, tumour recurrence in the whole patient series as well as in the ER-positive cohort. These findings support the novel data by Frietze and colleagues who found that CARM1 regulates oestrogen-stimulated breast cancer growth through up-regulation of E2F1 by subjecting its promoter to CARM1-dependent dimethylation on histone H3 arginine 17 (Frietze et al., 2008).

Supporting this further, our data showed the involvement of CARM1 in tumour proliferation as elevated expression in highly proliferative tumours, assessed by MIB1 and mitotic count was identified.

Supporting its poor prognostic role, we found a significant positive association between CARM1 and expression of basal CKs and p53. In contrast, we found an inverse relation between luminal CKs and steroid receptor expression which are markers of good prognosis in breast cancer.

We found a positive correlation between cyclin E and CARM1 protein expression in breast cancer in agreement with others (El Messaoudi et al.,

2006) who found CARM1 is required for proper activation of endogenous CCNE1 mRNA expression in mammalian cells.

Our results showed a positive association between CAMR1 protein expression and HER family members, EGFR1 and HER2 suggesting a crosstalk between this important growth signalling pathway and ER coregulators.

Furthermore, we have assessed the prognostic ability of CARM1 in ER-positive/luminal-like breast cancer patients. In this important group of patients, we found that CARM1 expression was significantly associated with shorter BCSS and shorter DFI which implies its role in subclassification of ER-positive groups into prognostic subgroups. These findings are also found in the patient group that did not receive systemic therapy to overcome the confounding effect of treatment on our survival analysis.

In conclusion, this study demonstrated the biological and prognostic role of CARM1 in breast cancer. CARM1 expression was an independent prognostic factor of shorter survival in breast cancer and the ER-positive luminal-like subtype. Overexpression of CARM1 is involved in the progression of breast cancer suggesting that targeting its expression in high proliferative breast cancer could be of potential in developing novel treatments for breast cancer.

### 4.5.2 PELP1

To date PELP1 has not been identified as a discriminating marker in the luminal subclasses of breast cancer. Our results, as discussed below imply that

PELP1 has the potential to stratify patients with ER positive breast cancer into biological subclasses with differing prognoses.

In this study, the status of the steroid ER co-regulator PELP1 was also investigated in a large cohort of patients with breast cancer to better understand its clinical and biological significance. We found a positive association between PELP1 and markers of poor prognosis and aggressive tumour behaviour including larger tumour size, higher histological grade, frequent development of tumour recurrence in the whole patient series. These findings support the emerging data that PELP1 interacts with many proteins and activates several oncogenes that are related to the aggressive tumour characteristics and metastatic behaviour, including SRC, phosphotidyl inositol 3 kinase (PI3K), and signal transducers and activators of transcription 3 (STAT3) (Nair and Vadlamudi, 2007).

In this study, we found a significant positive correlation between PELP1 and CARM1 which is necessary for the E2-induced proliferation of breast cancer cells via E2F1 and its target genes (Stallcup et al., 2003, Frietze et al., 2008). This positive correlation at the protein level suggests a possible synergistic action between PELP1 and CARM1, being both ER coactivators, in oestrogen induced proliferation of ER-positive breast cancer cells. This effect was confirmed by studying the effect of combined expression of PELP1/CARM1 on patient outcome indicating a significant reduction of survival.

The significance of non-genomic ER activity in mediating oestrogen signalling to promote cell proliferation and survival in breast cancer cells has been documented (Schiff et al., 2005). Many studies have highlighted the

importance of PELP1 in tumour progression through increasing oestrogen mediated cell proliferation possibly through its requirement to ER alpha interaction with SRC which leads to activation of MAPK pathway (Cheskis et al., 2008). Our data implicates the involvement of PELP1 in tumour proliferation as we identified elevated expressions in highly proliferative tumours, assessed by MIB1 and mitotic counts.

Supporting its poor prognostic role, we found significant positive associations between PELP1 and expression of P-cadherin and p53 which are more frequently expressed in basal-like breast cancer and are associated with poor prognosis.

The potential prognostic role of PELP1 in ER-positive/luminal-like breast cancer patients was investigated. PELP1 expression was significantly associated with shorter BCSS and shorter DFI which implies its role in subclassification of ER-positive groups into prognostic subgroups.

In conclusion, the results of this study demonstrated the biological and prognostic role of PELP1 in breast cancer which cannot be considered as a mere reflection of ER expression as evidenced by its role in the whole series of breast cancer as well as in the ER-positive/luminal like subclass.

CARM1 and PELP1 protein expression in breast cancer could have a role in clinical decision making and assessment of prognosis, particularly in the ER-positive group. Furthermore, improved understanding of their functional role and their mechanism of action in breast may reveal a role as therapeutic targets.

# 5 The protein expression of biomarkers with potential therapeutic implication and endocrine therapy response in ER-positive breast cancer

## 5.1 Introduction

ER-positive/luminal-like tumours are clearly not a homogenous group; some tumours respond to therapy and others do not. They are mostly low grade and more sensitive to endocrine therapy because of their ER-positive status especially in the Luminal A subclass. A proportion of ER-positive BC relapse after tamoxifen treatment, which is an important problem seen in clinical practice (Han et al., 2006).

To improve available therapies for ER-positive breast cancer, a better understanding of the mechanisms contributing to response and resistance to therapy is needed. It is a challenging task to discover new biomarkers that could be used to predict the hormonal therapy response or to be used as potential new therapeutic targets.

## 5.2 CD71

### 5.2.1 Introduction

In this study, in collaboration with Dr Julia Gee and Prof R Nicholson (Cardiff School of Pharmacy), it was proposed that assessment of CD71 expression could be used to stratify ER-positive patients to define subgroups with poor prognosis, high proliferation and resistance to hormonal therapy.

The transferrin receptor (TfR, CD71) is a type II transmembrane homodimer glycoprotein (180 kDa) involved in the cellular uptake of iron via internalization of iron-loaded transferrin (Ponka and Lok, 1999, Daniels et al., 2006a). Transferrin (Tf) is therefore an essential component of cell growth and

iron-requiring metabolic processes including DNA synthesis, electron transport, mitogenic signalling pathways and in turn, proliferation and cell survival. Consequently, rapidly growing cells require more iron for their growth than resting cells (Daniels et al., 2006a, Daniels et al., 2006b).

Not surprisingly, transferrin receptor is expressed at greater levels on cells with a high proliferation rate (Sutherland et al., 1981). Over-expression of endogenous transferrin receptor has also been described for various cancers including those of lung (Dowlati et al., 1997, Carbognani et al., 1996), and pancreas (Ryschich et al., 2004), reflecting increased cell proliferation. This observation can in part be attributed to the increased need for iron as a cofactor for the ribonucleotide reductase enzyme involved in DNA synthesis of rapidly dividing cells (Daniels et al., 2006a, Daniels et al., 2006b).

In breast cancer, transferrin receptor expression has been shown to be up to five times higher in the malignant component compared to normal tissue (Tonik et al., 1986), with expression relating closely to proliferative capacity in these tumours (Wrba et al., 1986). Moreover, within endocrine responsive breast cancer cell models such as MCF-7 (representative of the ER-positive luminal clinical phenotype) there is believed to be a possible association between CD71 and oestrogen receptor signalling. Studies have revealed 17β-estradiol (E2) can up-regulate CD71 expression in a dose-dependent manner, with E2 and iron showing synergistic effects in promoting proliferation (Dai et al., 2008). However, it remains unknown if transferrin/CD71 signalling is a prominent contributor to endocrine resistant breast cancer growth, and

therefore if it could provide a therapeutic target specifically for this undesirable disease state.

Previously, CD71 immunostaining (Wrba et al., 1986) showed elevated expression in poorly differentiated tumours, and a relationship with metastatic potential in animal mammary adenocarcinoma models (Cavanaugh et al., 1999).

The value of CD71 as a prognostic biomarker and a predictor of response to adjuvant treatment in the ER-positive/luminal-like breast cancer phenotype remain largely unexplored.

Therefore, in this study we assessed the biological and prognostic role of CD71 in breast cancer by: 1) Determining CD71 levels of expression in the endocrine responsive MCF-7 human breast cancer cell line as well as various sub-lines representative of acquired resistance to current endocrine agents (i.e. Tamoxifen, Faslodex or severe oestrogen deprivation). 2) Examining transferrin effects on in vitro tumour growth and its inhibition, by evaluating ER blockade, phosphoinositide-3 kinase (PI3K) inhibitor LY294002 and MAPK pathway inhibitor PD98059 treatment as discussed in(Habashy et al., 2010). 3) Studying the clinical relevance of CD71 protein expression in a large series of consecutive patients with invasive breast cancers using high throughput tissue microarrays (TMAs) and immunohistochemistry. In addition, we investigated if CD71 expression could be used to sub-classify ER-positive/ luminal-like cancers and its prognostic role in a subset of tamoxifen-only treated patients.

## 5.3 Material and Methods

### 5.3.1 Cell culture, PCR studies and Cell growth studies

*These methods were performed by Dr Julia Gee's group and were described in (Habashy et al., 2010).*

### 5.3.2 CD71 immunohistochemistry

The expression of CD71 was examined at the protein level using immunohistochemistry in various patient groups to assess its prognostic significance, as well as comparing staining across all the endocrine responsive and resistant in vitro breast cancer cell models.

The study population was derived from the Nottingham Tenovus Primary Breast Carcinoma Series of women aged 70 years or less, who presented with primary operable invasive breast carcinomas (with tumours of less than 5 cm diameter on clinical/pre-operative measurement, stage I and II) between 1988 and 1998. Tissue microarrays (TMAs) were used containing a series of 853 informative cases of unselected invasive breast carcinoma.

**Tissue microarray and immunostaining of the cell pellets and clinical breast cancer samples**

For paraffin-embedded pellet preparation, *the methods were performed by Dr Julia Gee's group and were described in (Habashy et al., 2010).*

For clinical material, arrayed samples comprised single representative 0.6mm tissue cores taken from each tumour block, sectioned at 4 μm thickness (Kononen et al., 1998).

Immunohistochemical staining of transferrin receptor (CD71) (clone 10F11, ab49517; Abcam, Cambridge, UK) using. signal localization (plasma membrane, cytoplasmic) and the staining intensity was quantified using H-score (histochemical score) analysis considering the invasive tumour component only (McCarty et al., 1985).

**Statistical analysis**

Statistical analysis was performed using SPSS statistical software (SPSS Inc., Chicago, USA). Cell line growth data obtained were log-transformed to compare growth rate at day 15, using ANOVA followed by a Bonferroni post-hoc test for analysis. H-scores were compared statistically between MCF-7 and the resistant models using Student's t-test with post-hoc testing. Association between the CD71 expression (categorised by the median of the H-score ≥5) and different clinicopathological parameters and biomarkers was evaluated using chi-square test. Survival curves were estimated by the Kaplan-Meier method with a log rank test to assess significance. Multivariate Cox regression analysis was used to evaluate any independent prognostic effect of the variables using 95% confidence intervalCD71 expression results

### 5.3.3 Endocrine responsive and resistant breast cancer cell line studies

Immunocytochemistry on the cell pellets revealed CD71 expression was increased in all cell lines derived from the luminal ER-positive MCF-7 model that had undergone progression to endocrine resistance **(Fig.5.1C&D)**, with increased immunoreactivity localised at the plasma membrane and cytoplasm. Of these models, total CD71 expression was most elevated for the MCF-7X line with ~5 fold increase versus MCF-7 (mean H-scores=144 versus 28.5).

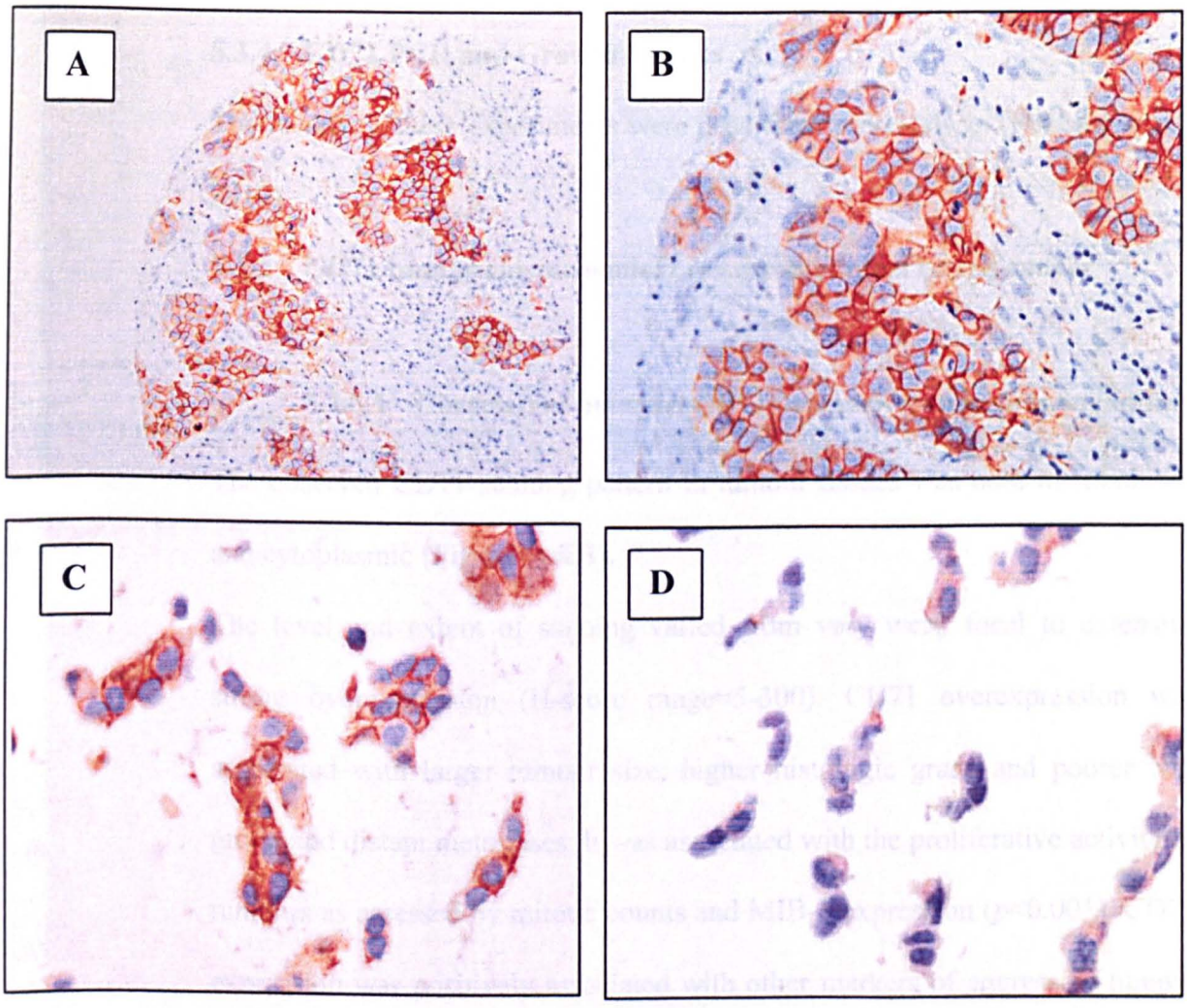


**Figure 5.1 :** CD71 expression in cell lines and clinical samples

(A&B) Grade 3 ductal carcinoma with positive membranous staining (Ax100, Bx200)

(C&D) Paraffin-embedded cell pellets: total CD71 expression was most elevated in the MCF-7X (C) (x200) in comparison to MCF-7 cells (D) (x200).

### 5.3.4 CD71 PCR and Growth Studies

The results of these experiments were described previously in (Habashy et al., 2010)

### 5.3.5 CD71 immunohistochemical results in clinical breast cancer

#### 5.3.5.1 Correlation between CD71 expression and other variables

The observed CD71 staining pattern in tumour tissues was both membranous and cytoplasmic **(Fig 5.1 A&B).**

The level and extent of staining varied from very weak focal to extensive strong overexpression (H-score range=5-300). CD71 overexpression was associated with larger tumour size, higher histologic grade and poorer NPI group and distant metastases. It was associated with the proliferative activity of tumours as assessed by mitotic counts and MIB-1 expression ($p$<0.001). CD71 expression was positively associated with other markers of aggressive tumour phenotype including basal CKs (CK14 and CK5/6), p53, EGFR, and HER2. In contrast, CD71 expression was inversely related to ER, progesterone receptor (PgR), androgen receptor (AR) and Bcl-2 expression. We found higher levels of expression of CD71 in medullary type cancer compared with others (89%, $p$<0.001) **(Tables 5.1 and 5.2).**

In the ER-positive/luminal-like tumours, CD71 expression showed a positive association with higher grade ($p$<0.001) and poorer NPI ($p$=0.004), distant metastasis ($p$=0.002) and high mitotic counts (p<0.001). CD71 expression was positively associated with p53 ($p$<0.001) and EGFR ($p$<0.001).

**Table 5.1:** Relation of CD71 protein expression to other clinicopathological variables in the whole series

| Variable | Negative CD71 N (%) | Positive CD71 N (%) | Total | $\chi^2$ | p value |
|---|---|---|---|---|---|
| **Age** | | | | | |
| <40 | 23(36.5) | 40(63.5) | 63 | 0.634 | 0.888 |
| 40-50 | 78(32.2) | 164(65.6) | 242 | | |
| 51-60 | 104(35) | 198(65) | 302 | | |
| >60 | 86(34.1) | 160(65.9) | 246 | | |
| **Size** | | | | | |
| ≤1.5 cm | 115(40.5) | 169(59.5) | 284 | 7.706 | 0.006 |
| >1.5 cm | 176(30.9) | 393(69.1) | 569 | | |
| **LN Stage** | | | | | |
| 1(Negative) | 192(35.5) | 351(64.5) | 543 | 1.238 | 0.539 |
| 2(1-3 LN) | 73(31.5) | 158(68.5) | 231 | | |
| 3(>3 LN) | 24(31.6) | 52(68.4) | 76 | | |
| **Grade** | | | | | |
| 1 | 81(50.3) | 80(49.7) | 161 | 78.847 | <0.001 |
| 2 | 128(47.1) | 144(52.9) | 273 | | |
| 3 | 82(19.5) | 338(80.5) | 420 | | |
| **NPI** | | | | | |
| Poor | 28(23) | 95(77) | 123 | 38.912 | <0.001 |
| Moderate | 144(29) | 348(71) | 492 | | |
| Good | 119(50) | 119(50) | 238 | | |
| **DM** | | | | | |
| No | 220(37.7) | 363(62.3) | 583 | 10.439 | 0.001 |
| Positive | 69(26.3) | 193(73.7) | 262 | | |
| **Recurrence** | | | | | |
| No | 172(36) | 306(63) | 478 | 1.855 | 0.173 |
| Positive | 113(31.5) | 246(68.5) | 359 | | |
| **VI** | | | | | |
| No | 179(35.9) | 319(64.1) | 498 | 1.787 | 0.409 |
| Probable | 27(31.8) | 58(68.2) | 85 | | |
| Definite | 84(31.5) | 183(68.5) | 267 | | |
| **Mitotic count** | | | | | |
| 1 | 153(55.6) | 122(44.4) | 275 | 98.89 | <0.001 |
| 2 | 52(35.1) | 96(64.9) | 148 | | |
| 3 | 72(18.5) | 317(81.5) | 389 | | |
| **Tumour type** | | | | | |
| Ductal/NST | 121(24.9) | 364(75.1) | 485 | 65.803 | <0.001 |
| Lobular | 56(60) | 38(40) | 94 | | |
| Tubular and Tubular mixed | 84(44.7) | 104(55.3) | 188 | | |
| Medullary | 3(10.7) | 25(89.3) | 28 | | |
| Other special types* | 8(53.3) | 7(46.7) | 15 | | |
| Mixed** | 19(44.2) | 24(55.8) | 43 | | |

*Includes Mucoid, invasive cribriform and invasive papillary carcinoma, ** Include ductal/NST mixed with lobular or special types

**Table 5.2:** Relation of the CD71 protein expression to other biomarkers in the whole series

| Variable | CD71 expression | | Total | χ 2 | p value |
|---|---|---|---|---|---|
| | Negative CD71 N (%) | Positive CD71 N (%) | | | |
| **CK5/6** | | | | 20.527 | <0.001 |
| Negative | 247(37.8) | 407(62.2) | 654 | | |
| Positive | 32(19.2) | 135(80.8) | 167 | | |
| **CK14** | | | | 10.694 | 0.001 |
| Negative | 239(35.8) | 428 (64.2) | 667 | | |
| Positive | 29(21.3) | 107(78.7) | 136 | | |
| **CK18** | | | | 0.280 | 0.579 |
| Negative | 142(33.8) | 278(66.2) | 420 | | |
| Positive | 111(35.5) | 200(64.5) | 311 | | |
| **HER2** | | | | 23.084 | <0.001 |
| Negative | 259(37.2) | 437(62.8) | 696 | | |
| Positive | 15(13.8) | 94(86.2) | 109 | | |
| **p53** | | | | 51.014 | <0.001 |
| Negative | 241(41.9) | 334(58.1) | 575 | | |
| Positive | 32(14.8) | 184(85.2) | 216 | | |
| **EGFR** | | | | 16.316 | <0.001 |
| Negative | 191(37.2) | 323(62.8) | 514 | | |
| Positive | 29(19.5) | 120(80.5) | 149 | | |
| **ER** | | | | 46.012 | <0.001 |
| Negative | 50(18.7) | 217(81.3) | 267 | | |
| Positive | 229(43) | 304(57) | 533 | | |
| **AR** | | | | 18.196 | <0.001 |
| Negative | 78(25.6) | 227(74.4) | 305 | | |
| Positive | 179(40.7) | 261(59.3) | 440 | | |
| **PgR** | | | | 24.011 | <0.001 |
| Negative | 90(25.6) | 261(74.4) | 351 | | |
| Positive | 186(42.4) | 253(57.6) | 439 | | |
| **MIB1** | | | | 12.289 | <0.001 |
| Low | 75(42.1) | 107(58.8) | 182 | | |
| High | 58(25) | 174(75) | 232 | | |
| **Bcl-2** | | | | 16.373 | 0.001 |
| Negative | 29(21.8) | 104(78.2) | 133 | | |
| Weak | 37(36.6) | 64(63.4) | 101 | | |
| Moderate | 69(44.2) | 87(55.8) | 156 | | |
| Strong | 21(38.2) | 34(61.8) | 55 | | |

#### 5.3.5.2 Correlation between CD71 protein expression and patient outcome

In the whole series, a significant correlation between CD71 expression and poorer BCSS was identified (Log Rank (LR)=14.833, $p<0.001$). In the ER-positive/luminal-like cohort, we also found a significant association (LR=14.044, $p<0.001$) **(Fig 5.2A)** However, no associations were found between CD71 expression and DFI neither in the whole series (LR=3.132, $p=0.077$) nor in the ER-positive patient group (LR=2.121, $p=0.145$).

In the group of ER-positive Tamoxifen only treated patients (n=180), we found that CD71 expression was associated with shorter BCSS (LR=10.345, $p=0.001$) **(Fig 5.2B)** and shorter DFI (LR=4.056, $p=0.044$) **(Fig 5.2C)** which may indicates poor response of CD71 expressing tumours to hormonal treatment. To support this finding, we examined the outcome in ER+CD71+ patients that received or did not receive tamoxifen, the group of patients who received tamoxifen showed a significant lower BCSS (LR=5.571, $p=0.018$) **(Fig 5.2D)** and shorter time to develop distant metastasis (LR=5.360, $p=0.021$).

#### 5.3.5.3 Multivariate analysis

A multivariate Cox hazard model analysis for predictors of BCSS was performed including CD71 expression, tumour size, histologic grade and lymph node stage. This analysis demonstrated that CD71 expression is an independent prognostic factor in the ER-positive/luminal-like patient group (HR =1.614, $p$ =0.016, 95% CI =1.092-2.384).

Importantly, in ER-positive patient who received tamoxifen only, CD71 was shown to be an independent prognostic factor of BCSS (HR=2.624, 95%CI =1.309-5.259 and $p$=0.007) **(Table 5.3)**, where patients with CD71 positive tumours showed shorter BCSS.

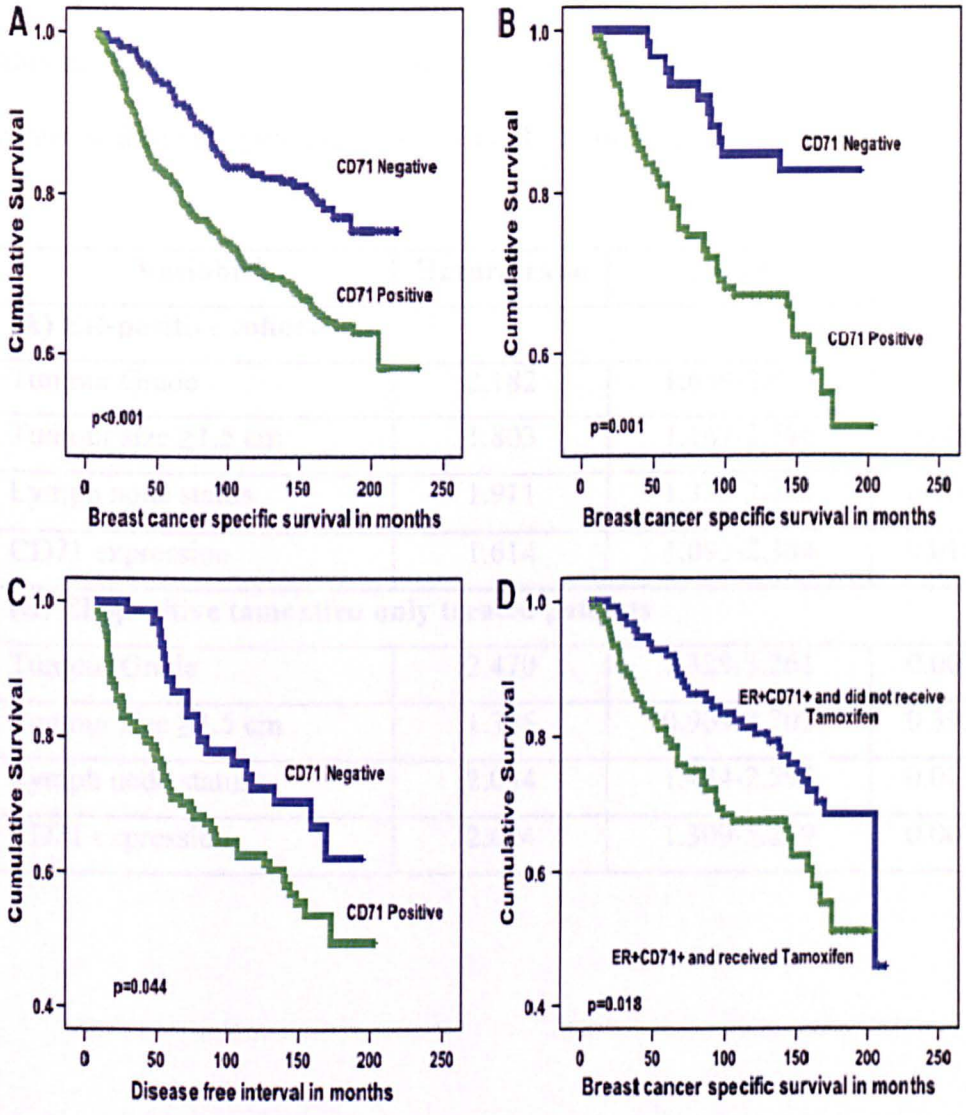


**Figure 5.2:** Kaplan Meier plots of CD71 expression groups in relation to BCSS and DFI

Kaplan Meier plots of CD71 expression and BCSS in (A) ER-positive cohort of unselected breast cancer patients, (B) ER-positive tamoxifen only treated patients. (C) Kaplan Meier plot of CD71 expression and DFI in ER-positive tamoxifen only treated patients. (D) Kaplan Meier plot of BCSS of patients received and did not receive tamoxifen in CD71+ER+ cohort.

**Table 5.3:** Cox proportional hazards analysis for predictors of BCSS: effects of tumour grade, size, lymph node stage, and CD71 expression in (A) ER-positive cohort and (B) ER-positive tamoxifen only treated patients

| Variable | Hazard ratio | 95% CI | p value |
|---|---|---|---|
| **(A) ER-positive cohort** | | | |
| Tumour Grade | 2.182 | 1.659-2.871 | <0.001 |
| Tumour size ≥1.5 cm | 1.803 | 1.167-2.786 | 0.008 |
| Lymph node status | 1.911 | 1.335-2.735 | 0.001 |
| CD71 expression | 1.614 | 1.092-2.384 | 0.016 |
| **(B) ER-positive tamoxifen only treated patients** | | | |
| Tumour Grade | 2.470 | 1.329-3.261 | 0.001 |
| Tumour size ≥1.5 cm | 1.335 | 0.909-2.703 | 0.393 |
| Lymph node status | 2.034 | 1.484-2.592 | 0.019 |
| CD71 expression | 2.624 | 1.309-5.259 | 0.007 |

## 5.4 FOXO3a expression in breast cancer as a downstream target of PIK3/Akt pathway

### 5.4.1 Introduction

Previous studies have highlighted the important role of Akt/PI3K pathway and its upstream and downstream targets in the biology and prognosis of luminal-like breast cancer (Zou et al., 2008) and this prompted us to study FOXO3a as one of the important downstream targets of this pathway. Zou and co-workers have reported that FOXO3a can suppress ER-dependent breast cancer cell proliferation and tumourigenesis in the MCF-7 breast cancer cell line, suggesting a crosstalk between FOXO3a and ER signalling pathways (Zou et al., 2008).

FOXO3a (FKHRL1) gene belongs to the forkhead family of transcription factors (Yang and Hung, 2009) and their activity is regulated by several post-translational modifications, including phosphorylation and acetylation (Tsai et al., 2007).

Other studies demonstrated FOXO3a as an important intracellular mediator of ER expression, suggesting possible therapeutic intervention (Guo and Sonenshein, 2004). Importantly, FOXO3a is a downstream target in the Akt/PI3K pathway and when phosphorylated, is prevented from translocating to the nucleus resulting in its loss of functional activity. In contrast, FOXO3a dephosphorylation leads to nuclear localisation and subsequent target gene activation (Brunet et al., 1999, Vara et al., 2004, Huang and Tindall, 2007, Yang et al., 2008). Therefore, as a target within the Akt/PI3K signalling

pathway FOXO3a regulates the expression of proapoptotic genes, cell cycle regulated genes, and genes that control cellular homeostasis (Brunet et al., 1999, Burgering and Kops, 2002). However, there is also evidence that an Akt-independent mechanism of FOXO3a regulation exists. In vitro co-transfection of FOXO3a and IKK resulted in strong inhibition of FOXO3a activity independent of Akt (Hu et al., 2004).

It was suggested that an efficient mitotic programme depends on downregulation of Akt/PI3K and consequent induction of FOXO3a transcriptional activity (Alvarez et al., 2001).

In breast cancer, FOXO3a activity has been shown to elevate p27 expression resulting in cell cycle arrest (Accili and Arden, 2004). Furthermore, nuclear FOXO3a can induce cellular apoptosis through upregulation of Fas ligand (Fas-L) (Brunet et al., 1999) and Bim (Burgering and Kops, 2002, Stahl et al., 2002) and has been implicated in resistance to oxidative stress and longevity (Kops et al., 2002). Other studies have highlighted the importance of FOXO3a for maintenance of the hematopoietic stem cell pool (Miyamoto et al., 2007, Tothova et al., 2007). Moreover, it has been reported that activation of FOXO3a could induce p53-dependent apoptosis even in cells with a transcriptionally inactive p53 (You et al., 2006).

FOXO3a may have therapeutic implications because some clinical anticancer treatments target FOXO3a through three main oncogenic kinases (Akt, IKK, and ERK) (Yang and Hung, 2009, Myatt and Lam, 2007). For instance, nuclear localization of FOXO3a could potentially improve the effectiveness of anti-EGFR agents such as gefitinib by mediating proliferative arrest (Krol et al.,

2007). Gefitinib treatment causes cell cycle arrest and induces apoptosis due to the effects of FOXO3a dephosphorylation and nuclear translocation at Akt sites. In contrast, resistant cells show inactive phosphorylated FOXO3a restricted to the cytoplasm (Krol et al., 2007). Furthermore, up-regulation of FOXO3a by paclitaxel has been reported to increase Bim mRNA and protein level with subsequent induction of apoptosis in breast cancer cells (Sunters et al., 2003).

The value of FOXO3a as a prognostic biomarker for ER-positive luminal-like breast cancer remains unclear. Therefore, in this study, we have investigated the clinical relevance and biological associations of FOXO3a protein expression in a large series of patients and in a subgroup of luminal-like ER-positive invasive breast cancers using high-throughput tissue microarrays (TMAs) and immunohistochemistry.

## 5.5 Material and Methods

Tissue microarrays were prepared and the immunohistochemical staining was performed using the streptavidin-biotin complex method using DakoCytomation Techmate 500 plus (DakoCytomation, Cambridge, UK) (see general material and methods chapter). To unmask the antigens, the sections were microwaved in citrate buffer pH 6 for 23 minutes. Rabbit polyclonal antibody to FOXO3a (Antibody 9467, Cell Signalling Technology, Danvers, MA, USA) was optimized at a working dilution of 1:50 using TMA sections and full-face sections of breast cancer tissue to assess the staining distribution. Negative controls were performed by omitting the primary antibody and

substitution with a diluent. Positive breast cancer cases were used as positive controls.

Western blotting was performed on breast cancer cell lysates of the human breast cancer cell line MCF-7 to confirm the specificity of the FOXO3a antibody used in immunohistochemistry. The specificity of the FOXO3a antibody was confirmed using Western blotting **(Fig 5.7)** (See General Material and Methods chapter). In normal breast tissue, FOXO3a expression was detected mainly in the nuclei of mammary epithelial cells. In the malignant tissues, FOXO3a showed both nuclear and cytoplasmic staining patterns but one pattern were obviously dominant. Examination of the TMAs has shown that some cases showed nuclear pattern and others were mainly cytoplasmic. Since the expression pattern and localisation of FOXO3a protein expression show variable biological functions, we categorised the positive cases according to whether they showed predominant nuclear (N) or predominant cytoplasmic (C) localisation. Both patterns scored separately using the percentage of the positive cells in each TMA core. Cases were categorised as showing a nuclear or cytoplasmic pattern in 50% or more of the informative TMA core provided that there is more than 20 % variation between both patterns. We have defined the cases with obvious overlap (less than 20 % variation, n=31) and were excluded to ensure a clear separation in their patterns of expression. The cases were scored without the knowledge of patient outcome.

## 5.6 FOXO3a expression results

The median age of the patients was 56 years (range 27-70). At the time of the primary diagnosis, Forty seven percent (47%) of patients had tumours less than 2 cm in size and 31.5% had grade 2 tumours. During follow-up, 30.6% of the patients developed metastatic disease.

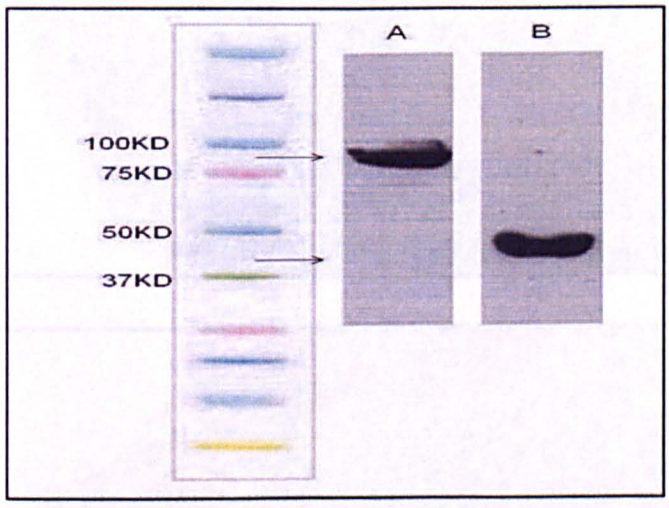


**Figure 5.3:** Western blotting analysis of MCF-7 cell lysates using the FOXO3a rabbit polyclonal antibody.

Enhanced chemiluminescence was used to visualize the membrane. The expected band size ranges from 82 -97 KD. Lane (A) is FOXO3a and lane (B) is β-actin.

In whole series, about 23 % showed predominant nuclear expression pattern and 34 % cytoplasmic pattern while 3 % showed both with less 20% difference in the predominant pattern. In ER-positive patient cohort, 26 % showed nuclear expression pattern **(Fig 5.4A)** and 31 showed % cytoplasmic pattern **(Fig 5.4B).**

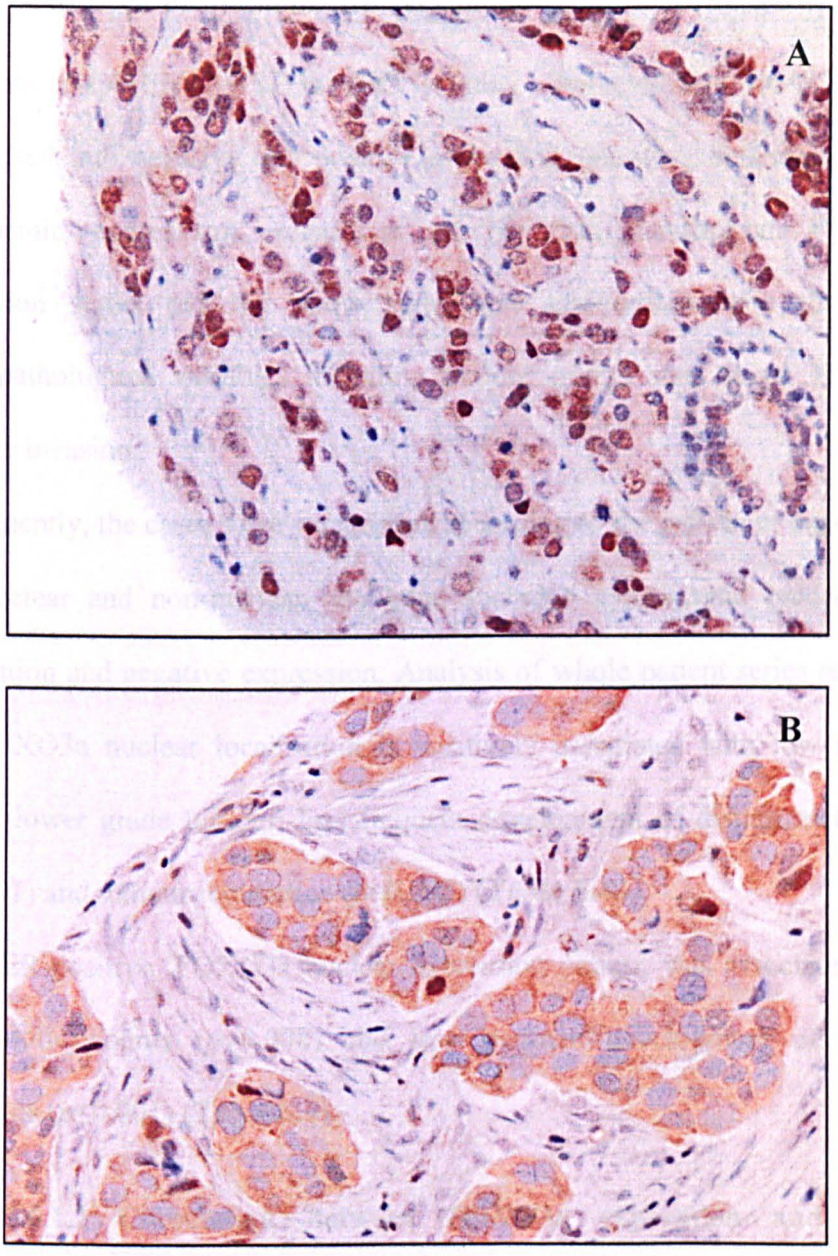


**Figure 5.4:** FOXO3a expression in breast cancer

(A) Predominant FOXO3a nuclear expression (x200)

(B) Predominant FOXO3a cytoplasmic expression (x200)

#### 5.6.1.1 Correlation between FOXO3a expression and other clinicopathological variables

The tumour-specific FOXO3a IHC staining characteristics were initially categorised into negative and positive expression (showing either nuclear or cytoplasmic expression), regardless of FOXO3a localisation. FOXO3a expression status did not show significant associations with the other clinicopathological variables including tumour grade, size, stage, NPI and vascular invasion.

Subsequently, the cases were categorised according to the pattern of expression into nuclear and non-nuclear, the latter included cytoplasmic predominant localisation and negative expression. Analysis of whole patient series revealed that FOXO3a nuclear localisation is positively associated with low mitotic counts, lower grade tumour, less frequent development of distant metastasis ($p$<0.001) and tumour recurrence ($p$=0.002) **(Table 5.4)**.

In the ER-positive, FOXO3a nuclear expression pattern was associated with low mitotic counts ($p$=0.008) and less frequent development of distant metastasis ($p$<0.001) **(Table 5.5)**.

#### 5.6.1.2 Correlation between FOXO3a expression and other biomarkers

In the whole series, the nuclear pattern showed significant positive associations with molecular biomarkers associated with good prognosis including PgR ($p$<0.001), FOXA1 ($p$<0.001) and p27 ($p$=0.001) expression. It also showed an inverse correlation with PIK3CA ($p$=0.001) **(Table 5.6)**.

In ER-positive cohort, the nuclear pattern showed significant positive associations with molecular biomarkers associated with good prognosis including PgR ($p$=0.004), FOXA1 ($p$<0.001) and p27 ($p$=0.004) expression. It also showed an inverse relation with PIK3CA ($p$=0.006) **(Table 5.7)**.

**Table 5.4:** Relation of FOXO3a immunostaining to other clinicopathological variables in the whole series

| Variable | Non-Nuclear localisation | Predominant Nuclear localisation | Total | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| **Age** | | | | | |
| <40 | 59(85.5) | 10(14.5) | 69 | 5.276 | 0.153 |
| 40-50 | 215(78.2) | 60(21.8) | 275 | | |
| 51-60 | 235(79.2) | 61(20.6) | 295 | | |
| >60 | 194(73.8) | 69(26.6) | 263 | | |
| **Tumour size** | | | | | |
| ≤2 cm | 335(78.1) | 94(21.9) | 429 | 0.032 | 0.873 |
| >2 cm | 367(77.6) | 106(22.4) | 473 | | |
| **Lymph node stage** | | | | | |
| 1(Negative) | 413(76.6) | 126(23.4) | 539 | 1.169 | 0.557 |
| 2(1-3 LN) | 219(79.1) | 58(20.9) | 277 | | |
| 3(>3 LN) | 69(81) | 16(19) | 84 | | |
| **Tumour grade** | | | | | |
| 1 | 97(71.3) | 39(28.7) | 136 | 9.115 | 0.010 |
| 2 | 214(74.6) | 73(25.4) | 287 | | |
| 3 | 391(81.6) | 88(18.4) | 479 | | |
| **Vascular invasion** | | | | | |
| No/Probable | 457(77.9) | 130(22.10 | 587 | 0.003 | 1.000 |
| Definite | 244(77.7) | 70(22.3) | 314 | | |
| **NPI** | | | | | |
| Good | 164(73.5) | 59(26.5) | 223 | 4.364 | 0.113 |
| Moderate | 408(78.3) | 113(21.7) | 521 | | |
| Poor | 131(82.4) | 28(17.6) | 159 | | |
| **Mitotic counts** | | | | | |
| **1** | 204(73.6) | 73(26.4) | 277 | 20.308 | <0.001 |
| **2** | 109(67.7) | 52(32.2) | 161 | | |
| **3** | 365(83.5) | 72(16.5) | 437 | | |
| **DM** | | | | | |
| No | 451(73.5) | 163(26.5) | 614 | 21.375 | <0.001 |
| Positive | 242(87.4) | 35(12.6) | 277 | | |
| **Recurrence** | | | | | |
| No | 374(73.9) | 132(26.1) | 506 | 10.000 | 0.002 |
| Positive | 314(82.8) | 65(17.2) | 379 | | |

**Table 5.5:** Relation of FOXO3a immunostaining to other clinicopathological variables in the ER-positive cohort

| Variable | Non-Nuclear localisation N (%) | Predominant Nuclear localisation N (%) | Total | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| **Age** | | | | | |
| <40 | 24(72.7) | 9(27.3) | 33 | 1.535 | 0.674 |
| 40-50 | 135(73.8) | 48(26.2) | 183 | | |
| 51-60 | 159(78.7) | 43(21.3) | 202 | | |
| >60 | 147(75.4) | 48(24.6) | 195 | | |
| **Tumour size** | | | | | |
| ≤2 cm | 231(75.2) | 76(24.8) | 307 | 0.126 | 0.777 |
| >2 cm | 234(76.5) | 72(23.5) | 306 | | |
| **Lymph node stage** | | | | | |
| 1(Negative) | 269(73.9) | 95(26.1) | 364 | 2.135 | 0.344 |
| 2(1-3 LN) | 153(77.7) | 44(22.3) | 197 | | |
| 3(>3 LN) | 41(82) | 9(18) | 50 | | |
| **Tumour grade** | | | | | |
| 1 | 88(71.5) | 35(28.5) | 123 | 4.905 | 0.086 |
| 2 | 185(73.5) | 67(26.5) | 253 | | |
| 3 | 191(80.6) | 46(19.34) | 237 | | |
| **Vascular invasion** | | | | | |
| No/Probable | 302(76.1) | 95(23.9) | 397 | 0.040 | 0.844 |
| Definite | 162(75.3) | 53(24.7) | 215 | | |
| **NPI** | | | | | |
| Good | 142(72.4) | 54(27.6) | 196 | 5.078 | 0.079 |
| Moderate | 247(75.5) | 80(24.5) | 327 | | |
| Poor | 77(84.6) | 14(15.40 | 91 | | |
| **Mitotic counts** | | | | | |
| **1** | 184(73.3) | 67(26.7) | 251 | 9.643 | 0.008 |
| **2** | 90(68.2) | 42(31.8) | 132 | | |
| **3** | 172(82.3) | 37(17.7) | 209 | | |
| **DM** | | | | | |
| No | 306(71.5) | 122(28.5) | 428 | 13.825 | <0.001 |
| Positive | 155(85.6) | 26(14.4) | 181 | | |
| **Recurrence** | | | | | |
| No | 251(72.3) | 96(27.7) | 347 | 4.627 | 0.035 |
| Positive | 207(79.9) | 52(20.1) | 259 | | |

**Table 5.6:** Relation of FOXO3a immunostaining to other biomarkers in the whole series

| Variable | Non-Nuclear localisation | Predominant Nuclear Localisation | Total | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| **ER** | | | | | |
| Negative | 202(83.8) | 39(16.2) | 241 | 6.356 | 0.013 |
| Positive | 466(75.9) | 148(24.1) | 614 | | |
| **PgR** | | | | | |
| Negative | 319(84.4) | 59(15.6) | 378 | 16.878 | <0.001 |
| Positive | 345(72.6) | 130(27.4) | 475 | | |
| **AR** | | | | | |
| Negative | 259(82.5) | 55(17.5) | 314 | 1.925 | 0.191 |
| Positive | 242(78.1) | 68(21.9) | 310 | | |
| **FOXA1** | | | | | |
| Negative | 319(85.1) | 56(14.9) | 375 | 15.145 | <0.001 |
| Positive | 234(73.1) | 86(26.9) | 320 | | |
| **CARM1** | | | | | |
| Negative/low | 159(80.7) | 38(19.30) | 197 | 1.806 | 0.405 |
| Moderate | 281(81.2) | 65(18.8) | 346 | | |
| Strong | 97(75.8) | 31(24.20 | 128 | | |
| **PELP1** | | | | | |
| Negative/low | 76(74.5) | 26(25.5) | 102 | 2.652 | 0.266 |
| Moderated | 413(80.7) | 99(19.3) | 512 | | |
| Strong | 69(75.8) | 22(24.2) | 91 | | |
| **p53** | | | | | |
| Negative | 474(77.8) | 135(22.2) | 609 | 0.080 | 0.856 |
| Positive | 196(78.8) | 53(21.3) | 249 | | |
| **MIB1** | | | | | |
| Low | 126(71.2) | 51(28.8) | 177 | 5.180 | 0.028 |
| High | 407(79.5) | 105(20.5) | 512 | | |
| **PIK3CA** | | | | | |
| Negative | 176(71) | 72(29) | 248 | 11.017 | 0.001 |
| Positive | 444(81.5) | 101(18.5) | 545 | | |
| **p27** | | | | | |
| Negative | 319(83.9) | 61(16.1) | 380 | 11.351 | 0.001 |
| Positive | 272(73.9) | 96(26.1) | 368 | | |
| **C-MYC** | | | | | |
| Negative | 88(88.9) | 11(11.1) | 99 | | |
| Low | 203(80.6) | 49(19.4) | 252 | 8.298 | 0.040 |
| Moderate | 203(75.5) | 66(24.5) | 269 | | |
| Strong | 113(80.10 | 28(19.9) | 141 | | |
| **Bcl-2** | | | | | |
| Negative | 236(80.8) | 56(19.2) | 292 | 1.896 | 0.193 |
| Positive | 312(76.5) | 96(23.5) | 408 | | |

**Table 5.7:** Relation of FOXO3a immunostaining to other biomarkers in the ER-positive cohort

| Variable | Non-Nuclear localisation N (%) | Predominant Nuclear localisation N (%) | Total | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| **PgR** | | | | | |
| Negative | 123(84.2) | 23(15.8) | 146 | 7.968 | 0.004 |
| Positive | 333(72.7) | 125(27.3) | 458 | | |
| **AR** | | | | | |
| Negative | 110(79.1) | 29(20.9) | 140 | 0.860 | 0.423 |
| Positive | 326(75.3) | 60(24.7) | 433 | | |
| **FOXA1** | | | | | |
| Negative | 183(84.7) | 33(15.3) | 216 | 14.177 | <0.001 |
| Positive | 180(70) | 77(30) | 257 | | |
| **CARM1** | | | | | |
| Negative/low | 121(78.1) | 34(21.9) | 155 | 2.128 | 0.345 |
| Moderate | 191(79.6) | 49(20.4) | 240 | | |
| Strong | 44(71) | 18(29) | 62 | | |
| **PELP1** | | | | | |
| Negative/low | 57(74) | 20(26) | 77 | 2.136 | 0.344 |
| Moderated | 278(79) | 74(21) | 352 | | |
| Strong | 40(71.4) | 16(28.6) | 56 | | |
| **p53** | | | | | |
| Negative | 374(76.8) | 113(23.2) | 487 | 0.036 | 0.545 |
| Positive | 86(74.1) | 30(25.9) | 116 | | |
| **MIB1** | | | | | |
| Low | 102(68.9) | 46(31.1) | 148 | 4.580 | 0.039 |
| High | 253(78.1) | 71(21.9) | 324 | | |
| **PIK3CA** | | | | | |
| Negative | 136(69.4) | 60(30.6) | 196 | 8.120 | 0.006 |
| Positive | 276(80.2) | 68(19.8) | 344 | | |
| **p27** | | | | | |
| Negative | 162(83.9) | 31(16.1) | 193 | 8.438 | 0.004 |
| Positive | 221(72.7) | 83(27.3) | 304 | | |
| **C-MYC** | | | | | |
| Negative | 56(88.9) | 7(11.1) | 63 | | |
| Low | 137(78.3) | 38(21.7) | 175 | 6.756 | 0.080 |
| Moderate | 138(73.4) | 50(26.60 | 188 | | |
| Strong | 69(79.3) | 18(20.7) | 87 | | |
| **BCL2** | | | | | |
| Negative | 92(78.6) | 25(21.4) | 117 | 0.351 | 0.121 |
| Positive | 275(76) | 87(24) | 362 | | |

### 5.6.1.3 Correlation between FOXO3a expression and patient outcome

**Univariate analysis**

In the whole patient series, initial univariate analysis of FOXO3a expression status (as positive versus negative) was not associated with BCSS (Log Rank (LR)=0.005, $p$=0.942) nor DMFI (LR=0.015, $p$=0.904) but when the localisation of expression was considered FOXO3a nuclear expression was associated with better outcome in terms of longer BCSS (LR=24.079, $p$<0.001) and longer DMFI (LR =15.996, $p$<0.001).

In the luminal-like ER-positive subgroup (n=633), (median follow up time=126 months), univariate analysis of survival showed no associations between FOXO3a expression status (as positive versus negative) and patient outcome in terms of breast cancer specific survival [BCSS] (LR=0.234, $p$=0.628) or distant metastasis free interval [DMFI] (LR=0.198, $p$=0.656).

However, FOXO3a nuclear localisation showed a significant association with both longer BCSS (LR=15.813, $p$<0.001) **(Fig 5.5)** and longer DMFI (LR=11.836, $p$=0.001) **(Fig 5.6)**.

Analysis of patient survival using categorisation of the cohort into three groups: predominant nuclear, predominant cytoplasmic and negative, our results showed that subcellular localisation differences of FOXO3a are associated with striking survival differences. Specifically there is a contrast between nuclear and cytoplasmic expression localisation where nuclear pattern

showed the most favourable BCSS (LR =18.279, $p$<0.001) **(Fig 5.7)** and DMFI (LR=14.775, $p$=0.001) **(Fig 5.82)** in ER-positive luminal-like cancer.

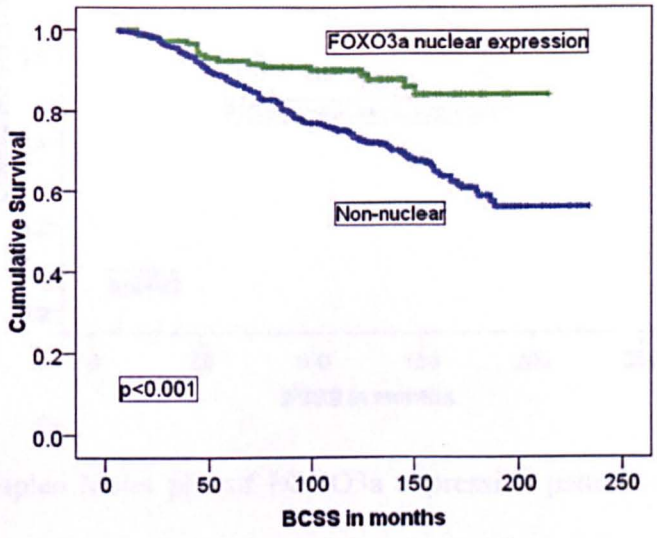


**Figure 5.5:** Kaplan Meier plot of FOXO3a nuclear verse non-nuclear protein expression in relation to BCSS in the ER-positive cohort

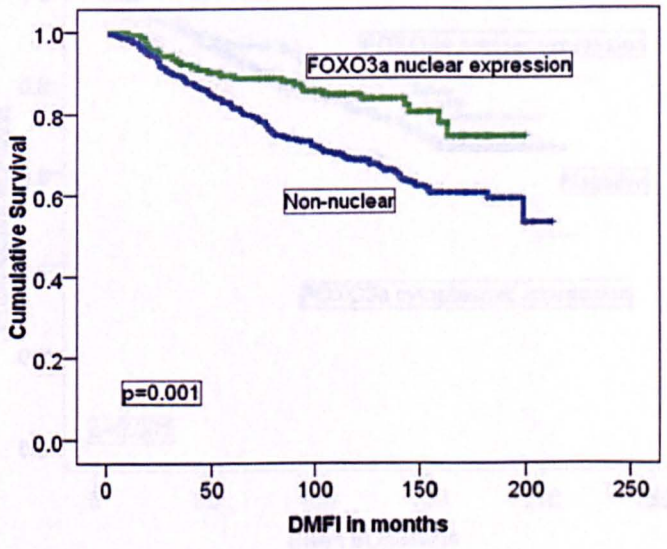


**Figure 5.6:** Kaplan Meier plot of FOXO3a nuclear expression in relation to DMFI in the ER-positive cohort

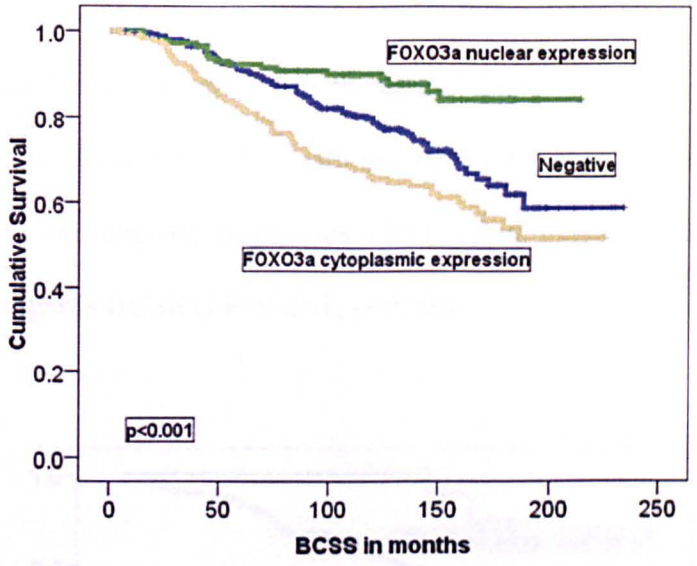


**Figure 5.7:** Kaplan Meier plot of FOXO3a expression patterns in relation to BCSS

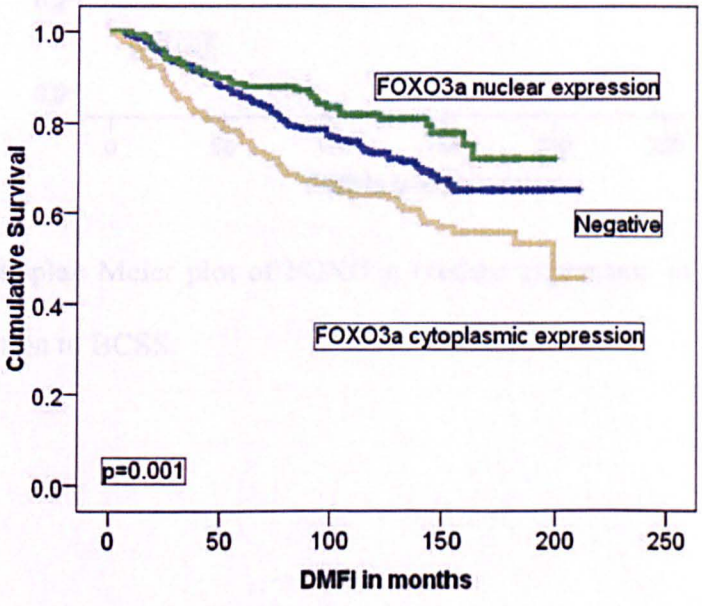


**Figure 5.8:** Kaplan Meier plot of FOXO3a expression patterns in relation to DMFI

**According to systemic therapy groups**

When systemic therapy was considered, similar associations of longer survival were found in the subgroup of ER-positive patients who did not receive adjuvant systemic therapy (n=222) with regards DMFI (LR=10.110, $p$=0.001) **(Fig 5.9)** and in the subgroup of patients who received tamoxifen monotherapy (n=221) with regards BCSS (LR=5.201, $p$=0.023).

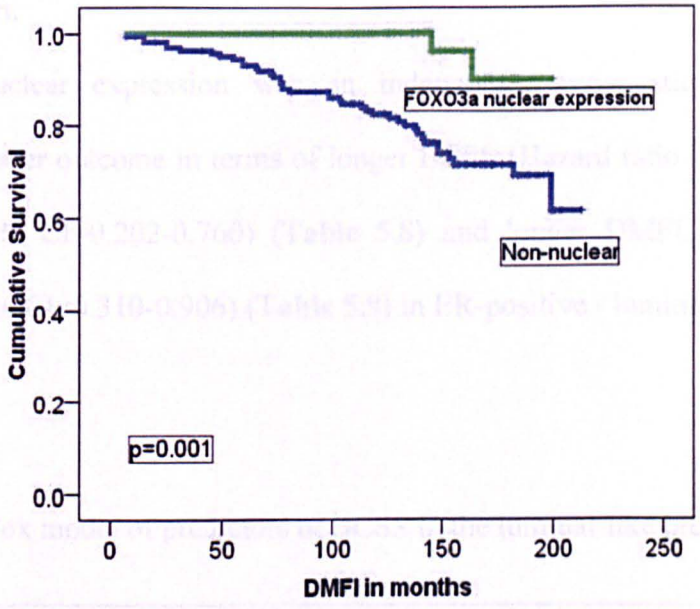


**Figure 5.9:** Kaplan Meier plot of FOXO3a nuclear expression in non-treated cohort in relation to BCSS.

**Multivariate analysis**

Since many potential prognostic factors may interact with specific therapies and therefore are compounded by the effect of adjuvant hormone therapy and chemotherapy, we have included the systemic therapy groups (given versus not given) in the multivariate analysis together with the other well established prognostic variables such as MIB1, PgR, tumour size, stage, grade to assess the prognostic independence of nuclear FOXO3a expression in the ER-positive patient cohort.

FOXO3a nuclear expression was an independent prognostic factor for predicting better outcome in terms of longer BCSS (Hazard ratio (HR)=0.392, $p$=0.006, 95% CI=0.202-0.760) **(Table 5.8)** and longer DMFI (HR=0.530, $p$=0.020, 95% CI=0.310-0.906) **(Table 5.9)** in ER-positive / luminal-like breast cancer.

**Table 5.8:** Cox model of predictors of BCSS in the luminal-like breast cancer

| Variable | P value | HR | 95% CI | |
|---|---|---|---|---|
| | | | Lower | Upper |
| **FOXO3a nuclear localisation** | 0.006 | 0.392 | 0.202 | 0.760 |
| **PgR expression** | 0.049 | 0.642 | 0.413 | 0.997 |
| **MIB1 expression** | 0.011 | 2.105 | 1.184 | 3.742 |
| **Tumour size** | 0.001 | 2.228 | 1.411 | 3.520 |
| **LN stage** | 0.000 | 1.746 | 1.290 | 2.363 |
| **Tumour grade** | 0.006 | 1.629 | 1.152 | 2.302 |
| **Endocrine therapy** | 0.502 | 0.846 | 0.519 | 1.379 |
| **Chemotherapy** | 0.305 | 0.715 | 0.377 | 1.358 |

**Table 5.9:** Cox model of predictors of DM in the luminal-like breast cancer

| Variable | p value | HR | 95% CI | |
|---|---|---|---|---|
| | | | Lower | Upper |
| **FOXO3a nuclear localisation** | 0.020 | 0.530 | 0.310 | 0.906 |
| **PgR expression** | 0.035 | 0.641 | 0.424 | 0.970 |
| **MIB1 expression** | 0.008 | 1.989 | 1.194 | 3.311 |
| **Tumour size** | 0.001 | 2.094 | 1.380 | 3.177 |
| **LN stage** | <0.001 | 1.836 | 1.380 | 2.442 |
| **Tumour grade** | 0.048 | 1.375 | 1.003 | 1.887 |
| **Endocrine therapy** | 0.889 | 0.967 | 0.604 | 1.548 |
| **Chemotherapy** | 0.938 | 0.977 | 0.536 | 1.779 |

## 5.7 AGTR1

### 5.7.1 Introduction

Angiotensin II is a pleiotropic hormone which could act as a neurotransmitter, growth factor, angiogenic factor, vasoconstrictor, and cytokine (Ladd et al., 2007). Via ligand-induced activity through the angiotensin II type 1 receptor (AGTR1), angiotensin II is converted from its precursor by the action of angiotensin 1-converting enzyme (ACE) (Koh et al., 2005). Angiotensinogen has anti-proliferative properties while, angiotensin II is a potent growth factor and it mediates its actions through AGTR1 (Ladd et al., 2007).

AGTR1 was found to be one of the most highly overexpressed genes in 10–20% of breast cancers across independent breast cancer microarray studies (Rhodes et al., 2009). It has been shown that AGTR1 overexpression defines a subset of ER-positive breast cancer that can benefit from AGTR1 antagonists. Specifically, AGTR1 was overexpressed only in tumours that were HER2-negative and ER-positive (Rhodes et al., 2009).

Angiotensin II has a carcinogenic effect via the AGTR1 which increases the risk of cancer development possibly via different mechanism. The first one is by promoting cell division and proliferation through the activation of mitogenic pathways especially EGFR (Greco et al., 2003). Another mechanism by which AGTR1 could induce its carcinogenic effect is the angiogenesis and promoting arterial smooth muscle cell proliferation via vascular endothelial growth factor in animal models (Egami et al., 2003).

Koh and co-workers investigated the genetic polymorphisms in ACE and AGTR1 genes by SNPs. Breast cancer patients possessing the low risk polymorphisms of AGTR1 and ACE had a lower breast cancer risk. This observation lends further support to the argument that gene variations within the renin angiotensin system may play a role in breast carcinogenesis (Koh et al., 2005).

This has a potential clinical importance because AGTR1 can be blocked by commonly prescribed antihypertensive agents especially those used for ACE inhibition. Inhibition of the angiotensin II effect by blockade of ACE and/or AGTR1 could be potential targets for the prevention and treatment of cancer especially breast cancer.

### 5.7.2 Material and Methods

#### 5.7.2.1 Identification of AGTR1 as a candidate luminal marker by gene expression analysis

The ANN methodology was discussed in details in general material and methods chapter. The luminal versus non-luminal data was used to divide the gene expression data into two groups to identify genes that can characterise the luminal phenotype. These data were bioinfomatically analysed in collaboration with Dr Graham Ball from Nottingham Trent University.

#### 5.7.2.2 AGTR1 immunohistochemistry

Tissue microarrays were prepared and the immunohistochemical staining was performed using the streptavidin-biotin complex method (LSAB) using DakoCytomation Techmate 500 plus (DakoCytomation, Cambridge, UK) (general material and methods chapter). Mouse monoclonal antibody [1E10-1A9] to Angiotensin II Type 1 Receptor (ab9391, Abcam, UK) was optimised at a working dilution of 1:100 with 6 hour primary antibody incubation time. Negative controls were performed by omitting the primary antibody and substitution with diluent. Positive breast cancer cases and kidney tissue were used as positive controls. The H-score (histochemical score) was used to assess the intensity of staining and the percentage of stained cells following immunohistochemistry (McCarty et al., 1985).

The X-tile (Camp et al., 2004) program was used to define optimal cut off points of AGTR1 H-score values (<30=negative/low, ≥30and<100=moderate and ≥100=strong expression).

### 5.7.3 AGTR1 expression results

At mRNA level, AGTR1 gene was identified as a characterizing gene of ER-positive luminal like subgroup using ANN analysis **(Table 5.10).**

**(Fig 5.10)** shows AGTR1 gene expression in luminal and non-luminal samples.

Of the whole series 1002 tumour cores were available for assessment. The expression was detected in the cytoplasm of tumour cells **(Fig 5.11)** with

decreased expression in normal acini. In the whole patient series, 23.3% of cases showed negative and low expression, 38% showed moderate expression and 38.7% of cases showed strong expression.

**Table 5.10:** ANN genes rank, summary of the first step

| Rank | Gene ID | Name | Selection error |
|---|---|---|---|
| 1 | GI_4885330-S | GPR42 | 0.384400019 |
| 2 | GI_14043065-A | **AGTR1** | 0.39510661 |
| 3 | GI_29728071-S | TBC1D9 | 0.397092733 |
| 4 | GI_22202636-A | MM-1 | 0.39841089 |
| 5 | GI_4507456-S | CD71 | 0.401191155 |
| 6 | GI_4758297-S | HER2 | 0.404675377 |
| 7 | Hs.499488-S | Hs.499488-S | 0.405384429 |
| 8 | GI_4503602-S | ESR1 | 0.4068704 |
| 9 | Hs.466852-S | Hs.466852-S | 0.407401035 |
| 10 | GI_5031906-S | MEF2A | 0.410688739 |
| 11 | GI_4759215-S | TCEAL1 | 0.413354317 |
| 12 | GI_21361616-S | FLJ20151 | 0.4139392 |
| 13 | GI_4885496-S | v-MYB | 0.415354654 |
| 14 | hmm23409-S | hmm23409-S | 0.41714708 |
| 15 | Hs.179115-S | Hs.179115-S | 0.417177662 |
| 16 | GI_42658625-S | KIAA1549 | 0.417591627 |
| 17 | GI_31377840-S | FLJ11280 | 0.418409403 |
| 18 | hmm20201-S | hmm20201-S | 0.419244839 |
| 19 | GI_42658619-S | NUP205 | 0.419958115 |
| 20 | GI_19913405-S | TOP2A | 0.420003738 |

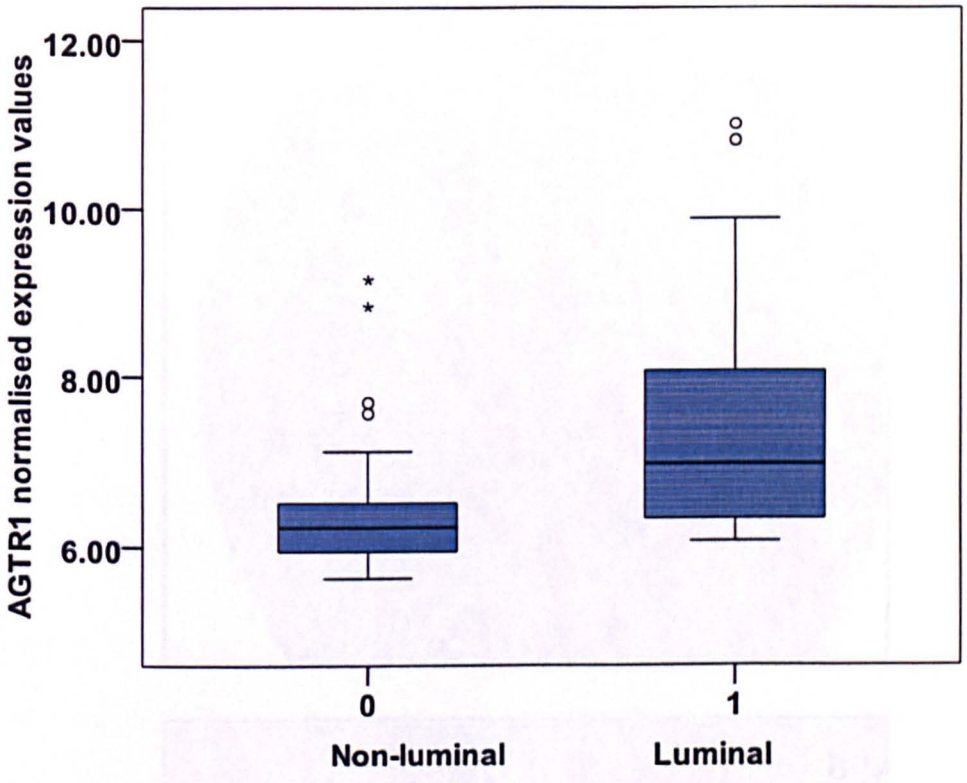


**Figure 5.10:** Boxplot of the AGTR1 normalised expression values in luminal vs. non-luminal samples

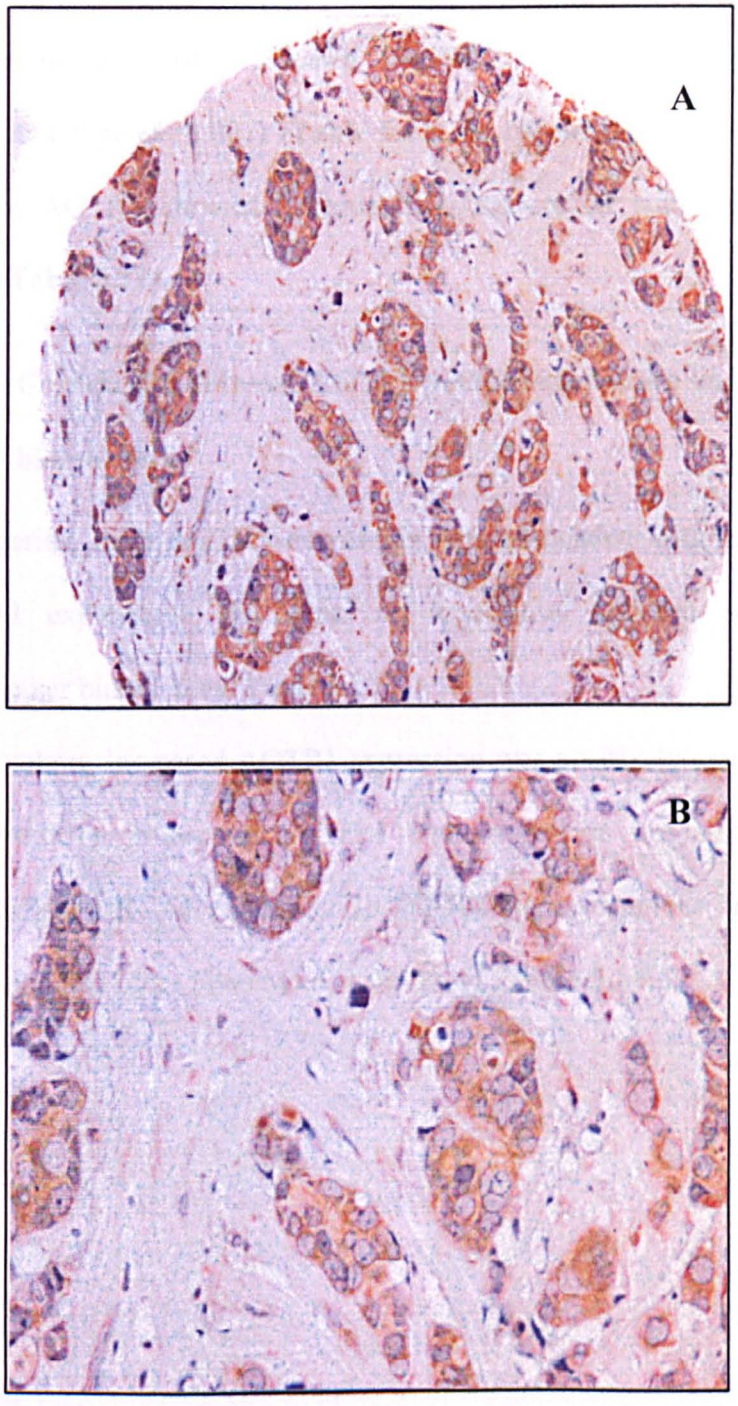


**Figure 5.11:** Grade 2 invasive ductal carcinoma with high expression of AGTR1 (A x100 & B x200)

#### 5.7.3.1 Correlation between AGTR1 protein expression and other clinicopathological variables

AGTR1 immunohistochemistry revealed significant association with the histological tumour type ($p$=0.007) **(Table 5.11)**. In ER-positive luminal-like patients' cohort, AGTR1 showed a border line association with increased mitotic counts **(Table 5.12)**.

#### 5.7.3.2 Correlation between AGTR1 protein expression and other biomarkers

In the whole series, high AGTR1 expression was associated with reduced nuclear BRCA1 expression. We found no association between AGTR1 expression and other biomarkers **(Table 5.13)**.

In ER-positive cohort, increased AGTR1 expression was positively associated with EGFR ($p$=0.001). In contrast, AGTR1 high expression was associated with reduced nuclear BRCA1 expression ($p$<0.001). No associations between AGTR1 expression and other biomarkers were found **(Table 5.14)**.

**Table 5.11:** Relation of AGTR1 immunostaining to other clinicopathological variables in the whole series

| Variable | AGTR1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | low | Moderate | Strong | | |
| **Age** | | | | 13.995 | 0.030 |
| <40 | 13(18.8) | 33(47.8) | 23(33.3) | | |
| 40-50 | 85(29) | 106(36.2) | 102(34.8) | | |
| 51-60 | 80(24) | 121(36.3) | 132(39.6) | | |
| >60 | 55(17.9) | 121(39.4) | 131(42.7) | | |
| **Size** | | | | 0.059 | 0.971 |
| ≤1.5 cm | 113(23) | 186(37.9) | 192(39.10 | | |
| >1.5 cm | 120(23.5) | 194(38) | 196(38.4) | | |
| **LN Stage** | | | | 4.285 | 0.369 |
| 1(Negative) | 145(24) | 233(38.6) | 225(37.3) | | |
| 2(1-3 LN) | 74(24.2) | 109(35.6) | 123(40.2) | | |
| 3(>3 LN) | 14(15.6) | 37(41.1) | 39(43.3) | | |
| **Grade** | | | | 5.592 | 0.232 |
| 1 | 34(20.4) | 73(43.7) | 60(35.9) | | |
| 2 | 87(26.5) | 121(36.9) | 120(36.6) | | |
| 3 | 112(22.1) | 186(36.8) | 208(41.1) | | |
| **NPI** | | | | 3.205 | 0.524 |
| Good | 66(23.8) | 114(41.2) | 97(35) | | |
| Moderate | 132(23.7) | 201(36.1) | 224(40.2) | | |
| Poor | 35(20.8) | 66(39.3) | 67(39.9) | | |
| **DM** | | | | 5.875 | 0.05 |
| No | 168(24.1) | 275(39.5) | 254(36.4) | | |
| Positive | 60(20.6) | 101(34.7) | 130(44.7) | | |
| **Recurrence** | | | | 1.358 | 0.507 |
| No | 140(24.4) | 218(38) | 215(37.5) | | |
| Positive | 87(21.4) | 156(38.4) | 163(40.1) | | |
| **VI** | | | | 7. 343 | 0.119 |
| No | 133(24.6) | 217(40.1) | 191(35.3) | | |
| Probable | 25(21.4) | 46(39.3) | 46(39.3) | | |
| Definite | 73(21.4) | 117(34.4) | 151(44.3) | | |
| **Tumour type** | | | | 24.302 | 0.007 |
| Ductal/NST | 129(21.8) | 209(35.3) | 254(42.9) | | |
| Lobular | 27(27.3) | 51(51.5) | 21(21.2) | | |
| Tubular and Tubular mixed | 53(25.6) | 82(39.6) | 72(34.8) | | |
| Medullary | 9(32.1) | 8(28.6) | 11(39.3) | | |
| Other special types* | 6(35.3) | 6(35.3) | 5(29.4) | | |
| Mixed** | 9(15.3) | 25(42.4) | 25(42.4) | | |
| **Mitosis** | | | | 8.957 | 0.062 |
| 1 | 81(24) | 138(40.9) | 118(35) | | |
| 2 | 50(28.2) | 67(37.9) | 60(33.9) | | |
| 3 | 98(21.3) | 163(35.4) | 199(43.3) | | |

*Includes Mucoid, invasive cribriform and invasive papillary carcinoma, ** Include ductal/NST mixed with lobular or special type

**Table 5.12:** Relation of AGTR1 immunostaining to other clinicopathological variables in the ER-positive cohort

| Variable | AGTR1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | low | Moderate | Strong | | |
| **Age** | | | | 5.445 | 0.488 |
| <40 | 5(17.9) | 13(46.4) | 10(35.7) | | |
| 40-50 | 54(28) | 74(38.3) | 65(33.7) | | |
| 51-60 | 54(23.9) | 85(37.6) | 87(38.5) | | |
| >60 | 47(20) | 91(38.7) | 97(41.3) | | |
| **Size** | | | | 1.074 | 0.584 |
| ≤1.5 cm | 88(24.9) | 137(38.7) | 129(36.4) | | |
| >1.5 cm | 72(220 | 126(38.4) | 130(39.6) | | |
| **LN Stage** | | | | 5.651 | 0.227 |
| 1(Negative) | 98(24.4) | 161(40) | 143(35.6) | | |
| 2(1-3 LN) | 54(23.8) | 77(33.9) | 96(42.3) | | |
| 3(>3 LN) | 8(15.7) | 24(47.10 | 19(37.3) | | |
| **Grade** | | | | 8.476 | 0.076 |
| 1 | 29(19.7) | 63(42.9) | 55(37.4) | | |
| 2 | 83(28.7) | 104(36) | 102(35.3) | | |
| 3 | 48(19.5) | 96(39) | 102(41.5) | | |
| **NPI** | | | | 3.356 | 0.500 |
| Poor | 62(25.6) | 97(40.10 | 83(34.3) | | |
| Moderate | 79(23.1) | 125(36.5) | 138(40.4) | | |
| Good | 19(19.4) | 41(41.8) | 38(38.8) | | |
| **DM** | | | | 4.372 | 0.112 |
| No | 119(24.1) | 198(40.2) | 176(35.7) | | |
| Positive | 38(20.9) | 63(34.6) | 81(44.5) | | |
| **Recurrence** | | | | 0.870 | 0.647 |
| No | 99(24.60 | 152(37.7) | 152(37.7) | | |
| Positive | 58(21.6) | 108(40.3) | 102(38.1) | | |
| **VI** | | | | 6.170 | 0.187 |
| No | 90(25.5) | 142(40.2) | 121(34.3) | | |
| Probable | 19(19.8) | 40(41.7) | 37(38.5) | | |
| Definite | 49(21.2) | 81(35.1) | 101(43.7) | | |
| **Tumour type** | | | | 16.933 | 0.076 |
| Ductal/NST | 73(21.3) | 126(36.7) | 144(42) | | |
| Lobular | 26(28.3) | 45(48.90 | 21(22.80 | | |
| Tubular and Tubular mixed | 47(26.3) | 66(36.9) | 66(36.9) | | |
| Medullary | 1(25) | 1(25) | 2(50) | | |
| Other special types | 5(41.7) | 4(33.3) | 3(25) | | |
| Mixed | 8(15.4) | 21(40.4) | 23(44.2) | | |
| **Mitosis** | | | | 10.821 | 0.029 |
| 1 | 74(24.6) | 123(40.90 | 104(34.6) | | |
| 2 | 43(29.7) | 53(36.6) | 49(33.8) | | |
| 3 | 39(18.1) | 78(36.3) | 98(45.9) | | |

**Table 5.13:** Relation of AGTR1 expression to other biomarkers in the whole series

| Variable | AGTR1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | Moderate | Strong | | |
| **CK5/6** | | | | 2.463 | 0.292 |
| Negative | 177(22.2) | 302(37.9) | 317(39.8) | | |
| Positive | 47(27.2) | 66(38.2) | 60(34.7) | | |
| **CK14** | | | | 0.868 | 0.648 |
| Negative | 197(23.7) | 306(36.9) | 327(39.4) | | |
| Positive | 30(25) | 48(40) | 42(35) | | |
| **CK19** | | | | 5.319 | 0.070 |
| Negative | 29(31.9) | 26(28.6) | 36(39.6) | | |
| Positive | 198(22.6) | 340(38.80 | 338(38.6) | | |
| **CK18** | | | | 5.168 | 0.075 |
| Negative | 38(30.2) | 39(31) | 49(38.9) | | |
| Positive | 166(21.7) | 297(38.8) | 302(39.5) | | |
| **ER** | | | | 0.581 | 0.748 |
| Negative | 62(22.90 | 99(36.5) | 110(40.6) | | |
| Positive | 160(23.5) | 263(38.6) | 259(38) | | |
| **PgR** | | | | 2.012 | 0.366 |
| Negative | 94(22.10 | 156(36.7) | 175(41.2) | | |
| Positive | 130(24.70 | 203(38.5) | 194(36.8) | | |
| **AR** | | | | 0.745 | 0.689 |
| Negative | 77(22.6) | 125(36.8) | 138(40.6) | | |
| Positive | 140(25) | 206(36.7) | 215(38.3) | | |
| **p53** | | | | 1.225 | 0.542 |
| Negative | 161(23.2) | 268(38.6) | 266(38.3) | | |
| Positive | 62(23.9) | 90(34.7) | 107(41.3) | | |
| **BRCA1** | | | | 15.282 | <0.001 |
| Negative | 18(14.4) | 40(32) | 67(53.6) | | |
| Positive | 173(25.2) | 268(39) | 246(35.8) | | |
| **MIB1** | | | | 5.869 | 0.053 |
| low | 56(25.1) | 94(42.2) | 73(32.7) | | |
| High | 128(22.9) | 196(35.1) | 234(41.9) | | |
| **P-cadherin** | | | | 2.052 | 0.358 |
| Negative | 85(23.4) | 146(40.1) | 133(36.5) | | |
| Positive | 107(24) | 158(35.4) | 181(40.6) | | |
| **E-cadherin** | | | | 3.500 | 0.174 |
| Negative | 79(22.1) | 149(41.6) | 130(36.3) | | |
| Positive | 146(24.4) | 213(35.6) | 240(40.1) | | |
| **HER2** | | | | 8.630 | 0.013 |
| Negative | 201(24) | 320(38.1) | 318(37.9) | | |
| Positive | 18(14.6) | 43(35) | 62(50.4) | | |
| **EGFR** | | | | 8.805 | 0.012 |
| Negative | 163(24.1) | 267(39.5) | 246(36.4) | | |
| Positive | 34(20.2) | 52(31) | 82(48.8) | | |

**Table 5.14:** Relation of AGTR1 expression to other biomarkers in the ER-positive cohort

| Variable | AGTR1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | Moderate | Strong | | |
| **CK5/6** | | | | 0.195 | 0.907 |
| Negative | 143(23) | 240(38.6) | 239(38.4) | | |
| Positive | 12(25) | 19(39.6) | 17(35.4) | | |
| **CK14** | | | | 1.238 | 0.539 |
| Negative | 148(24.4) | 229(37.7) | 230(37.9) | | |
| Positive | 9(18) | 19(38) | 22(44) | | |
| **CK19** | | | | 1.603 | 0.449 |
| Negative | 10(28.6) | 10(28.6) | 15(42.9) | | |
| Positive | 148(23.2) | 250(39.1) | 241(37.7) | | |
| **CK18** | | | | 2.813 | 0.245 |
| Negative | 6(24) | 6(24) | 13(52) | | |
| Positive | 138(22.6) | 242(39.7) | 230(37.7) | | |
| **PgR** | | | | 1.073 | 0.585 |
| Negative | 36(21.4) | 63(37.5) | 69(41.1) | | |
| Positive | 123(24.3) | 197(38.9) | 187(36.9) | | |
| **AR** | | | | 4.737 | 0.094 |
| Negative | 27(17.6) | 63(41.2) | 36(41.2) | | |
| Positive | 127(26.3) | 177(36.6) | 179(37.1) | | |
| **p53** | | | | 0.577 | 0.757 |
| Negative | 126(22.7) | 217(39) | 213(38.30 | | |
| Positive | 30(25.9) | 43(37.1) | 43(37.1) | | |
| **BRCA1** | | | | 17.027 | <0.001 |
| Negative | 1(1.8) | 24(43.6) | 30(54.5) | | |
| Positive | 135(25.5) | 206(38.9) | 189(35.7) | | |
| **MIB1** | | | | 6.343 | 0.042 |
| low | 48(25.7) | 79(42.2) | 60(32.1) | | |
| High | 79(22.7) | 119(34.2) | 150(43.1) | | |
| **P-cadherin** | | | | 2.175 | 0.337 |
| Negative | 77(23.1) | 137(41.1) | 119(35.7) | | |
| Positive | 60(24.1) | 88(35.3) | 101(40.6) | | |
| **E-cadherin** | | | | 6.895 | 0.032 |
| Negative | 50(20.4) | 111(45.3) | 84(34.3) | | |
| Positive | 108((25.1) | 151(35.1) | 171(39.8) | | |
| **HER2** | | | | 3.598 | 0.165 |
| Negative | 146(23.7) | 237(38.5) | 233(37.8) | | |
| Positive | 6(12) | 22(44) | 22(44) | | |
| **EGFR** | | | | 10.821 | 0.001 |
| Negative | 124(23.9) | 213(41.1) | 181(34.9) | | |
| Positive | 15(17.4) | 25(29.1) | 46(53.5) | | |

#### 5.7.3.3 Correlation between AGTR1 protein expression and patient outcome

In the whole series, AGTR1 expression was associated with shorter breast cancer specific survival (BCSS) (LR=14.524, $p$=0.001) and shorter distant metastasis free interval (DMFI) (LR=9.558, $p$=0.008). In ER-positive luminal like patient cohort, strong AGTR1 expression was associated with shorter BCSS (LR=12.713, $p$=0.002) **(Fig 5.12)** and shorter distant metastasis free interval DMFI (LR=6.841, $p$=0.039) **(Fig 5.13)**.

Multivariate COX analysis model including tumour grade, size and LN stage showed that strong AGTR1 expression can independently predict increased breast cancer specific death risk (HR=1.788, $p$=0.008) **(Table 5.15)**.

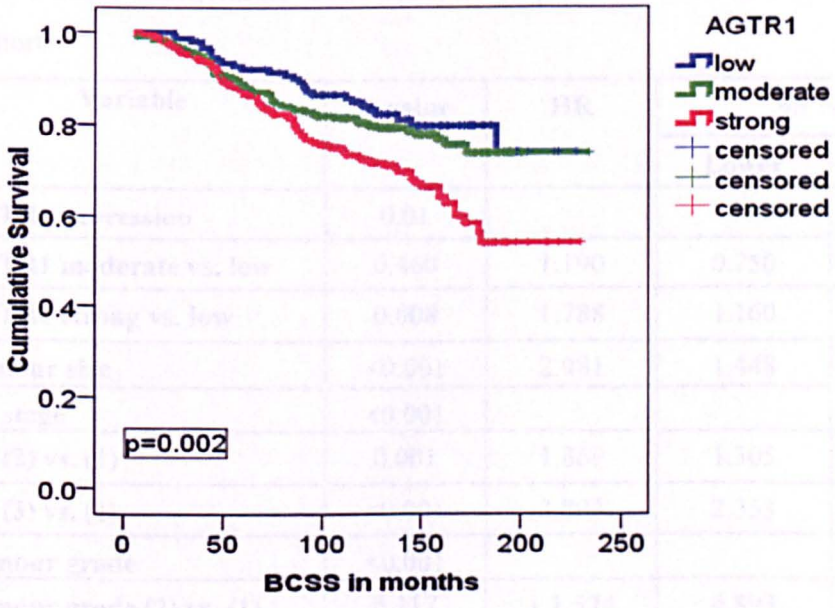


**Figure 5.12:** Kaplan Meier plot of AGTR1 expression in relation to BCSS in ER-positive luminal-like cohort

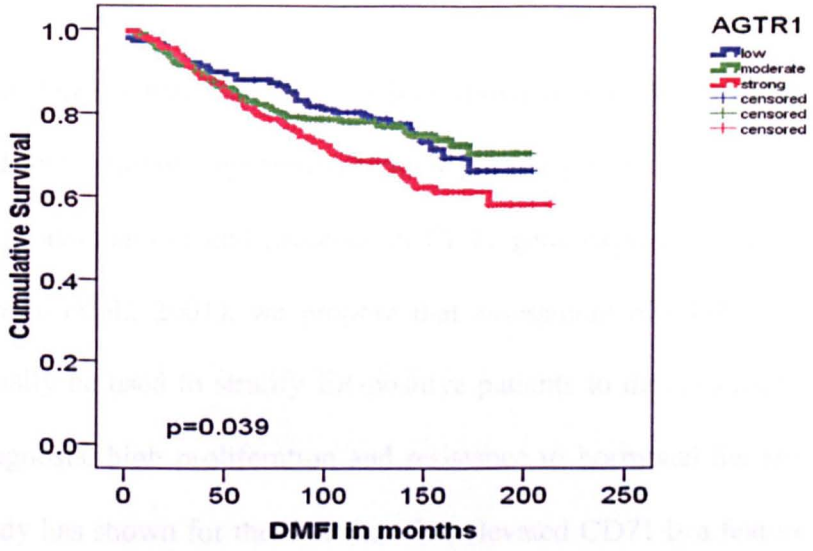


**Figure 5.13:** Kaplan Meier plot of AGTR1 expression in relation to DMFI in ER-positive luminal-like cohort

**Table 5.15:** Multivariate COX analysis of predictor of BCSS in ER-positive cohort

| Variable | P value | HR | 95 % CI | |
|---|---|---|---|---|
| | | | Lower | Upper |
| **AGTR1 expression** | 0.01 | | | |
| **AGTR1 moderate vs. low** | 0.460 | 1.190 | 0.750 | 1.887 |
| **AGTR1 strong vs. low** | 0.008 | 1.788 | 1.160 | 12.755 |
| **Tumour size** | <0.001 | 2.081 | 1.448 | 2.991 |
| **LN stage** | <0.001 | | | |
| **LN (2) vs. (1)** | 0.001 | 1.869 | 1.305 | 2.678 |
| **LN (3) vs. (1)** | <0.001 | 3.803 | 2.353 | 6.145 |
| **Tumour grade** | <0.001 | | | |
| **Tumour grade (2) vs. (1)** | 0.117 | 1.1.574 | 0.893 | 2.773 |
| **Tumour grade (3) vs. (1)** | <0.001 | 3.196 | 1.846 | 5.535 |

## 5.8 Discussion

### 5.8.1 CD71

Transferrin acting via CD71 has been shown to alter during disease progression and may promote aggressive tumour growth (Inoue et al., 1993). Because of these associations and presence of CD71 gene expression in luminal group C (Sorlie et al., 2001), we propose that assessment of CD71 expression might equally be used to stratify ER-positive patients to define subgroups with poor prognosis, high proliferation and resistance to hormonal therapy. The present study has shown for the first time that elevated CD71 is a feature of endocrine resistant breast cancer, as evidenced by immunostaining of acquired endocrine resistant sub-lines derived from luminal-like MCF-7 cells. Furthermore, we showed that the model for resistance to severe oestrogen deprivation, MCF-7X, over-expresses CD71 at the gene and protein level in the presence of exogenous transferrin, leading to increased growth and this could represent a prominent mitogenic mechanism for endocrine resistant cells in the presence of circulating transferrin. Studies in breast cancer models are promising with antisense inhibition of CD71 or selective antibodies to this receptor, where these inhibit cell survival and proliferation confirming a fundamental growth importance of CD71 to such cells (Yang et al., 2001a, Yang et al., 2001b). Peng and colleagues (Peng et al., 2007) suggested the use of intracellular antibody technology targeted against CD71 in CD71-overexpressing cancer. The use of monoclonal antibodies against transferrin receptor and ascorbate to inhibit both cell proliferation and the pro-angiogenic hypoxia inducible factor

HIF-1α may also be of therapeutic use giving them a selective growth advantage (Jones et al., 2006). Transferrin/CD71 trafficking has been closely associated with PI3K where inhibitors of this intracellular kinase appear able to deplete cell surface CD71 level (Jess et al., 1996). Our finding that Faslodex and the phosphoinositide-3 kinase inhibitor (LY294002) can partially deplete transferrin-induced growth of MCF-7X cells implies mechanistic cross-talk between transferrin/CD71 mitogenic signalling, ER and PI3K (in contrast to an apparent lack of CD71 interplay with MAP kinase) in ER-positive endocrine resistant cells.

Our retrospective tissue studies supported the concept that there is a need for increased iron uptake mediated through elevated CD71 protein levels in high grade breast tumours, characterised by poor NPI, large size and, as predicted, high mitotic activity. Consequently, CD71 expression was more frequently increased in medullary carcinoma and basal-like tumours (CK5/6+ & CK14+) that show these features (Rakha et al., 2006). Furthermore, CD71 expression was also significantly associated with other markers of aggressive phenotype and endocrine treatment resistance including p53, HER2 and EGFR (Tsutsui et al., 2002).

Tumours with elevated CD71 expression had a shorter BCSS in the whole patient series and in the ER-positive/luminal-like patient cohort. These results confirmed that CD71 expression can define poor clinical outcome in the ER-positive patient group. Supporting this, CD71 expression was found to be an independent prognostic marker in ER-positive cohort. In considering ER-positive tamoxifen-only treated patients, increased CD71 expression was

associated with shorter BCSS and DFI suggestive that there might (as in vitro) be a relationship between CD71 expression and adverse endocrine response.

We found that CD71 positive patients who were given tamoxifen, in comparison to those who did not take treatment have poorer prognosis. A possible explanation of this might be related to the function of transferrin/CD71 as a transporter for iron, needed for enzyme function and hence potentially mitogenic pathways. One would envisage that this would be beneficial in tumour cells and thus contribute to resistance, whether to oestrogen deprivation or tamoxifen. CD71 has been shown previously to interact with PI3 Kinase signalling, and this is certainly a prominent contributor in tamoxifen resistant cell growth experimentally. It is feasible that CD71 crosstalks with such growth factor signalling (e.g. EGFR, HER2, MAPK, PI3K) (Knowlden et al., 2003, Nicholson and Gee, 2000, Nicholson et al., 2005, Nicholson et al., 2004a, Nicholson et al., 2004b) which is prevalent in tamoxifen resistance, and actively permits adverse agonistic behaviour of the endocrine agent and thus adverse growth and invasion promotion in the presence of the antihormones.

In conclusion, the present study demonstrated that prominent expression of CD71 protein is a feature of breast cancers with poor prognosis and as such, we propose that transferrin receptor expression may have implications for diagnosis and prognosis. CD71 protein expression could be of value in characterizing a subset of ER-positive/luminal-like tumours with poor prognosis in clinical practice, as well as defining patients less likely to respond

to endocrine therapy. Therapies of current interest in breast cancer (e.g. Faslodex, PI3K-inhibitors) appear able to partially impact on transferrin/CD71-promoted growth, but further investigation of this important mitogenic mechanism may assist in designing new therapeutic strategies to target highly proliferative, endocrine resistant breast cancers. Therapies targeting iron delivery or CD71 itself, may have therapeutic benefits in treating CD71+ ER-positive breast cancer phenotype in the clinic.

### 5.8.2 FOXO3a

Akt/PI3K pathway regulates the sub-cellular localization of FOXO3a by phosphorylation and prevents the protein from translocating to the nucleus to regulate transcription (Brunet et al., 1999). This indicates that absence of nuclear FOXO3a expression, with either complete absence or cytoplasmic localisation due to its phosphorylation by Akt, may represent an important biological mechanism responsible in part for poor prognosis in ER-positive breast cancer, thus removing a constraint to cellular proliferation and potentially to tumourigenesis through an active Akt/PI3K pathway. This proposal is supported by our findings showing that absence of nuclear expression of FOXO3a was associated with poorer outcome and showed a significant association with PIK3CA as a marker strongly related to Akt. Other breast cancer studies confirmed the association between Akt/PI3K activation and cytoplasmic FOXO3a expression pattern with decreased patient survival, in agreement with our findings (Hu et al., 2004).

In this study we did not find a significant association with survival when patients were categorised into either negative or positive FOXO3a expressers *per se*. Instead, we found that subcellular localisation indicates functional relevance as evidenced here by more favourable outcome in patients with predominant nuclear expression. Supporting these findings, previous studies have shown that nuclear FOXO3a induces the expression of genes that inhibit cell cycle progression such as the CDK inhibitors (Brunet et al., 1999, Zou et al., 2008). Subsequently, we found a significant positive association between nuclear FOXO3a and the expression of the cell cycle inhibitor p27 implying a role in the induction of cell cycle arrest.

In this patient series including ER-positive/luminal-like subtype, nuclear localisation of FOXO3a was associated with markers of good prognosis such as PgR (Bardou et al., 2003), and FOXA1 expression which is required for the expression of 50% of ER-regulated genes (Thorat et al., 2008). Furthermore, we have also shown that nuclear FOXO3a expression is significantly associated with longer BCSS and DMFI which implies its role in stratification of ER-positive groups into prognostic subgroups, possibly explained by a tumour suppressor function associated with cell cycle arrest.

Previous studies have shown that loss of FOXO3a function by its absence or by cytoplasmic localisation is positively associated with proliferation (Accili and Arden, 2004). We have found that BC especially luminal-like cases expressing nuclear FOXO3a are characterised by low proliferation as indicated by negative correlation with mitosis. Taken together, our findings support the interaction of FOXO3a as a downstream target of Akt/PI3K pathway with

markers related to proliferation and cell cycle, a role which is independent of the systemic therapy as shown here by our multivariate analysis results. Our results demonstrated the biological and prognostic role of FOXO3a protein expression and its subcellular localization in BC. Promoting FOXO3a nuclear localisation could be a potential therapeutic target. Loss of nuclear FOXO3a expression could tilt the balance in favour of proliferation and poor outcome in luminal-like breast cancers through active Akt/PI3K pathway highlighting the importance of cellular proliferation in their biological stratification.

### 5.8.3 AGTR1

Recently, it has been shown that AGTR1 overexpression defines a subset of ER-positive breast cancer that can benefit from AGTR1 antagonists (Ateeq et al., 2009, Rhodes et al., 2009). In this study, AGTR1 gene expression was associated with the luminal phenotype at mRNA level while our protein expression study has shown that increased AGTR1 expression characterised an aggressive ER-positive phenotype with shorter survival.

The poor prognosis of AGTR1+ER+ phenotype could be explained in part by loss of BRCA1 tumour suppressor function that we have shown in the current study.

Our results showed a positive correlation between AGTR1 and EGFR implying that AGTR1 can perform its function through the activation of mitogenic signalling pathways supporting the results of a previous study that showed AGTR1 possible regulation of mitogenic signalling pathways by two simultaneous mechanisms, one involved conventional PKCs and the other involved EGFR transactivation (Greco et al., 2003).

This study demonstrated an increase of DM with AGTR1 overexpression which could be explained by the role played by AGTR1 through angiotensin II in promoting tumour cell invasion. A previous study (Rhodes et al., 2009) has shown that overexpression of AGTR1 due to angiotensin stimulation significantly promoted cell invasion in a AGTR1 transfected breast carcinoma cells in comparison to the negative control. Importantly, AGTR1 and angiotensin mediated invasion was decreased in a dose-dependent manner with addition of the AGTR1 blocker, losartan (An ACE inhibitor).

The non-significant association between ER and AGTR1 protein expression could be attributed to a post translational modification event that could alter the protein product of the gene, further study are warranted to clarify this point.

In conclusion, AGTR1 expression in the luminal-like breast cancer characterises an aggressive phenotype with shorter survival. Evaluation the potential application of AGTR1 blockade as a novel targeted therapy in breast cancer is warranted.

# 6 Prognostic and biological significance of cellular proliferation and its role in oestrogen receptor positive breast cancer subgrouping

## 6.1 Introduction

The importance of cellular proliferation in subclassifying luminal cancer is recognised. Many studies have demonstrated a strong relationship between cellular proliferation and poor prognosis in breast cancer particularly in the oestrogen receptor (ER)-positive/luminal-like molecular subtype (Cheang et al., 2009).

Several proliferation markers have been proposed to be of clinical importance for assessing prognosis in breast cancer, among them is the pan proliferation marker Ki67 (MIB1), cyclins, PCNA, S-phase fraction, thymidine kinase and others (Stuart-Harris et al., 2008, Colozza et al., 2005).

In this chapter, we studied the biological and prognostic implication of proliferation using cell cycle phase specific proteins and compared their association with the pan proliferation marker MIB1.

In addition, we have studied the protein expression of p27 and Bcl-2 and their roles in the biology and outcome of breast cancer with particular emphasis on the ER-positive subgroup.

## 6.2 Prognostic and biological significance of the cell-cycle associated proteins, cyclin B1 and thymidine kinase 1 (TK1) in breast cancer and luminal-like subtype

### 6.2.1 Introduction

TK1 is an enzyme involved in the synthesis of thymidine triphosphate needed by the proliferating cells to enter S phase (Gasparri et al., 2009). Structurally, human TK1 has a molecular mass of 25.4 kDa, and consists of 234 amino acids (Welin et al., 2004). Phosphorylation of thymidine is catalyzed by two thymidine kinases: the cytoplasmic TK1 which is absent in non-dividing cells and the mitochondrial TK2 (Munch-Petersen et al., 1995). TK1 is activated in the G1/S phase of the cell cycle to perform its function, and this activity has been shown to correlate with the proliferative activity of tumour cell (Hallek et al., 1992).

TK1 usually increases earlier than Ki67 and represents a unique marker of G1 phase in the cell cycle while Ki67 on the other hand is expressed maximally in mid and late S-phase (Gasparri et al., 2009).

Previous studies have shown that serum TK1 appears to have some clinical value in solid tumours as prostate cancer, breast cancer, and small-cell lung cancer (Hallek et al., 1992).

TK1 serum level could be used with success in predicting increased risk of recurrence after surgery in patients with early breast cancer (He et al., 2006). These findings highlight the importance of proliferation related genes in ER-positive luminal-like breast cancer.

Cyclin B1 is the regulatory subunit of cyclin-dependent kinase 1 (Cdk1). It is virtually undetectable in cells from G0/G1 phase to mid S phase, but became visible in the cytoplasm in late S phase. As cells proceed within G2 phase, the level of cyclin B1 rapidly increased in the perinuclear region of the cytoplasm then appears in the nucleus at the mitotic phase (Kakino et al., 1996, Winters et al., 2001). In other words, cyclin B1 is translocated to the nucleus from the cytoplasm, and plays an essential role in cell proliferation through promotion of mitosis. Breast epithelial cells express cyclin B1 in their cytoplasm in the G2 phase and in their nuclei in the M phase (Kawamoto et al., 1997).

Proper regulation of cyclin B1 is essential for the initiation of mitosis as it regulates the G2-M transition of the cell cycle and its expression is higher in premalignant and malignant than normal breast lesions (Aaltonen et al., 2009, Yuan et al., 2004). Previous cell line studies have demonstrated that downregulation of cyclin B1 with small interfering RNA (siRNA) inhibited proliferation of several breast and cervical cancer cell lines including MCF-7, BT-474, SK-BR-3, MDA-MB-231 and HeLa cells (Androic et al., 2008, Yuan et al., 2004).

Cyclin B1 protein has been found to be expressed in many cancers and shown to be associated with high grade tumours and advanced stage, as well as poor prognosis, including oesophageal squamous cell carcinoma (Takeno et al., 2002), small cell lung cancer (Yoshida et al., 2004, Cooper et al., 2009) and B-cell lymphoma (Obermann et al., 2005).

Clinical *in vivo* studies showed that cyclin B1 was an independent predictor of poor overall survival among premenopausal (Kühling et al., 2003). Previously,

it has been demonstrated that CCNB1 gene clustered in Luminal B subtype which is characterised by low ER expression and high expression of proliferation related genes (Hu et al., 2006).

Cyclin B1 expression tends to increase in tumours with co-occurrence of TP53 mutations and MYC amplification, a combination that seems to characterize basal-like and poor prognosis Luminal B tumours (Agarwal et al., 2009).

Confirming the clinical importance of both TK1 and cyclin B1, several prognostic gene signatures were previously developed for ER-positive/luminal-like breast cancer composed of many cell-cycle and proliferation associated genes including both TK1 and cyclin B1 (Chanrion et al., 2008).

In this study, we assessed the biological and clinical relevance of these cell-cycle stage specific markers, both individually and in combination, and compared their expression to the pan-proliferation marker MIB1 in the whole patient series and in the ER-positive breast luminal-like cohort.

### 6.2.2 Material and Methods

Mouse monoclonal antibodies to TK1 (ab57757; Abcam, Cambridge, UK) and cyclin B1 (ab72; Abcam, Cambridge, UK) were optimized at a working dilution of 5μg/ml and 0.3μg/ml respectively using optimisation tumour TMAs and full-face sections of breast carcinoma; tonsil tissue was used as positive control tissue. To unmask the antigens, the sections were microwaved in citrate buffer pH 6 for 23 minutes at 700W. An indirect labelled streptavidin avidin biotin technique (LSAB) technique with DAB chromogen was performed using a DakoCytomation Techmate 500 Plus (DakoCytomation, Cambridge, UK) automatic immunostainer as previously discussed in the Material and methods chapter. Negative controls were performed by omitting the primary antibody and substitution with diluent.

For TK1, the informative cores were scored using the percentage of positive cells stained in each core. The data was categorised using the median of the TK1 percentage (8%).

For cyclin B1 H-score was used for assessment. The median of the H score > 0 cutoff was used to categorise the data of cyclin B1.

### 6.2.3 Results

In breast cancer tissue, protein expression was detected in the cytoplasm of malignant cells for TK1 and cyclin B1 **(Fig 6.1)**.

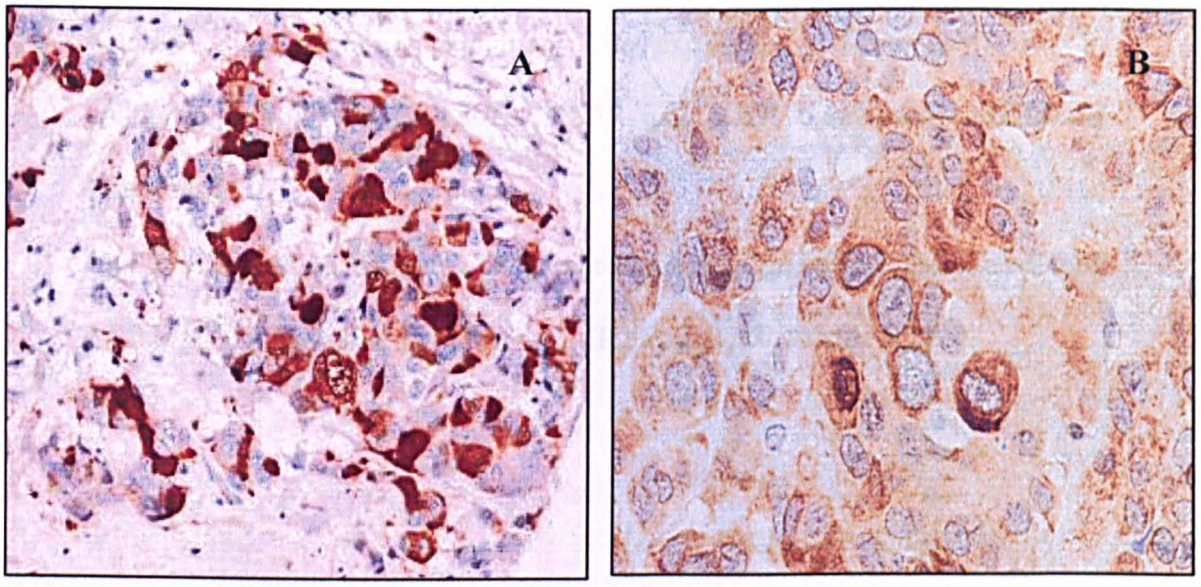


**Figure 6.1 :** Expression of TK1 and cyclin B1 in breast cancer
(A) TK1 expression in high grade invasive ductal carcinoma (x200). (B) Cyclin B1 expression in high grade invasive ductal carcinoma (x400).

**TK1**

In the whole series, high TK1 expression (≥ median 8%) was found in 51.5% of patients and was positively associated with younger age group ($p$=0.001), large tumour size ($p$=0.001), high tumour grade ($p$<0.001), raised mitotic counts ($p$<0.001), poor NPI group, DM and recurrence. The positive expression was noted in medullary type cancer and ductal NST with very low expression in lobular cancer ($p$<0.001).

In the ER-positive group, TK1 was associated with younger age group ($p$<0.001), high tumour grade ($p$<0.001), LN stage ($p$=0.004), mitotic counts ($p$<0.001), poor NPI group ($p$<0.001) and vascular invasion ($p$=0.010).

In the whole series, there was an inverse association between TK1 expression and ER, PgR, AR, Bcl-2, CK18 and CK19. In contrast, positive associations between TK1 expression and basal CKs expression (CK5/6; ($p$<0.001), P-cadherin ($p$<0.001), p53, HER2, EGFR and MIB1 expression were found **(Table 6.1)**.

In the ER-positive patients, TK1 expression was positively associated with MIB1, p53, P-cadherin, E-cadherin, HER2 and EGFR **(Table 6.2)**.

**Table 6.1:** Relation of TK1 expression to other biomarkers in the whole series of breast cancer patients

| Variable | TK1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Negative | Positive | Total | | |
| **CK5/6** | | | | 18.751 | <0.001 |
| Negative | 398(51) | 383(49) | 781 | | |
| Positive | 40(30.5) | 91(69.5) | 131 | | |
| **CK14** | | | | 3.882 | 0.049 |
| Negative | 398(49.2) | 411(50.8) | 809 | | |
| Positive | 34(38.2) | 55(61.8) | 89 | | |
| **CK18** | | | | 26.814 | <0.001 |
| Negative | 31(25.8) | 89(74.2) | 120 | | |
| Positive | 382(51.3) | 363(48.7) | 745 | | |
| **CK19** | | | | 11.608 | 0.001 |
| Negative | 29(31.5) | 63(68.5) | 92 | | |
| Positive | 411(50.2) | 407(49.8) | 818 | | |
| **ER** | | | | 51.343 | <0.001 |
| Negative | 69(28.3) | 175(71.7) | 244 | | |
| Positive | 359(55.1) | 292(44.9) | 651 | | |
| **PgR** | | | | 37.090 | <0.001 |
| Negative | 139(36.5) | 242(63.5) | 381 | | |
| Positive | 288(57.1) | 216(42.9) | 504 | | |
| **AR** | | | | 28.793 | <0.001 |
| Negative | 107(36) | 190(64) | 297 | | |
| Positive | 308(55.3) | 249(44.7) | 557 | | |
| **p53** | | | | 34.504 | <0.001 |
| Negative | 351(54) | 299(46) | 650 | | |
| Positive | 78(32) | 166(68) | 244 | | |
| **Bcl-2** | | | | 28.207 | <0.001 |
| Negative | 100(37.2) | 169(62.8) | 269 | | |
| Positive | 254(57.7) | 186(42.3 | 440 | | |
| **MIB1** | | | | 91.57 | <0.001 |
| low | 149(76.4) | 46(23.6) | 195 | | |
| High | 203(36.6) | 351(63.4) | 554 | | |
| **P-cadherin** | | | | 32.477 | <0.001 |
| Negative | 217(32.477) | 166(43.3) | 383 | | |
| Positive | 152(36.5) | 264(63.5) | 416 | | |
| **E-cadherin** | | | | 7.680 | 0.006 |
| Negative | 182(54.3) | 153(45.7) | 335 | | |
| Positive | 253(44.8) | 312(55.2) | 565 | | |
| **HER2** | | | | 38.779 | <0.001 |
| Negative | 425(52.3) | 388(47.7) | 813 | | |
| Positive | 21(20) | 84(80) | 105 | | |
| **EGFR** | | | | 37.198 | <0.001 |
| Negative | 353(52.2) | 323(47.8) | 676 | | |
| Positive | 40(25.3) | 118(74.7) | 158 | | |

**Table 6.2:** Relation of TK1 expression to other biomarkers in ER-positive cohort

| Variable | TK1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Negative | Positive | Total | | |
| **CK5/6** | | | | 0.024 | 0.607 |
| Negative | 333(55.1) | 271(44.9) | 604 | | |
| Positive | 18(50) | 18(50) | 36 | | |
| **CK14** | | | | 0.015 | 1.000 |
| Negative | 327(54.9) | 269(45.1) | 596 | | |
| Positive | 21(53.8) | 18(46.2) | 39 | | |
| **CK18** | | | | 1.658 | 0.215 |
| Negative | 10(41.7) | 14(58.3) | 24 | | |
| Positive | 329(55) | 269(45) | 598 | | |
| **CK19** | | | | 1.383 | 0.307 |
| Negative | 17(45.9) | 20(54.1) | 37 | | |
| Positive | 339(55.80 | 268(44.2) | 607 | | |
| **PgR** | | | | 1.193 | 0.311 |
| Negative | 81(51.3) | 77948.7) | 158 | | |
| Positive | 270(56.20 | 210(43.8) | 480 | | |
| **AR** | | | | 0.717 | 0.419 |
| Negative | 65(52.4) | 59(47.6) | 124 | | |
| Positive | 277(56.6) | 212(43.3) | 489 | | |
| **p53** | | | | 13.220 | <0.001 |
| Negative | 304(58.1) | 219(41.9) | 523 | | |
| Positive | 44(39.3) | 68(60.7) | 112 | | |
| **Bcl-2** | | | | 4.961 | 0.033 |
| Negative | 56(47.5) | 62(52.5) | 118 | | |
| Weak | 224(59.1) | 155(40.9) | 379 | | |
| **MIB1** | | | | 56.292 | <0.001 |
| low | 131(78) | 37(22) | 176 | | |
| High | 154(43) | 204(57) | 355 | | |
| **P-cadherin** | | | | 6.798 | 0.009 |
| Negative | 203(58) | 147(42) | 350 | | |
| Positive | 105(46.9) | 119(53.1) | 224 | | |
| **E-cadherin** | | | | 14.416 | <0.001 |
| Negative | 150(64.9) | 81(35.1) | 231 | | |
| Positive | 202(49.4) | 207(50.6) | 409 | | |
| **HER2** | | | | 39.515 | <0.001 |
| Negative | 347(58.7) | 244(41.3) | 591 | | |
| Positive | 5(10.9) | 41(89.1) | 49 | | |
| **EGFR** | | | | 23.032 | <0.001 |
| Negative | 302(58.2) | 217(41.8) | 519 | | |
| Positive | 24(29.6) | 57(70.4) | 81 | | |

**Cyclin B1**

The invasive breast cancer tissue showed expression of cyclin B1 in cytoplasm and with perinuclear localisation **(Fig 6.1)**. 42.7% of the tumours were positive for cyclin B1 expression.

**Correlation with the clinicopathological variables**

There was a positive association between cyclin B1 and P-cadherin expression ($p$=0.01).

In the ER-positive subtype, cyclin B1 expression was positively associated with mitotic counts and distant metastasis. Positive expression was noted in medullary type cancer and NST. We found significant positive associations between cyclin B1 expression and P-cadherin and p53 **(Tables 6.3 and 6.4).**

**Table 6.3**: Relation of cyclin B1 expression to other clinicopathologic variables in the ER-positive cohort

| Variable | cyclin B1 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Negative | Positive | Total | | |
| **Patients' age** | | | | 1.743 | 0.627 |
| <40 | 26(63.4) | 15(36.6) | 41 | | |
| 40-50 | 117(59.4) | 80(40) | 197 | | |
| 51-60 | 127(58.8) | 89(41.2) | 216 | | |
| >60 | 128(54.7) | 106(45.3) | 234 | | |
| **Tumour size** | | | | 2.693 | 0.109 |
| **≤1.5 cm** | 155(62) | 95(38) | 250 | | |
| >1.5 cm | 244(55.6) | 195(44.4) | 439 | | |
| **Lymph node stage** | | | | 0.338 | 0.844 |
| 1(Negative) | 246(58.3) | 176(41.7) | 422 | | |
| 2(1-3 LN) | 125(57.9) | 91(42.1) | 216 | | |
| 3(>3 LN) | 27(54) | 23(46) | 50 | | |
| **Tumour Grade** | | | | 7.713 | 0.021 |
| 1 | 77(56.6) | 59(43.4) | 136 | | |
| 2 | 186(63.7) | 106(36.3) | 292 | | |
| 3 | 399(57.9) | 290(42.1) | 261 | | |
| **NPI** | | | | 6.353 | 0.042 |
| Good | 152(63.9) | 86(36.1) | 238 | | |
| Moderate | 190(53.5) | 165(46.5) | 355 | | |
| Poor | 57(59.4) | 39(40.6) | 96 | | |
| **DM** | | | | 6.321 | 0.012 |
| No | 296(60.8) | 191(39.2) | 487 | | |
| Positive | 98(50.3) | 97(49.7) | 195 | | |
| **Recurrence** | | | | 7.685 | 0.007 |
| No | 250(61.9) | 154(38.1) | 404 | | |
| Positive | 138(51.1) | 132(48.9) | 270 | | |
| **VI** | | | | 2.886 | 0.236 |
| No | 213(59.3) | 146(40.7) | 359 | | |
| Probable | 48(50) | 48(50) | 96 | | |
| Definite | 137(59.1) | 95(40.9) | 232 | | |
| **Histologic tumour type** | | | | 7.701 | 0.173 |
| Ductal/NST | 204(55.4) | 164(44.6) | 368 | | |
| Lobular | 58(69.9) | 25(30.1) | 83 | | |
| Tubular and Tubular mixed | 101(59.4) | 69(40.6) | 170 | | |
| Medullary | 3(75) | 1(25) | 4 | | |
| Other special types* | 5(52.8) | 6(47.2) | 11 | | |
| Mixed** | 28(52.8) | 25(47.2) | 53 | | |
| **Mitosis** | | | | 10.073 | 0.006 |
| 1 | 191(64.7) | 104(35.3) | 295 | | |
| 2 | 85(56.3) | 66(43.7) | 151 | | |
| 3 | 115(51.1) | 110(48.9) | 225 | | |
| **Menopausal status** | | | | 1.135 | 0.294 |
| Premenopausal | 146(60.6) | 95(39.4) | 241 | | |
| Postmenopausal | 250(56.4) | 290(42.2) | 447 | | |

*Includes Mucoid, invasive cribriform and invasive papillary carcinoma, ** Include ductal/NST mixed with lobular or special type

**Table 6.4:** Relation of cyclin B1 expression to other biomarkers in the ER-positive cohort

| Variable | cyclin B1 Expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Negative | Positive | Total | | |
| **CK5/6** | | | | 0.866 | 0.352 |
| Negative | 369(58.2) | 265(41.8) | 634 | | |
| Positive | 23(51.1) | 22(48.9) | 45 | | |
| **CK14** | | | | 4.888 | 0.027 |
| Negative | 368(59.1) | 255(40.9) | 623 | | |
| Positive | 19(42.2) | 26(57.8) | 45 | | |
| **CK18** | | | | 0.294 | 0.705 |
| Negative | 16(53.3) | 14(46.7) | 30 | | |
| Positive | 364(58.3) | 260(41.7) | 624 | | |
| **CK19** | | | | 0.373 | 0.601 |
| Negative | 22(62.9) | 13(37.1) | 35 | | |
| Positive | 374(57.4) | 275(42.4) | 649 | | |
| **PgR** | | | | 0.008 | 0.928 |
| Negative | 97(57.7) | 71(42.3) | 168 | | |
| Positive | 297(58.1) | 214(41.9) | 511 | | |
| **AR** | | | | 0.014 | 0.924 |
| Negative | 87(58.8) | 61(41.2) | 148 | | |
| Positive | 296(59.3) | 203(40.7) | 499 | | |
| **p53** | | | | 7.035 | 0.010 |
| Negative | 330(60.1) | 219(39.9) | 549 | | |
| Positive | 61(47.3) | 68(52.7) | 129 | | |
| **Bcl-2** | | | | 1.552 | 0.220 |
| Negative | 67(52.3) | 61(47.7) | 128 | | |
| Positive | 235(58.6) | 166(41.4) | 401 | | |
| **MIB1** | | | | 3.752 | 0.050 |
| Low | 110(62.9) | 65(37.1) | 175 | | |
| High | 205(54.1) | 174(45.9) | 379 | | |
| **P-cadherin** | | | | 14.283 | <0.001 |
| Negative | 231(63.5) | 133(36.5) | 364 | | |
| Positive | 115(47.9) | 125(52.1) | 240 | | |
| **E-cadherin** | | | | 0.417 | 0.567 |
| Negative | 131(56.2)) | 102(43.8) | 233 | | |
| Positive | 264(58.8) | 185(41.2) | 449 | | |
| **HER2** | | | | 0.230 | 0.680 |
| Negative | 356(57.8) | 260(42.2) | 616 | | |
| Positive | 36(61) | 23(39) | 59 | | |

### 6.2.4 Survival analysis

**Univariate analysis**

**TK1**

In the whole series, a significant correlation between TK1 expression and poorer BCSS was identified (Log Rank (LR)=11.623, $p$=0.001). In the ER-positive/luminal-like cohort, we also found a significant association between TK1 and shorter BCSS (LR=11.835, $p$=0.001) **(Fig 6.2A).**

A significant relation between TK1 expression and shorter DMFI in the whole series (LR=7.225, $p$=0.007) and in the ER-positive patient group (LR=9.518, $p$=0.002) was found **(Fig 6.2B).**

**DMFI according to systemic therapy group**

***Tamoxifen only treated patients in ER-positive patient group (n=237)***

Patients with tumours expressing high TK1 protein showed a significant shorter DMFI (LR= 6.581, $p$=0.010).

***Chemotherapy only treated patients in ER- patient group (n=119)***

We found no significant relation between TK1 expression and DMFI in ER negative chemotherapy treated patients (LR= 0.271, $p$=0.603).

**Cyclin B1**

We found no significant association between cyclin B1 expression and BCSS in the whole series (LR= 2.399, $p$=0.121). In contrast, the ER-positive/luminal-like cohort showed a significant association with shorter BCSS (LR=7.606, $p$=0.004) **(Fig 6.2C).**

No significant association was found between cyclin B1 expression and DMFI in the whole series (LR= 2.059, $p$=0.151). In contrast, the ER-positive/luminal-

like cohort showed a significant association with shorter DMFI (LR= 6.694, $p$=0.021) **(Fig.6.2D).**

**DMFI according to systemic therapy group**

***Tamoxifen only treated patients in ER-positive patient group (n=237)***

Patients with tumours expressing high cyclin B1 protein showed a significant shorter DMFI (LR= 5.438, $p$=0.010).

***Chemotherapy only treated patients in ER- patient group (n=127)***

We found a significant relation between cyclin B1 expression and longer DMFI in ER negative chemotherapy treated patients (LR= 4.128, $p$=0.042) **(Fig.6.2E).**

The effect of combined expression of both proteins on the DMFI in luminal-like ER-positive cohort was tested and we found significantly shorter DMFI in patients expressing both markers (LR=8.194, $p$=0.017) than patients expressing only one marker in univariate analysis **(Fig.6.2F).**

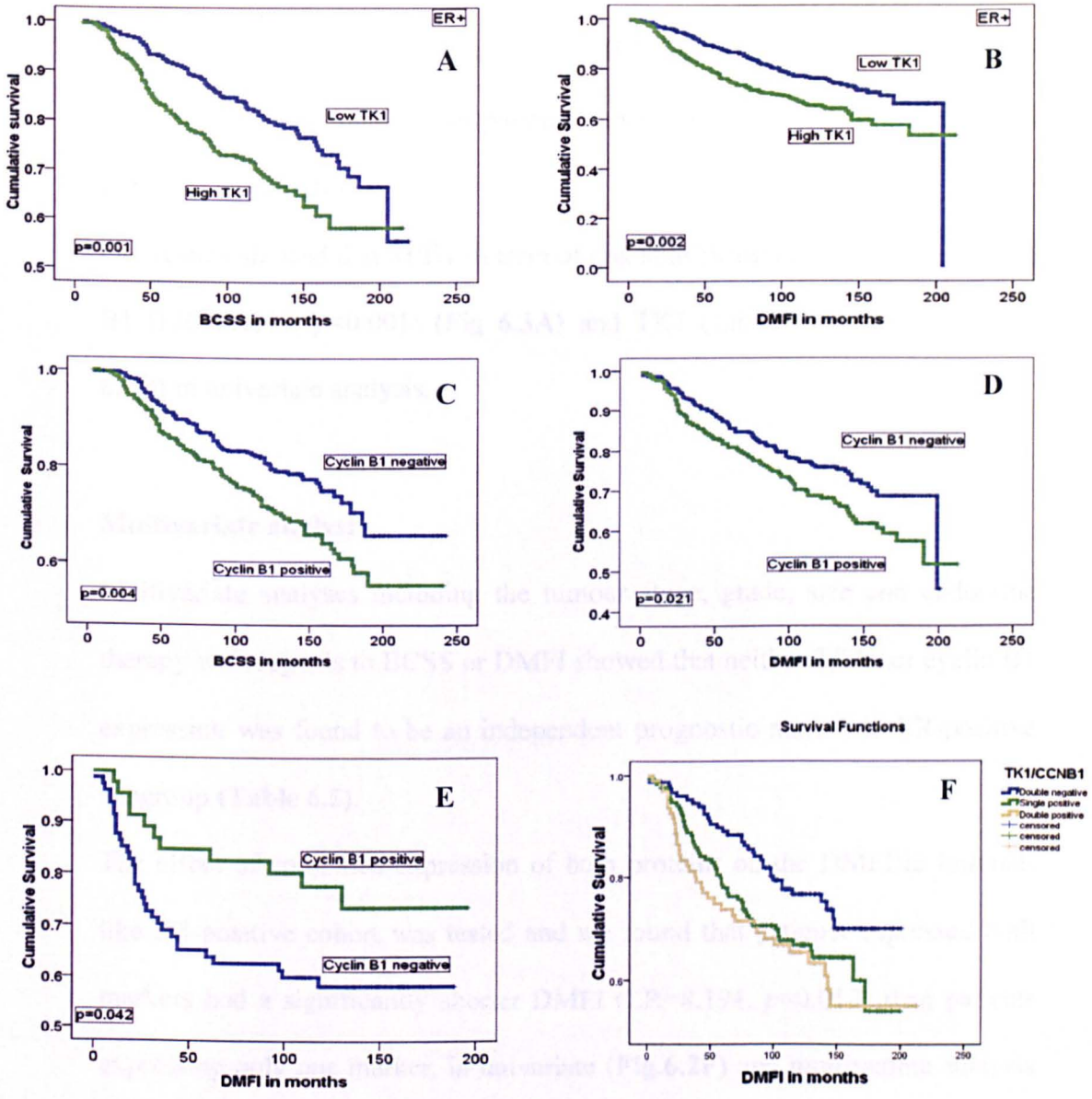


**Figure 6.2:** Kaplan Meier plots of TK1 expression

(A) Kaplan Meier plot of TK1 expression in relation to BCSS in ER-positive luminal like cohort. (B) Kaplan Meier plot of TK1 expression in relation to DMFI in ER-positive luminal like cohort. (C) Kaplan Meier plot of cyclin B1 expression in relation to BCSS in ER-positive luminal like cohort. (D) Kaplan Meier plot of cyclin B1 expression in relation to DMFI in ER-positive luminal like cohort. (E) Kaplan Meier plot of cyclin B1 expression in relation to BCSS in ER negative chemotherapy treated patients. (F) Kaplan Meier plot of TK1 and cyclin B1 combined expression in relation to DMFI in ER-positive luminal like cohort.

**Survival analysis in relation to MIB1 expression**

To compare the combined expression of MIB1 and TK1 or cyclin B1, we have studied the clinical outcome of patients expressing both markers (MIB1/TK1) or (MIB1/cyclin B1).

Our results showed that MIB1 in term of risk stratification, is superior to cyclin B1 (LR=21.817, $p<0.001$) **(Fig 6.3A)** and TK1 (LR=27.900, $p<0.001$) **(Fig 6.3B)** in univariate analysis.

**Multivariate analysis**

Multivariate analyses including the tumour stage, grade, size and endocrine therapy with regards to BCSS or DMFI showed that neither TK1 nor cyclin B1 expression was found to be an independent prognostic marker in ER-positive subgroup **(Table 6.5)**.

The effect of combined expression of both proteins on the DMFI in luminal-like ER-positive cohort was tested and we found that patients expressed both markers had a significantly shorter DMFI (LR=8.194, $p=0.017$) than patients expressing only one marker, in univariate **(Fig.6.2F)** and multivariate analysis (HR= 1.1738, $p= 0.040$, 95% CI=1.025–2.946) **(Table 6.6)**.

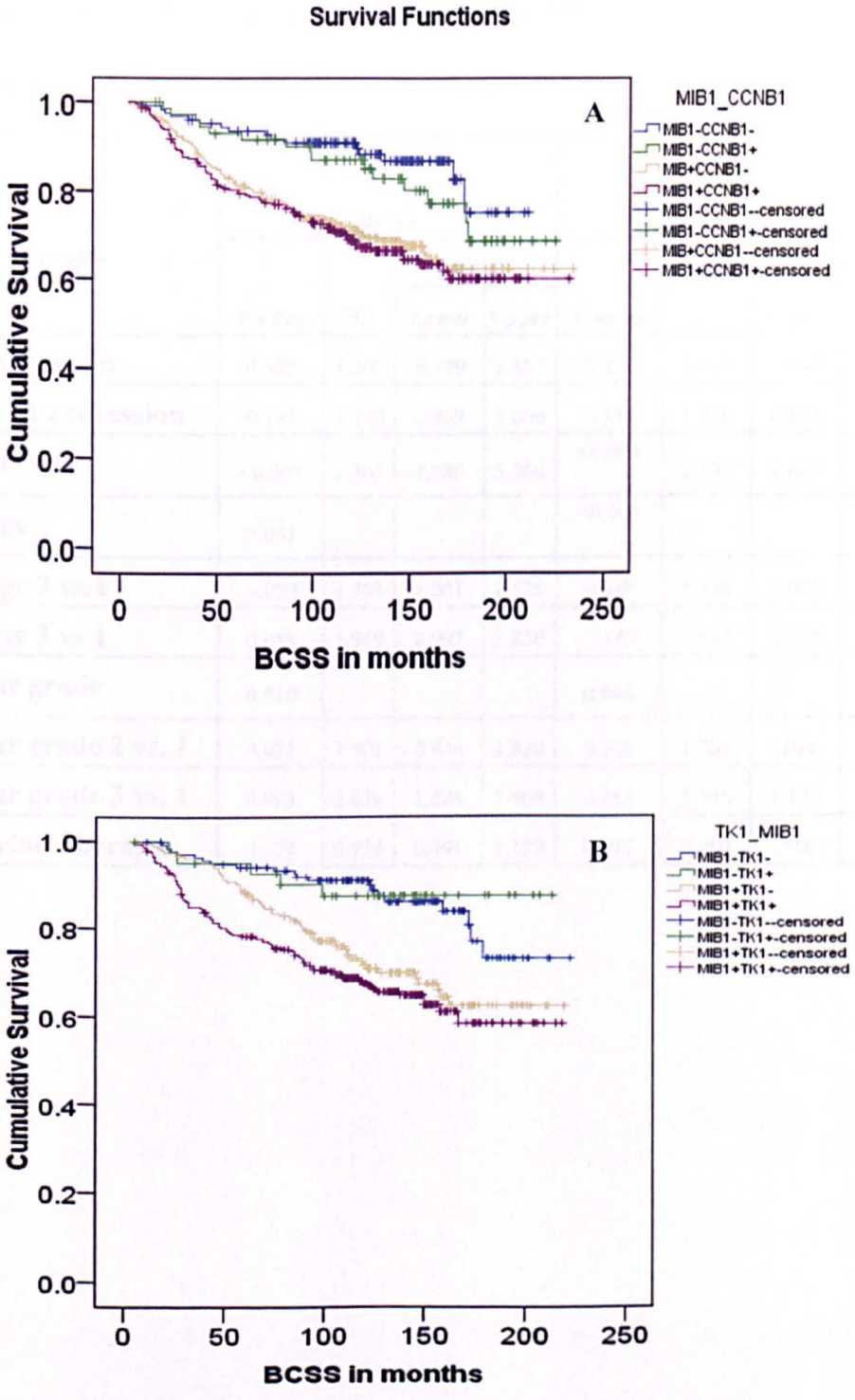
Survival Functions
A
MIB1_CCNB1
MIB1-CCNB1-
MIB1-CCNB1+
MIB+CCNB1-
MIB1+CCNB1+
MIB1-CCNB1--censored
MIB1-CCNB1+-censored
MIB+CCNB1--censored
MIB1+CCNB1+-censored
Cumulative Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
250
BCSS in months
B
TK1_MIB1
MIB1-TK1-
MIB1-TK1+
MIB1+TK1-
MIB1+TK1+
MIB1-TK1--censored
MIB1-TK1+-censored
MIB1+TK1--censored
MIB1+TK1+-censored
Cumulative Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
50
100
150
200
250
BCSS in months

**Figure 6.3:** Kaplan Meier plots of the combined expression of (A) cyclin B1 and (B) TK1 with MIB1 in relation to BCSS

**Table 6.5:** COX model of predictors of BCSS and DMFI in ER-positive patients

| Variable | BCSS | | | | DMFI | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 95% CI | | | HR | 95% CI | |
| | P value | HR | Lower | Upper | P value | | Lower | Upper |
| **TK1 expression** | 0.399 | 1.207 | 0.779 | 1.871 | 0.090 | 1.435 | 0.945 | 2.180 |
| **Cyclin B1 expression** | 0.193 | 1.320 | 0.869 | 2.006 | 0.330 | 1.216 | 0.821 | 1.801 |
| **Tumour size** | <0.001 | 2.503 | 1.580 | 3.966 | <0.001 | 2.469 | 1.607 | 3.792 |
| **LN stage** | 0.061 | | | | <0.001 | | | |
| **LN stage 2 vs.1** | 0.050 | 1.591 | 1.001 | 2.529 | 0.049 | 1.563 | 1.002 | 2.437 |
| **LN stage 3 vs.1** | 0.053 | 1.949 | 0.992 | 3.830 | <0.001 | 3.614 | 2.025 | 6.449 |
| **Tumour grade** | 0.010 | | | | 0.044 | | | |
| **Tumour grade 2 vs. 1** | 0.071 | 1.901 | 0.946 | 3.820 | 0.102 | 1.702 | .899 | 3.221 |
| **Tumour grade 3 vs. 1** | 0.003 | 2.876 | 1.424 | 5.808 | 0.014 | 2.248 | 1.180 | 4.282 |
| **Endocrine Therapy** | 0.198 | 0.754 | 0.491 | 1.159 | 0.197 | 0.769 | .516 | 1.146 |

**Table 6.6:** COX model of the combined expression of TK1/cyclin B1 with regards DMFI in whole series and ER-positive patients

| Variable | Whole series | | | | ER-positive patients | | | |
|---|---|---|---|---|---|---|---|---|
| | P value | HR | 95% CI | | P value | HR | 95% CI | |
| | | | Lower | Upper | | | Lower | Upper |
| **Tumour size** | <0.001 | 2.030 | 1.437 | 2.868 | <0.001 | 2.435 | 1.586 | 3.738 |
| **LN stage** | <0.001 | | | | <0.001 | | | |
| **LN stage 2 vs.1** | 0.040 | 1.442 | 1.016 | 2.047 | .048 | 1.564 | 1.004 | 2.436 |
| **LN stage 3 vs.1** | <0.001 | 4.620 | 3.015 | 7.080 | <0.001 | 3.580 | 2.019 | 6.347 |
| **Tumour grade** | 0.038 | | | | 0.039 | | | |
| **Tumour grade 2 vs. 1** | 0.139 | 1.583 | 0.861 | 2.908 | 0.102 | 1.702 | 0.900 | 3.218 |
| **Tumour grade 3 vs. 1** | 0.015 | 2.047 | 1.149 | 3.648 | 0.012 | 2.261 | 1.194 | 4.282 |
| **Endocrine Therapy** | 1.000 | 1.000 | 0.728 | 1.373 | 0.246 | 0.788 | 0.527 | 1.178 |
| **TK1/CCNB1(double -ve)** | 0.408 | | | | 0.096 | | | |
| **TK1/CCNB1(single +ve vs. double –ve)** | 0.180 | 1.306 | 0.884 | 1.931 | 0.080 | 1.526 | 0.951 | 2.450 |
| **TK1/CCNB1(double +ve vs. double –ve)** | 0.416 | 1.207 | 0.767 | 1.901 | 0.040 | 1.738 | 1.025 | 2.946 |

### 6.2.5 Discussion

Many studies showed the clinical importance of TK1 and cyclin B1 (Chen et al., Chanrion et al., 2008) among gene signatures that can predict the disease relapse in breast cancer patients. Cyclin B1 is included in the 21 gene commercially available recurrence signature for predicting the relapse in ER-positive lymph node negative patients (Oncotype DX) (Paik et al., 2004).

Some authors have previously reported that TK1 is a more useful proliferation marker than Ki-67 and PCNA in breast, lung and colorectal carcinoma (Guan et al., 2009).

In this study, we found a highly significant positive association between TK1 and high proliferation as assessed by MIB1 protein expression, high mitosis and higher tumour grades indicating a high reliability of TK1 in assessing the activated G1 state in the cell cycle (Gasparri et al., 2009, Zhang et al., 2001, He et al., 2006, Broet et al., 2001).

TK1 usually increases earlier than Ki67 and represents a unique marker for the activated G1 phase in the cell cycle (Gasparri et al., 2009). The results of this study showed that MIB1 expression is more powerful than TK1 or cyclin B1 in assessing the patient outcome contrasting with the results of another investigator who previously reported that TK1 was a more useful proliferation marker than Ki-67 and PCNA in breast, lung and colorectal carcinoma (Guan et al., 2009).

Our results showed that ER-positive tumours expressing high levels of TK1 and cyclin B1 proteins are liable to metastasize. Dai and colleagues identified a subset of patients characterized by relatively high oestrogen receptor

expression for their age, the occurrence of metastases was strongly predicted by a homogeneous gene expression pattern almost entirely consisting of cell cycle genes among which TK1 gene was included (Dai et al., 2005). Others have shown that serum TK1 concentration was higher in patients developing distant and/or loco-regional recurrence within 3 months after surgery (He et al., 2006).

Supporting its role as a marker of poor prognosis in breast cancer, we found significant positive associations between TK1 and other clinicopathological variables of unfavourable outcome including younger age group, high tumour grade, high mitotic counts and poor NPI group in agreement with others (Gasparri et al., 2009, Zhang et al., 2001, He et al., 2006, Broet et al., 2001).

Our results showed that high cyclin B1 expression was associated with mutated p53 protein expression in luminal-like breast cancer. Previous study has shown that in case of expression of the mutated p53, the cells tend to express cyclin B1 more than those with wild type (Taylor and Stark, 2001). Cyclin B1 expression tends to increase in tumours with co-occurrence of TP53 mutations and MYC amplification, a combination that seems to characterize basal-like and Luminal B tumours (Agarwal et al., 2009).

Clinical in vivo studies showed that cyclin B1 was an independent predictor of poor overall survival among premenopausal (Kühling et al., 2003). In this study, although cyclin B1 protein expression was found to be associated with shorter BCSS and DMFI in univariate analysis in ER-positive cohort, it was not an independent prognostic marker. However, interestingly cyclin B1 was found to be associated with longer BCSS in chemotherapy only treated ER

negative patients indicating its potential role in assessing the chemotherapy response, especially in ER negative patients.

There are suggestions that the difference between different luminal subclasses is due to its variable expression of proliferation associated genes (Sotiriou et al., 2003). Our results are consistent with these findings and showed that increased cellular proliferation, as assessed by high TK1 and cyclin B1 expression, was associated with poor outcome in the ER-positive cohort. Although TK1 is a predictor of outcome and not cyclin B1 in the whole series, using both markers in combination produced better stratification in terms of DMFI than each one separately.

In conclusion, overexpression of cyclin B1 and TK1 is involved in the progression of ER-positive breast cancer suggesting that targeting their expression in high proliferative breast cancer could offer novel treatment of breast cancer. Using both markers in combination produced better stratification in terms of outcome than each one separately. Increased cellular proliferation occurs in some luminal cancers and appears to form a biological and clinically distinct subclass of ER-positive breast cancer patients.

## 6.3 The prognostic and biological significance of p27 and Bcl-2 expression in breast cancer and ER-positive subtype

### 6.3.1 Introduction

Cellular proliferation has a significant impact on ER-positive breast cancer biology. The cell cycle is regulated by many mechanisms and is affected by the interaction of multiple pathways that either enhance or delay its progression. In addition to its control by cyclins and cyclin dependant kinases and cell cycle regulators, many studies have highlighted the role of Bcl-2 in controlling the cell cycle independent of its antiapoptotic function by causing retarded entry into the cell cycle (Vairo et al., 2000, Huang et al., 1997). In Bcl-2 transgenic cells, delayed cycle entry correlated with increased expression of p27 (Vairo et al., 2000). Elevated Bcl-2 levels are associated with decreased proliferation and a favourable prognosis in many malignancies, including breast and colorectal cancer (Mazel et al., 1996). Further evidence that Bcl-2 can regulate cell cycle, was provided by Deng et el (Deng et al., 2003) who found that Bcl2 may regulate G1/ S transition by a novel signalling mechanism that couples regulation of intracellular reactive oxygen species (ROS) with p27 and Cdk2. Phosphorylation of Bcl2 may functionally link its antiapoptotic, cell cycle retardation, and antioxidant properties(Deng et al., 2003, Maddika et al., 2007). Also, Greider and co-workers suggested that one possible mechanism by which BCL2 can delay cell cycle entry may be the inhibition of C-MYC activity through the elevation of p27 (Greider et al., 2002). These observations have

also been found in other organs where Bcl-2 delays hepatocyte cell cycle progression during liver regeneration (Vail et al., 2002). Furthermore, Bcl-2-induced inhibition of hormonal dependent apoptosis was associated with an inhibition of Rb protein downregulation, a constant level of p21 protein, and a inhibition of cell cycle (Truchet et al., 2000).

### 6.3.2 p27 (kip1)

p27 is a cell cycle inhibitor involved in G1 arrest of cell cycle due to its potent inhibition of cyclin E and cyclin A-CDK2 complexes. In addition, it is a positive regulator of cyclin D-dependent kinases such as CDK4 (Han et al., 2003). Low expression of p27 and its degradation correlate with increased cellular proliferation and poor prognosis in many cancers including lung, breast, colon, ovary, oesophagus, thyroid and prostate cancer (Han et al., 2003). p27 gene transfection in malignant human brain tumour cells blocked cellular proliferation in G1 phase of the cell cycle (Chen et al., 1996). In addition, subcellular localization of p27 has an important role in p27 regulation. p27 needs to be translocated to the nucleus to function in G1 and its degradation is linked to nuclear export (Tomoda et al., 1999).

### 6.3.3 Bcl-2

Bcl-2 is an antiapoptotic protein that promotes cell survival, but also may block cellular proliferation, and contrary to expectation, its expression is associated with good prognosis oestrogen receptor (ER) positivity in breast cancer patients (Callagy et al., 2008).

Deregulation of normal programmed cell death mechanisms plays an important role in the pathogenesis of breast cancer by two major pathways. The intrinsic pathway (mitochondrial) involves changes in mitochondrial membrane permeability, release of cytochrome c, exposure of phosphatidylserine on the outer part of the plasma membrane, and loss of plasma membrane integrity. The other mechanism, the extrinsic pathway is dependent on extracellular signals including tissue necrosis factor-alpha (TNF), Fas ligand, and TNF-related ligand TRAIL. The intrinsic and extrinsic pathways activate caspases, which cleave DNA and catabolise the cytoskeleton (Zhivotovsky and Kroemer, 2004, Brown and Attardi, 2005, Youle and Strasser, 2008) **(Fig 6.4)**.

Bcl-2 inhibits most kinds of programmed cell death and provides a selective growth advantage to various cell types. This function is achieved by controlling the mitochondrial membrane permeability and suppressing apoptosis by inhibiting caspase activity either by preventing the release of cytochrome C from the mitochondria or by binding to the apoptosis-activating factor (APAF-1) (Youle and Strasser, 2008).

Supporting its clinical applications, Bcl-2 was included as one of a panel of sixteen genes whose expression can predict tumour recurrence in tamoxifen-treated node-negative breast cancer (Paik et al., 2004).

Many studies have demonstrated the relation between Bcl-2 and p27. Overexpression of Bcl-2 may lead to retardation of the G0/S transition by sustaining the expression level of cyclin-dependent kinase inhibitor p27 (Linette et al., 1996). Others have demonstrated that p27 may mediate the effects of Bcl-2 on cellular proliferation (Cheng et al., 2008).

In this study, we hypothesized that the interaction between Bcl-2 and p27 can significantly retard the cell proliferation and enhance the cell cycle arrest which could possibly reflect on patient prognosis and tumour biology.

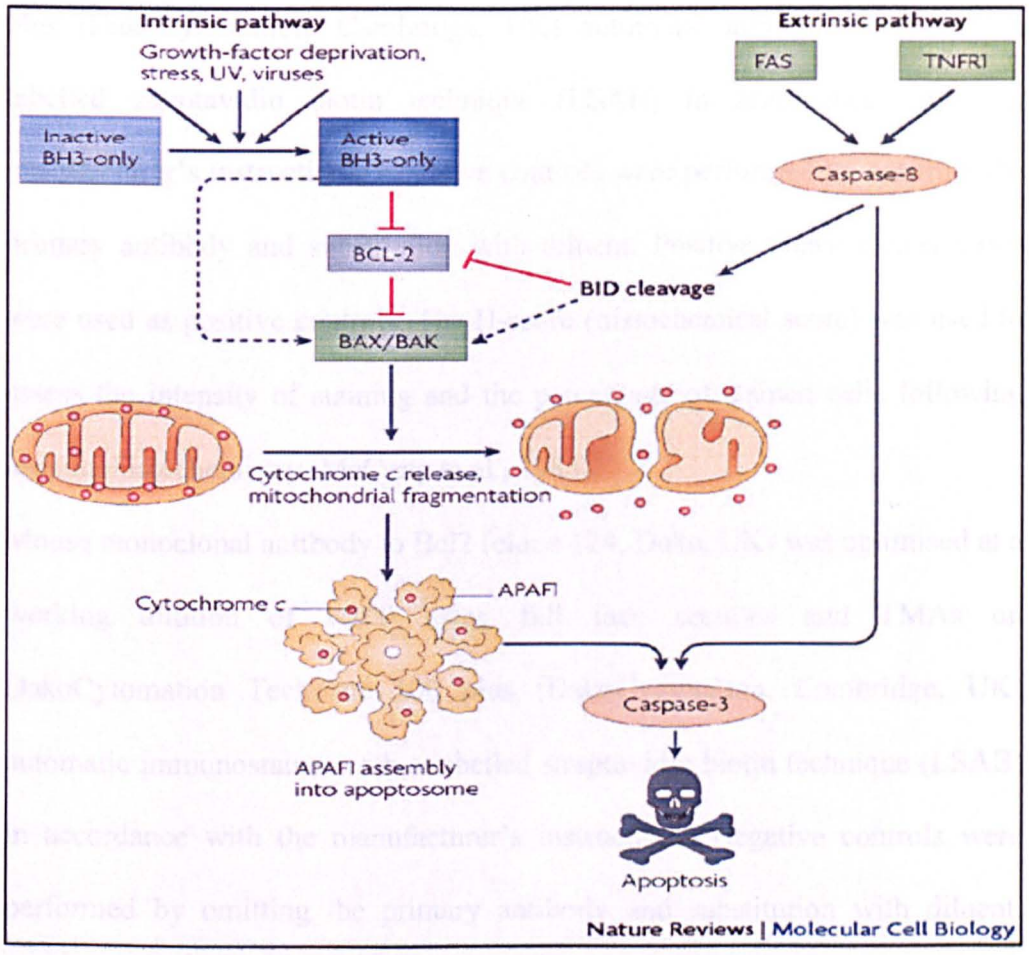


**Figure 6.4**: Intrinsic and extrinsic pathways of apoptosis.
Adapted from (Youle and Strasser, 2008)

### 6.3.4 Material and Methods

Tissue microarrays were prepared and the immunohistochemical staining was performed using the streptavidin-biotin complex method using DakoCytomation Techmate 500 plus (DakoCytomation, Cambridge, UK)

(general material and methods chapter). To unmask the antigens, the sections were microwaved in citrate buffer pH 6 for 23 minutes. p27 mouse monoclonal antibody (clone SX53G8, Dako, UK) was optimized at a working dilution of 1:40 using full face sections and TMAs on DakoCytomation Techmate 500 plus (DakoCytomation, Cambridge, UK) automatic immunostainer with a labelled streptavidin biotin technique (LSAB) in accordance with the manufacturer's instructions. Negative controls were performed by omitting the primary antibody and substitution with diluent. Positive breast cancer cases were used as positive controls. The H-score (histochemical score) was used to assess the intensity of staining and the percentage of stained cells following immunohistochemistry (McCarty et al., 1985).

Mouse monoclonal antibody to Bcl2 (clone 124, Dako, UK) was optimised at a working dilution of 1:100 using full face sections and TMAs on DakoCytomation Techmate 500 plus (DakoCytomation, Cambridge, UK) automatic immunostainer with a labelled streptavidin biotin technique (LSAB) in accordance with the manufacturer's instructions. Negative controls were performed by omitting the primary antibody and substitution with diluent. Positive breast cancer cases were used as positive controls.

Cut-offs were chosen using (≥10%) positive cells (Abdel-Fatah et al.).

### 6.3.5 p27 expression results

The optimization, scoring and staining process have been discussed in details in the general material and methods chapter.

The pattern of staining in breast carcinoma was nuclear **(Fig 6.5)**, with increased expression in the normal breast acini. After excluding the uninformative TMA cores from the study, 1029 tumours were available for assessment.

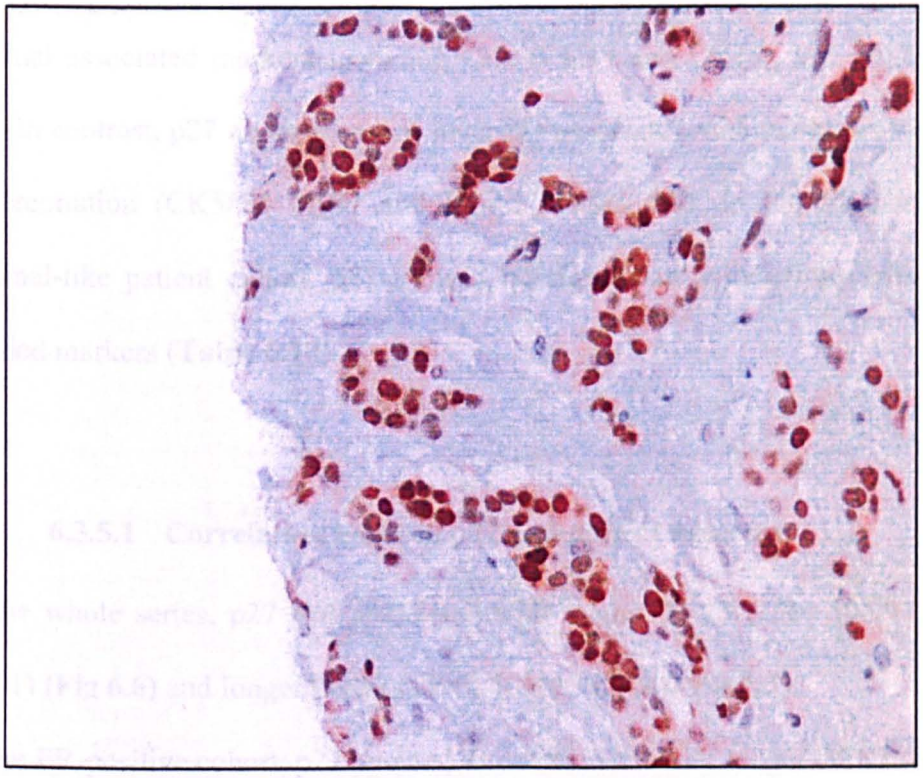

**Figure 6.5:** TMA core with positive p27 expression (x200)

#### 6.3.5.1 Correlation between p27 expression and other clinicopathological variables

p27 expression was associated with small tumour size (p=0.003), low grade tumours ($p$<0.001), good NPI group ($p$<0.001), lower mitotic index (p<0.001) and less frequent development of distant metastasis. No associations were found between p27 expression and LN stage or vascular invasion. A significant decrease of p27 expression was found in medullary cancer **(Table 6.7).**

In the ER-positive luminal-like patient cohort, p27 retained its associations with low grade tumours, good NPI group and lower mitotic index **(Table 6.8)**.

#### 6.3.5.2 Correlation between p27 expression and other biomarkers

In the whole series, we found significant positive associations between p27 and luminal associated markers including CKs (CK19 and CK18), ER, PgR and AR. In contrast, p27 expression was inversely associated with markers of basal differentiation (CK5/6), MIB1 and HER2 **(Table 6.9)**. In the ER-positive luminal-like patient cohort, p27 showed no significant associations with the studied markers **(Table 6.10)**.

#### 6.3.5.1 Correlation between p27 and patient outcome

In the whole series, p27 was associated with longer BCSS (LR= 10.991, $p$= 0.001) **(Fig 6.6)** and longer DMFI (LR=8.404, $p$=0.004) **(Fig 6.7)**.

In the ER-positive cohort, p27 retained its associations with longer BCSS (LR= 5.959 and $p$= 0.015) **(Fig 6.8)** and longer DMFI (LR=4.725 and $p$=0.030) **(Fig 6.9)**.

**Table 6.7**: Relation of p27 expression to other clinicopathological variables in the whole series

| Variable | p27 Expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | High | Total | | |
| **Patients' age** | | | | 3.350 | 0.341 |
| <40 | 58(79.5) | 15(20.5) | 73 | | |
| 40-50 | 214(69.7) | 93(30.3) | 307 | | |
| 51-60 | 241(70.5) | 101(29.5) | 342 | | |
| >60 | 211(68.7) | 96(31.3) | 307 | | |
| **Tumour size** | | | | 8.989 | 0.003 |
| **≤1.5 cm** | 211(64.1) | 118(35.9) | 329 | | |
| >1.5 cm | 513(73.3) | 187(26.7) | 700 | | |
| **Lymph node stage** | | | | 3.625 | 0.163 |
| 1(Negative) | 431(68.6) | 197(31.4) | 628 | | |
| 2(1-3 LN) | 220(71.4) | 88(28.6) | 308 | | |
| 3(>3 LN) | 71(78) | 20(22) | 92 | | |
| **Tumour Grade** | | | | 63.331 | <0.001 |
| 1 | 91(53.5) | 79(46.5) | 170 | | |
| 2 | 207(62) | 127(38) | 334 | | |
| 3 | 425(81.1) | 99(18.9) | 524 | | |
| **NPI** | | | | 45.731 | <0.001 |
| Good | 154(54.8) | 127(45.2) | 281 | | |
| Moderate | 434(75.3) | 142(24.7) | 576 | | |
| Poor | 136(79.1) | 36(20.9) | 172 | | |
| **DM** | | | | 8.042 | 0.005 |
| No | 474(67.6) | 227(32.4) | 701 | | |
| Positive | 248(76.3) | 77(23.7) | 325 | | |
| **Recurrence** | | | | 4.288 | 0.039 |
| No | 397(67.9) | 188(32.1) | 585 | | |
| Positive | 327(73.8) | 116(26.2) | 443 | | |
| **VI** | | | | 0.776 | 0.678 |
| No | 393(69.3) | 174(30.7) | 567 | | |
| Probable | 86(71.7) | 34(28.3) | 120 | | |
| Definite | 243(71.9) | 95(28.1) | 338 | | |
| **Histologic tumour type** | | | | 50.402 | <0.001 |
| Ductal/NST | 480(76.8) | 145(23.2) | 625 | | |
| Lobular | 55(58.5) | 39(41.5) | 94 | | |
| Tubular and Tubular mixed | 110(55.3) | 89(44.7) | 199 | | |
| Medullary | 28(93.3) | 2(6.7) | 30 | | |
| Other special types* | 13(59.1) | 9(40.9) | 22 | | |
| Mixed** | 38(64.4) | 21(35.6) | 59 | | |
| **Mitosis** | | | | 66.247 | <0.001 |
| 1 | 178(54.8) | 147(45.2) | 325 | | |
| 2 | 117(66.5) | 59(33.5) | 176 | | |
| 3 | 398(81.2) | 92(18.8) | 490 | | |
| **Menopausal status** | | | | 0.026 | 0.889 |
| Premenopausal | 281(70.1) | 120(29.9) | 401 | | |
| Postmenopausal | 443(70.5) | 185(29.5) | 628 | | |

*Includes Mucoid, invasive cribriform and invasive papillary carcinoma, ** Include ductal/NST mixed with lobular or special type

**Table 6.8**: Relation of p27 expression to other clinicopathological variables in the ER-positive cohort

| Variable | p27 Expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | High | Total | | |
| **Patients' age** | | | | 0.305 | 0.959 |
| <40 | 20(64.5) | 11(35.5) | 31 | | |
| 40-50 | 120(60.9) | 77(39.1) | 197 | | |
| 51-60 | 151(63.2) | 88(36.8) | 239 | | |
| >60 | 142(62.3) | 86(37.7) | 228 | | |
| **Tumour size** | | | | 2.343 | 0.138 |
| **≤1.5 cm** | 139(58.4) | 99(41.6) | 238 | | |
| >1.5 cm | 294(64.3) | 163(35.7) | 457 | | |
| **Lymph node stage** | | | | 4.566 | 0.102 |
| 1(Negative) | 248(59.2) | 171(40.8) | 419 | | |
| 2(1-3 LN) | 144(65.8) | 75(34.2) | 219 | | |
| 3(>3 LN) | 39(70.9) | 16(29.1) | 55 | | |
| **Tumour Grade** | | | | 14.465 | 0.001 |
| 1 | 80(53) | 71(47) | 151 | | |
| 2 | 172(59.5) | 117(40.5) | 289 | | |
| 3 | 180(70.9) | 74(29.1) | 254 | | |
| **NPI** | | | | 13.498 | 0.001 |
| Poor | 132(53.7) | 114(46.3) | 246 | | |
| Moderate | 231(65.6) | 121(34.4) | 352 | | |
| Good | 70(72.2) | 27(27.8) | 97 | | |
| **DM** | | | | 3.761 | 0.060 |
| No | 291(60) | 194(40) | 485 | | |
| Positive | 141(67.8) | 67(32.2) | 208 | | |
| **Recurrence** | | | | 1.907 | 0.177 |
| No | 244(60.2) | 161(39.8) | 405 | | |
| Positive | 189(65.4) | 100(34.6) | 289 | | |
| **VI** | | | | 2.121 | 0.346 |
| No | 222(60) | 148(40) | 370 | | |
| Probable | 64(66.7) | 32(33.3) | 96 | | |
| Definite | 146(64.6) | 80(35.4) | 226 | | |
| **Histologic tumour type** | | | | 12.906 | 0.024 |
| Ductal/NST | 241(67.1) | 118(32.9) | 359 | | |
| Lobular | 50(60.2) | 33(39.8) | 83 | | |
| Tubular and Tubular mixed | 96(53.6) | 83(46.4) | 184 | | |
| Medullary | 4(100) | 0(0) | 4 | | |
| Other special types | 8(50) | 8(50) | 16 | | |
| Mixed | 34(63) | 20(37) | 54 | | |
| **Mitosis** | | | | 16.820 | <0.001 |
| 1 | 153(53.3) | 134(46.7) | 287 | | |
| 2 | 92(63) | 54(37) | 146 | | |
| 3 | 165(70.8) | 68(29.2) | 233 | | |
| **Menopausal status** | | | | 1.796 | 0.189 |
| Premenopausal | 142(58.9) | 99(41.1) | 241 | | |
| Postmenopausal | 291(64.1) | 163(35.9) | 454 | | |

**Table 6.9**: Relation of p27 expression to other biomarkers in the whole series

| Variable | p27 Expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | High | Total | | |
| **CK5/6** | | | | 12.316 | <0.001 |
| Negative | 555(67.8) | 264(32.2) | 819 | | |
| Positive | 142(81.1) | 33(18.9) | 175 | | |
| **CK14** | | | | 4.109 | 0.045 |
| Negative | 577(69) | 259(31) | 836 | | |
| Positive | 107(77.5) | 31(22.5) | 138 | | |
| **CK18** | | | | 23.522 | <0.001 |
| Negative | 114(88.4) | 15(11.6) | 129 | | |
| Positive | 525(67.3) | 255(32.7) | 780 | | |
| **CK19** | | | | 10.629 | <0.001 |
| Negative | 76(85.4) | 13(14.6) | 89 | | |
| Positive | 621(68.8) | 281(31.2) | 902 | | |
| **ER** | | | | 74.483 | <0.001 |
| Negative | 251(90.3) | 27(9.7) | 278 | | |
| Positive | 433(62.3) | 262(37.7) | 695 | | |
| **PgR** | | | | 58.170 | <0.001 |
| Negative | 360(82.6) | 76(17.4) | 436 | | |
| Positive | 320(60) | 213(40) | 533 | | |
| **p53** | | | | 8.460 | 0.004 |
| Negative | 474(67.4) | 229(32.6) | 703 | | |
| Positive | 210(76.9) | 63(23.1) | 273 | | |
| **AR** | | | | 23.144 | <0.001 |
| Negative | 282(79.2) | 74(20.8) | 356 | | |
| Positive | 366(64.3) | 203(35.7) | 569 | | |
| **MIB1** | | | | 9.353 | 0.003 |
| low | 138(63.9) | 78(36.1) | 216 | | |
| High | 429(74.9) | 144(25.1) | 573 | | |
| **P-cadherin** | | | | 9.906 | 0.002 |
| Negative | 234(64.5) | 129(35.5) | 363 | | |
| Positive | 338(74.6) | 115(25.4) | 453 | | |
| **E-cadherin** | | | | 0.320 | 0.616 |
| Negative | 267(70.8) | 110(29.2) | 377 | | |
| Positive | 414(69.1) | 185(30.2) | 599 | | |
| **HER2** | | | | 8.274 | 0.004 |
| Negative | 587(68.6) | 269(31.4) | 856 | | |
| Positive | 106(80.9) | 25(19.1) | 131 | | |
| **EGFR** | | | | 0.818 | 0.406 |
| Negative | 467(68.7) | 211(31.1) | 678 | | |
| Positive | 126(72.4) | 48(27.6) | 174 | | |

**Table 6.10**: Relation of p27 expression to other biomarkers in the ER-positive cohort

| Variable | p27 expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Low | High | Total | | |
| **CK5/6** | | | | 0.371 | 0.548 |
| Negative | 394(62.3) | 238(37.7) | 632 | | |
| Positive | 29(58) | 21(42) | 50 | | |
| **CK14** | | | | 0.037 | 0.889 |
| Negative | 380(62.3) | 230(37.7) | 610 | | |
| Positive | 36(61) | 23(39) | 59 | | |
| **CK18** | | | | 1.946 | 0.230 |
| Negative | 21(75) | 7(25) | 28 | | |
| Positive | 381(62) | 234(38) | 615 | | |
| **CK19** | | | | 3.022 | 0.102 |
| Negative | 26(76.5) | 8(23.5) | 34 | | |
| Positive | 402(61.7) | 250(38.3) | 652 | | |
| **PgR** | | | | 3.968 | 0.054 |
| Negative | 115(68.9) | 52(31.1) | 167 | | |
| Positive | 311(60.3) | 205(39.7) | 516 | | |
| **AR** | | | | 1.054 | 0.354 |
| Negative | 109(65.7) | 57(34.3) | 166 | | |
| Positive | 298(61.2) | 189(38.8) | 487 | | |
| **p53** | | | | 0.270 | 0.684 |
| Negative | 343(61.8) | 212(38.2) | 555 | | |
| Positive | 81(64.3) | 45(35.7) | 126 | | |
| **MIB1** | | | | 1.950 | 0.182 |
| low | 110(60.8) | 71(39.2) | 181 | | |
| High | 240(66.9) | 119(33.1) | 359 | | |
| **E-cadherin** | | | | 0.083 | 0.806 |
| Negative | 158(62.9) | 93(37.1) | 251 | | |
| Positive | 269(61.8) | 166(38.2) | 435 | | |
| **P-cadherin** | | | | 0.178 | 0.730 |
| Negative | 211(63.4) | 122(36.6) | 333 | | |
| Positive | 156(61.7) | 97(38.3) | 253 | | |
| **HER2** | | | | 1.721 | 0.242 |
| Negative | 384(61.3) | 242(38.7) | 626 | | |
| Positive | 38(70.4) | 16(29.6) | 54 | | |
| **EGFR** | | | | 3.265 | 0.079 |
| Negative | 333(63.8) | 189(36.2) | 522 | | |
| Positive | 49(53.8) | 42(46.2) | 91 | | |

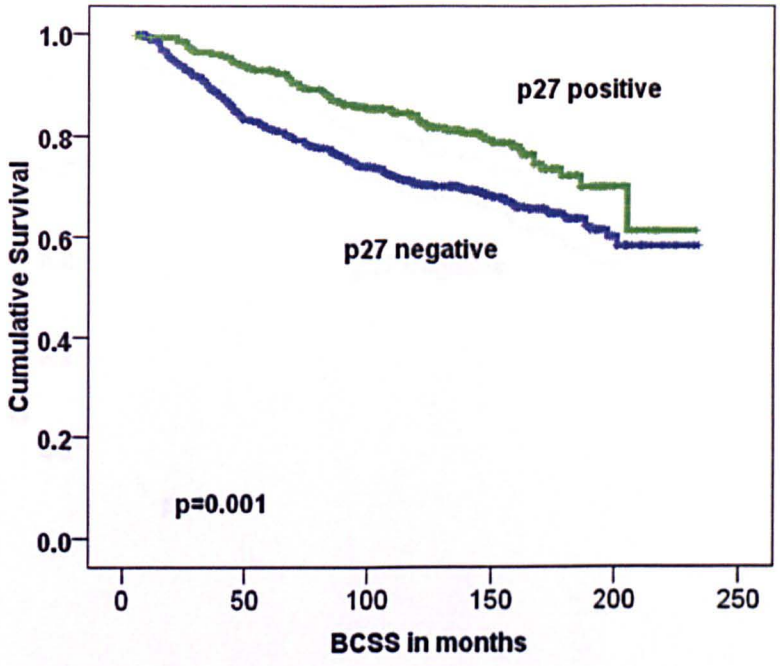


**Figure 6.6:** Kaplan Meier plot of p27 expression in relation to BCSS in the whole series

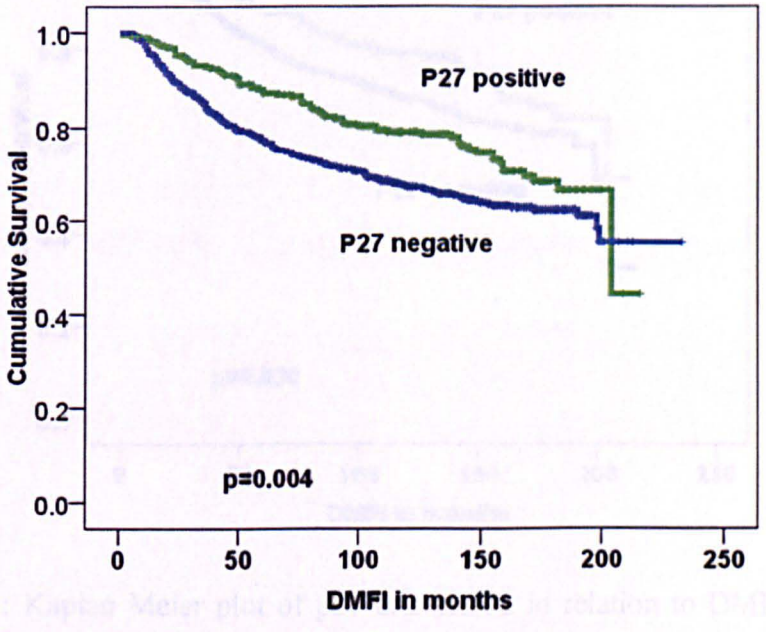


**Figure 6.7:** Kaplan Meier plot of p27 expression in relation to DMFI in the whole series

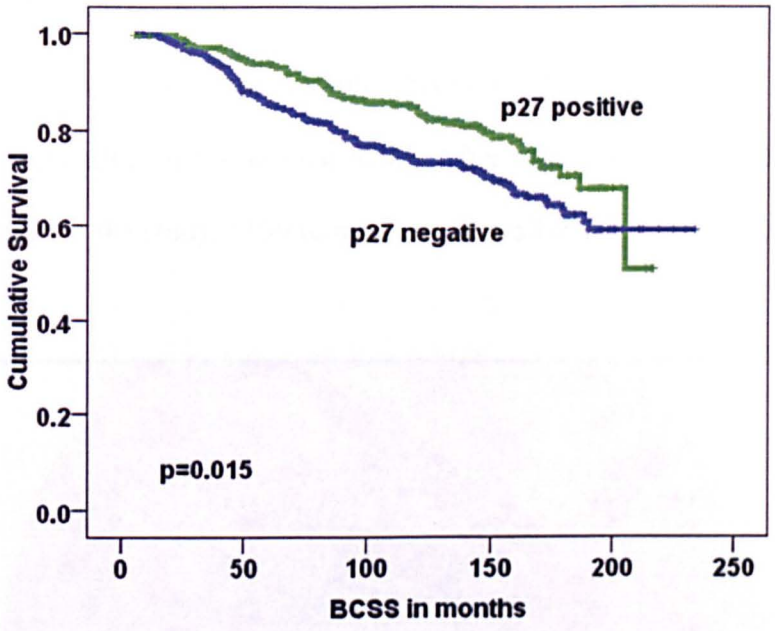


**Figure 6.8** Kaplan Meier plot of p27 expression in relation to BCSS in the ER-positive luminal-like cohort

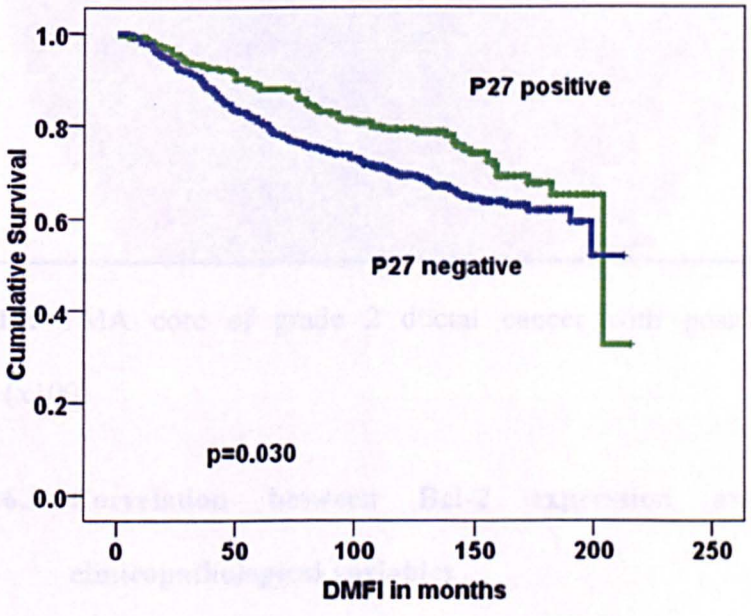


**Figure 6.9:** Kaplan Meier plot of p27 expression in relation to DMFI in the ER-positive luminal-like cohort

### 6.3.6 Bcl-2 expression results

The pattern of staining in breast carcinoma was cytoplasmic **(Fig 6.10)**, with increased expression in the normal acini. After excluding the uninformative TMA cores from the study, 1190 tumours were available for assessment.

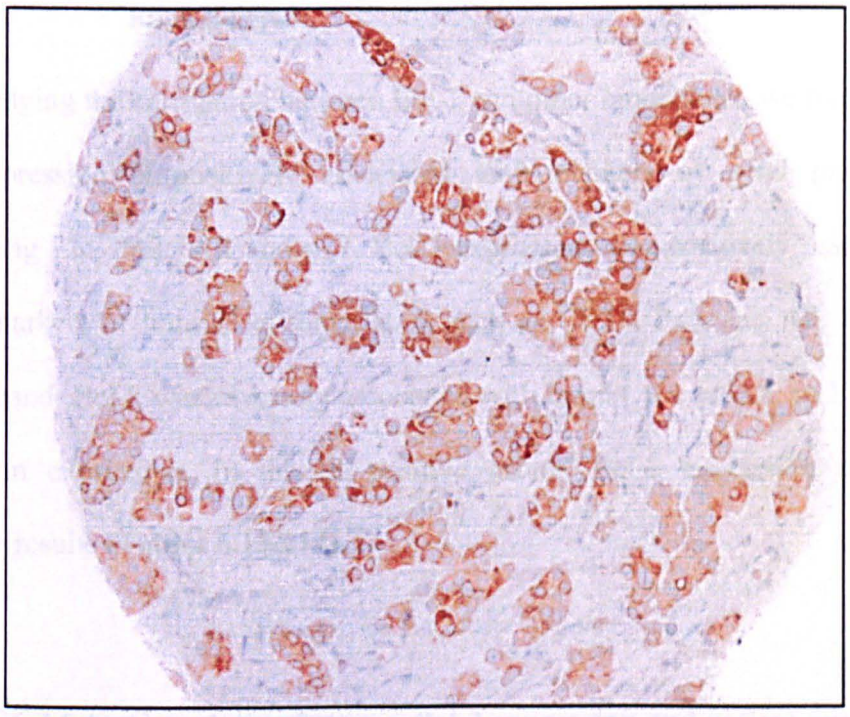

**Figure 6.10:** TMA core of grade 2 ductal cancer with positive Bcl-2 expression (x100)

#### 6.3.6.1 Correlation between Bcl-2 expression and other clinicopathological variables

In the whole patient series, Bcl-2 was associated with postmenopausal status and older age group, small tumour size, LN negativity, low grade tumours, lobular and tubular histologic type **(Table 6.11).**

In the ER-positive cohort, Bcl-2 was significantly associated with low tumour grade, low mitotic index, good NPI group, absence of DM and more frequent expression in lobular and lobular histological types with decreased expression in medullary cancer **(Table 6.12)**.

#### 6.3.6.2 Correlation between Bcl-2 expression and other biomarkers

On studying the correlation between Bcl-2 and other biomarkers, we found that its expression is positively associated with markers of good prognosis including ER, PgR, AR and p27. Bcl-2 expression was positively associated with markers of luminal differentiation such as CK18, PgR and AR. On the other hand, Bcl-2 was inversely associated with basal CKs, HER2, p53 and P-cadherin expression. In the ER-positive group, Bcl-2 expression showed similar results **(Tables 6.13&14)**.

#### 6.3.6.1 Correlation between Bcl-2 expression and patient outcome

In the whole series, Bcl-2 was associated with longer BCSS (LR= 32.911 and $p< 0.001$) **(Fig 6.11)** and longer DMFI (LR=25.150 and $p< 0.001$) **(Fig 6.12)**.
In the ER-positive breast cancer, Bcl-2 retained its associations with longer BCSS (LR= 12.220 and $p<0.001$) **(Fig 6.13)** and longer DMFI (LR=12.167 and $p< 0.001$) **(Fig 6.14)**.

**Table 6.11:** Relation of Bcl-2 expression to other clinicopathological variables in the whole series

| Variable | Bcl-2 Expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Negative | Positive | Total | | |
| **Patients' age** | | | | 29.095 | <0.001 |
| <40 | 49(55.7) | 39(44.3) | 88 | | |
| 40-50 | 93(26.9) | 253(71.3) | 253 | | |
| 51-60 | 128(31.4) | 280(68.6) | 408 | | |
| >60 | 98(28.2) | 249(71.8) | 347 | | |
| **Tumour size** | | | | 18.729 | <0.001 |
| **≤1.5 cm** | 94(22.9) | 316(77.1) | 410 | | |
| >1.5 cm | 274(35.1) | 506(64.9) | 780 | | |
| **Lymph node stage** | | | | 14.299 | 0.001 |
| 1(Negative) | 229(30) | 535(70) | 764 | | |
| 2(1-3 LN) | 93(28.4) | 235(71.6) | 328 | | |
| 3(>3 LN) | 46(47.9) | 50(52.1) | 96 | | |
| **Tumour Grade** | | | | 183.9 | <0.001 |
| 1 | 20(8.5) | 214(91.5) | 234 | | |
| 2 | 62(16.5) | 314(83.5) | 376 | | |
| 3 | 286(49.4) | 293(50.6) | 579 | | |
| **NPI** | | | | 107.3 | <0.001 |
| Good | 38(10.6) | 322(89.4) | 360 | | |
| Moderate | 246(37.6) | 409(62.4) | 655 | | |
| Poor | 84(48) | 91(52) | 175 | | |
| **DM** | | | | 26.429 | <0.001 |
| No | 225(26.5) | 624(73.5) | 849 | | |
| Positive | 139(41.9) | 193(58.1) | 332 | | |
| **Recurrence** | | | | 17.746 | <0.001 |
| No | 187(26.3) | 525(73.7) | 712 | | |
| Positive | 174(37.4) | 285(62.1) | 459 | | |
| **VI** | | | | 2.165 | 0.339 |
| No | 199(29.4) | 478(70.6) | 677 | | |
| Probable | 43(30.9) | 96(69.1) | 139 | | |
| Definite | 126(33.8) | 247(66.2) | 373 | | |
| **Histologic tumour type** | | | | 130.9 | <0.001 |
| Ductal/NST | 282(41.2) | 402(58.8) | 684 | | |
| Lobular | 27(22.3) | 94(77.7) | 121 | | |
| Tubular and Tubular mixed | 22(8.4) | 241(91.6) | 263 | | |
| Medullary | 21(70) | 9(30) | 30 | | |
| Other special types* | 6(24) | 19(76) | 25 | | |
| Mixed** | 10(14.9) | 57(85.1) | 67 | | |
| **Mitosis** | | | | 186.5 | <0.001 |
| 1 | 50(11.8) | 373(88.2) | 423 | | |
| 2 | 40(19.6) | 164(80.4) | 204 | | |
| 3 | 269(51.4) | 254(48.6) | 523 | | |
| **Menopausal status** | | | | 1.291 | 0.256 |
| Premenopausal | 149(32.9) | 304(67.1) | 453 | | |
| Postmenopausal | 219(29.8) | 517(70.2) | 736 | | |

**Table 6.12**: Relation of Bcl-2 expression to other biomarkers in the ER-positive cohort

| Variable | Bcl-2 Expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Negative | Positive | Total | | |
| **Patients' age** | | | | 4.744 | 0.191 |
| <40 | 7(19.4) | 29(80.6) | 36 | | |
| 40-50 | 24(10.7) | 201(89.3) | 225 | | |
| 51-60 | 49(16.7) | 244(83.3) | 293 | | |
| >60 | 35(13.6) | 223(86.4) | 258 | | |
| **Tumour size** | | | | 4.929 | 0.026 |
| **≤1.5 cm** | 33(10.7) | 276(89.3) | 309 | | |
| >1.5 cm | 82(16.3) | 422(83.7) | 504 | | |
| **Lymph node stage** | | | | 6.443 | 0.040 |
| 1(Negative) | 71(13.7) | 447(86.3) | 518 | | |
| 2(1-3 LN) | 29(12.4) | 204(87.6) | 233 | | |
| 3(>3 LN) | 15(25) | 45(75) | 60 | | |
| **Tumour Grade** | | | | 20.305 | <0.001 |
| 1 | 14(6.8) | 193(93.2) | 207 | | |
| 2 | 43(13.1) | 286(86.9) | 329 | | |
| 3 | 58(21) | 218(79) | 276 | | |
| **NPI** | | | | 11.870 | 0.003 |
| Poor | 30(9.4) | 290(90.6) | 320 | | |
| Moderate | 63(16.1) | 328(83.9) | 391 | | |
| Good | 22(21.6) | 80(78.4) | 102 | | |
| **DM** | | | | 9.061 | 0.004 |
| No | 172(12) | 526(88) | 598 | | |
| Positive | 43 (20.5) | 167(79.5) | 210 | | |
| **Recurrence** | | | | 6.283 | 0.016 |
| No | 60(11.9) | 443(88.1) | 503 | | |
| Positive | 55(18.3) | 245(81.7) | 300 | | |
| **VI** | | | | 4.685 | 0.096 |
| No | 55(12.4) | 390(87.6) | 445 | | |
| Probable | 18(16.5) | 91(83.5) | 109 | | |
| Definite | 42(16.3) | 216(83.7) | 258 | | |
| **Histologic tumour type** | | | | 23.037 | <0.001 |
| Ductal/NST | 69(17.7) | 320(82.3) | 389 | | |
| Lobular | 22(20.8) | 84(79.2) | 106 | | |
| Tubular and Tubular mixed | 15(6.3) | 222(93.7) | 237 | | |
| Medullary | 1(50) | 1(50) | 2 | | |
| Other special types* | 2(10) | 18(90) | 20 | | |
| Mixed** | 6(10.2) | 53 (89.8) | 59 | | |
| **Mitosis** | | | | 23.349 | <0.001 |
| 1 | 37(9.9) | 336(90.1) | 373 | | |
| 2 | 18(10.8) | 149(89.2) | 167 | | |
| 3 | 56(23.2) | 185(76.8) | 241 | | |
| **Menopausal status** | | | | 4.909 | 0.032 |
| Premenopausal | 28(10.3) | 243(89.7) | 271 | | |
| Postmenopausal | 87(16.1) | 454(83.9) | 251 | | |

*Includes Mucoid, invasive cribriform and invasive papillary carcinoma, ** Include ductal/NST mixed with lobular or special type

**Table 6.13**: Relation of Bcl-2 expression to other biomarkers in the whole series of breast cancer patients

| Variable | Bcl-2 Expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Negative | Positive | Total | | |
| **CK5/6** | | | | 105.4 | <0.001 |
| Negative | 235(24.7) | 716(75.3) | 951 | | |
| Positive | 121(62.1) | 74(37.9) | 195 | | |
| **CK14** | | | | 37.931 | <0.001 |
| Negative | 270(27.6) | 710(72.4) | 980 | | |
| Positive | 80(52.3) | 73(47.7) | 153 | | |
| **CK18** | | | | 99.302 | <0.001 |
| Negative | 95(66.4) | 48(33.6) | 143 | | |
| Positive | 226(25.1) | 676(74.9) | 902 | | |
| **CK19** | | | | 3.999 | 0.046 |
| Negative | 40(40) | 60(60) | 100 | | |
| Positive | 317(30.3) | 729(69.7) | 1046 | | |
| **ER** | | | | 384.3 | <0.001 |
| Negative | 234(74.3) | 81(25.7) | 315 | | |
| Positive | 115(14.1) | 698(85.9) | 813 | | |
| **PgR** | | | | 278.2 | <0.001 |
| Negative | 276(57.4) | 205(42.6) | 481 | | |
| Positive | 67(10.7) | 561(89.3) | 628 | | |
| **p53** | | | | 118.6 | <0.001 |
| Negative | 175(21.5) | 638(78.5) | 813 | | |
| Positive | 169(55.2) | 137(44.8) | 306 | | |
| **AR** | | | | 120.2 | <0.001 |
| Negative | 202(51.1) | 193(48.9) | 395 | | |
| Positive | 126(18.9) | 539(81.1) | 665 | | |
| **MIB1** | | | | 41.641 | <0.001 |
| low | 44(15.9) | 233(84.1) | 277 | | |
| High | 234(37.4) | 392(62.6) | 626 | | |
| **P-cadherin** | | | | 116.3 | <0.001 |
| Negative | 55(12.9) | 373(87.1) | 428 | | |
| Positive | 230(45.5) | 276(54.5) | 506 | | |
| **E-cadherin** | | | | 5.125 | 0.024 |
| Negative | 142(34.5) | 270(65.5) | 412 | | |
| Positive | 196(28) | 504(72) | 700 | | |
| **HER2** | | | | 89.083 | <0.001 |
| Negative | 258(26) | 735(74) | 993 | | |
| Positive | 90(65.7) | 47(34.3) | 137 | | |
| **EGFR** | | | | 36.275 | <0.001 |
| Negative | 206(26) | 585(74) | 791 | | |
| Positive | 92(48.4) | 98(51.6) | 190 | | |
| **p27** | | | | 47.890 | <0.001 |
| Negative | 209(41.8) | 291(58.2) | 500 | | |
| Positive | 37(15.9) | 196(84.1) | 233 | | |

**Table 6.14**: Relation of Bcl-2 expression to other biomarkers in the ER-positive cohort

| Variable | Bcl-2 Expression | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Negative | Positive | Total | | |
| **CK5/6** | | | | 8.601 | 0.006 |
| Negative | 98(13.2) | 642(86.8) | 740 | | |
| Positive | 16(27.1) | 43(72.9) | 59 | | |
| **CK14** | | | | 6.674 | 0.018 |
| Negative | 95(13) | 636(87) | 731 | | |
| Positive | 15(25) | 45(75) | 60 | | |
| **CK18** | | | | 9.174 | 0.007 |
| Negative | 9(33.3) | 18(66.7) | 27 | | |
| Positive | 93(12.9) | 626(87.1) | 719 | | |
| **CK19** | | | | 0.037 | 1.000 |
| Negative | 5(13.2) | 33(86.8) | 38 | | |
| Positive | 109(14.3) | 655(85.7) | 764 | | |
| **PgR** | | | | 36.495 | <0.001 |
| Negative | 51(27.1) | 137(72.9) | 188 | | |
| Positive | 58(9.7) | 541(90.3) | 599 | | |
| **AR** | | | | 11.841 | 0.001 |
| Negative | 38(22.2) | 133(77.8) | 171 | | |
| Positive | 69(11.8) | 516(88.2) | 585 | | |
| **p53** | | | | 18.779 | <0.001 |
| Negative | 75(11.5) | 579(88.5) | 654 | | |
| Positive | 34(25.8) | 98(74.2) | 132 | | |
| **MIB1** | | | | 3.936 | 0.047 |
| low | 24(10) | 215(90) | 239 | | |
| High | 60(15.6) | 324(84.4) | 384 | | |
| **P-cadherin** | | | | 15.906 | <0.001 |
| Negative | 36(9.2) | 355(90.8) | 391 | | |
| Positive | 56(19.9) | 255(80.1) | 281 | | |
| **E-cadherin** | | | | 4.109 | 0.051 |
| Negative | 47(17.4) | 223(82.6) | 270 | | |
| Positive | 63(12.1) | 456(87.9) | 519 | | |
| **HER2** | | | | 19.905 | <0.001 |
| Negative | 89(12.2) | 640(87.8) | 729 | | |
| Positive | 19(33.3) | 38(66.7) | 57 | | |
| **EGFR** | | | | 5.058 | 0.032 |
| Negative | 79(13) | 529(87) | 608 | | |
| Positive | 22(21.4) | 81(78.6) | 103 | | |
| **p27** | | | | 3.778 | 0.05 |
| Negative | 54(18.1) | 245(81.9) | 299 | | |
| Positive | 23(11.6) | 175(88.4) | 198 | | |

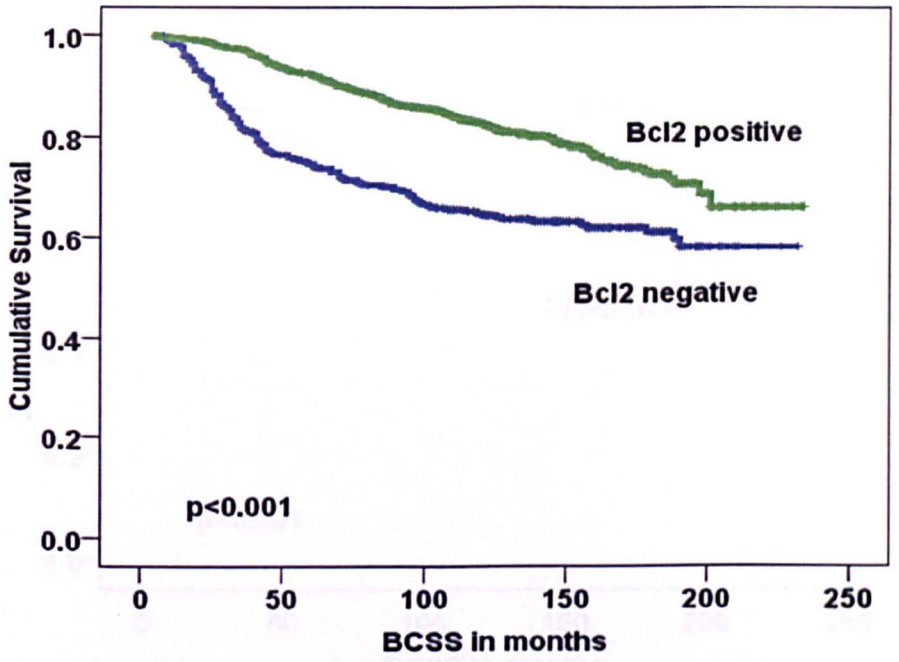


**Figure 6.11:** Kaplan Meier plot of Bcl-2 expression in relation to BCSS in the whole series.

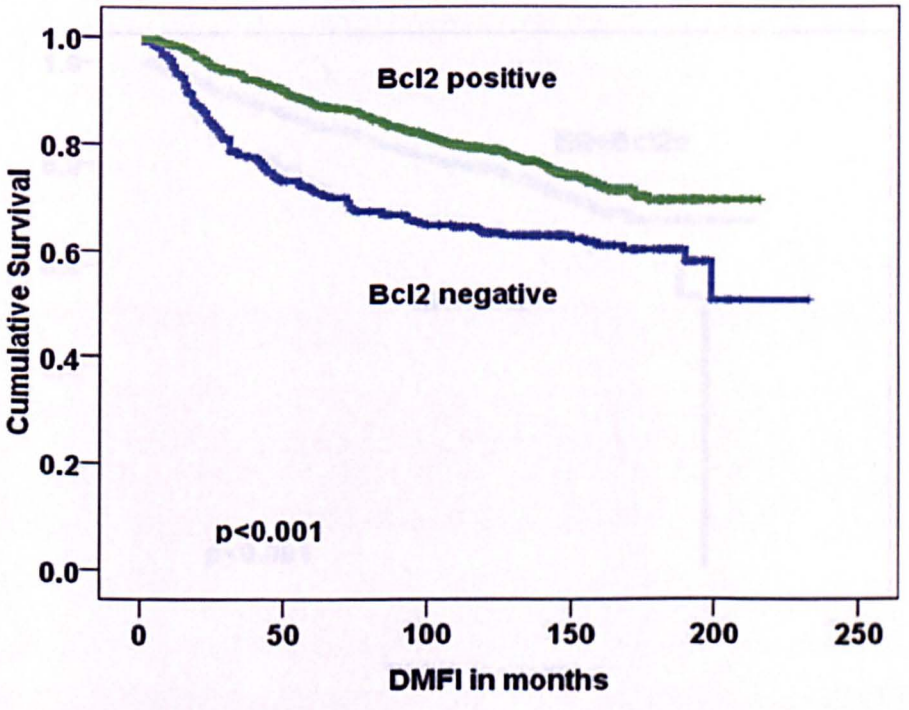


**Figure 6.12:** Kaplan Meier plot of Bcl-2 expression in relation to DMFI in the whole series

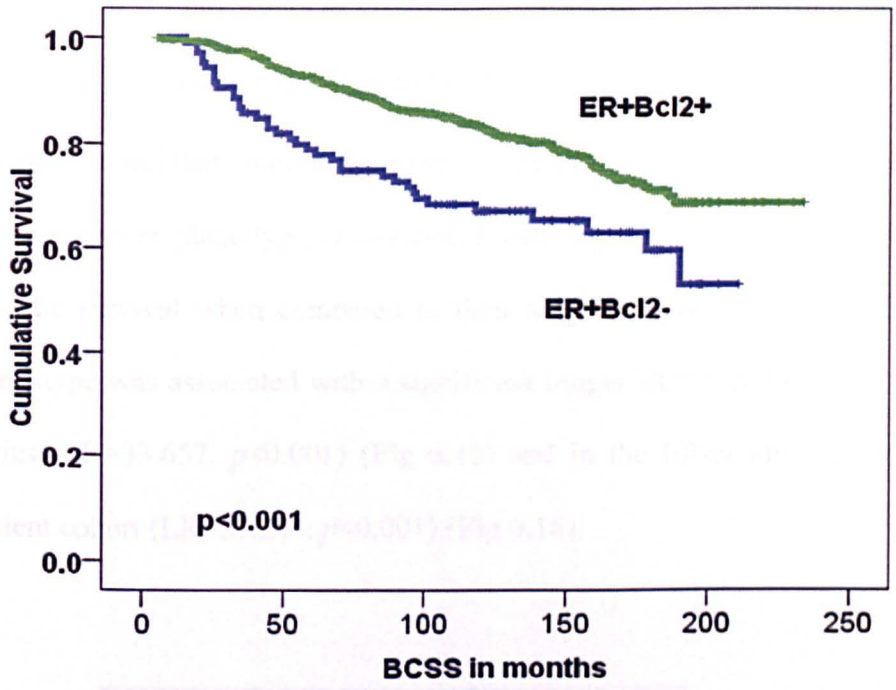


**Figure 6.13** Kaplan Meier plot of Bcl-2 expression in relation to BCSS in the ER-positive luminal-like cohort

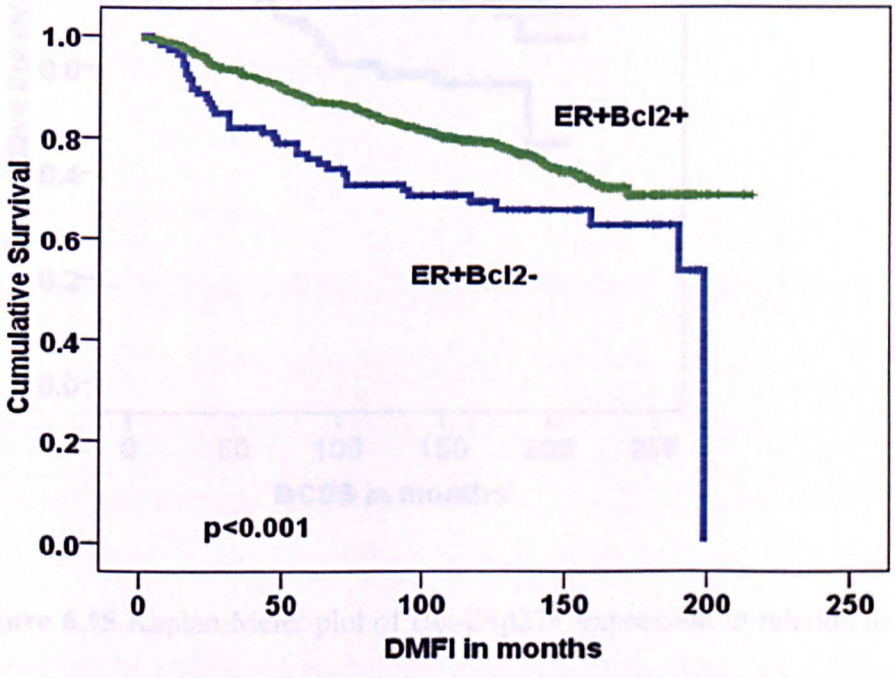


**Figure 6.14:** Kaplan Meier plot of Bcl-2 expression in relation to DMFI in the ER-positive luminal-like cohort

### 6.3.6.2 The effect of combined Bcl-2 and p27 expression on breast cancer spesific survival

We have found that combined positive expression of Bcl-2 and p27 in the Bcl-2+p27+ cancer phenotype is associated with significant longer breast cancer specific survival when compared to their single phenotype. ER+Bcl-2+p27+ phenotype was associated with a significant longer BCSS in the whole patient series (LR=33.657, $p<0.001$) **(Fig 6.15)** and in the ER-positive luminal-like patient cohort (LR=29.244, $p<0.001$) **(Fig 6.16)**.

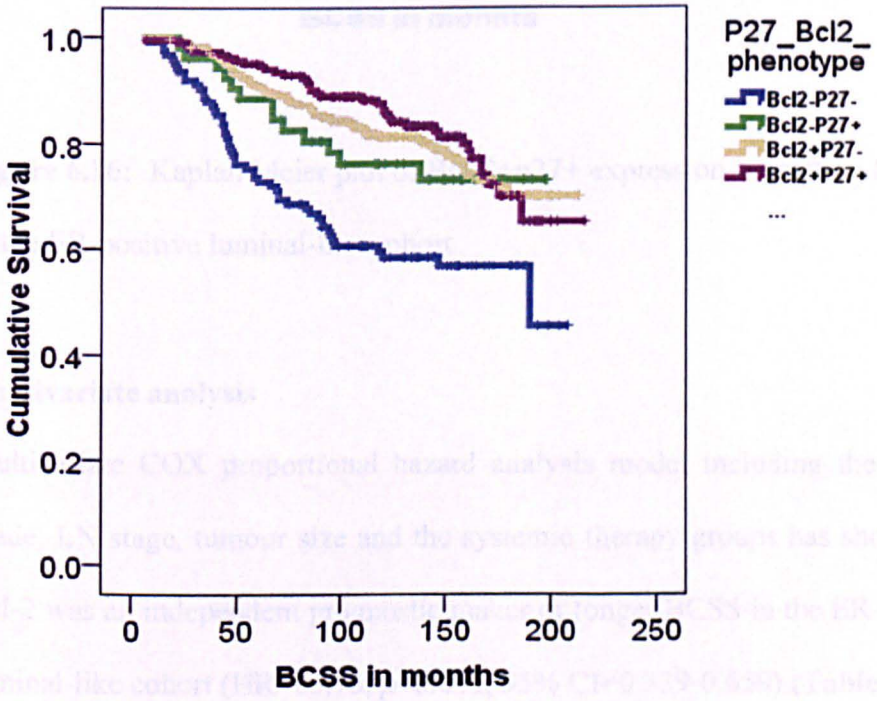


**Figure 6.15** Kaplan Meier plot of Bcl-2+p27+ expression in relation to BCSS in the whole patients series

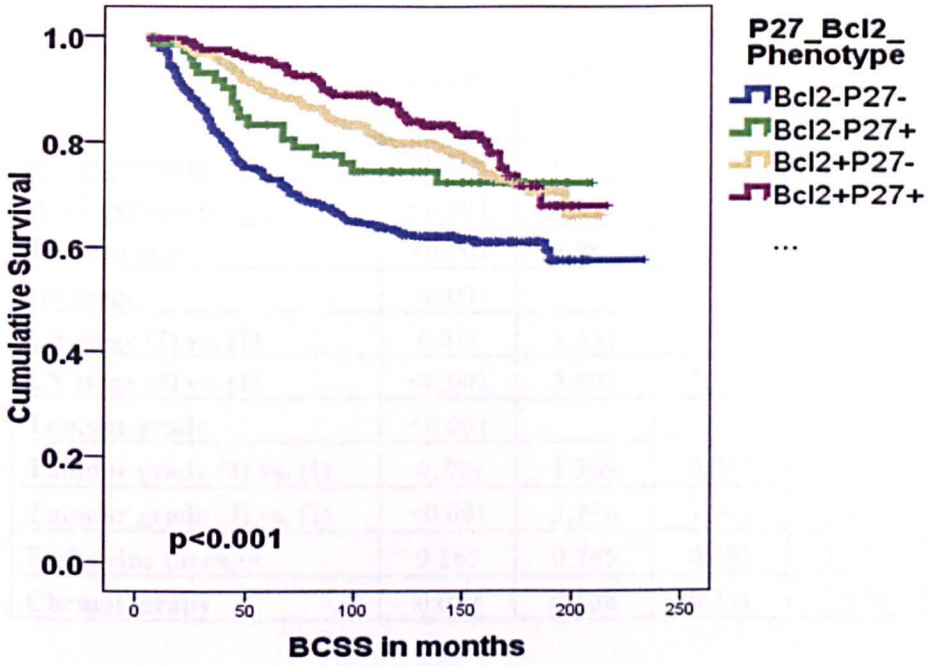


**Figure 6.16:** Kaplan Meier plot of Bcl-2+p27+ expression in relation to BCSS in the ER-positive luminal-like cohort

**Multivariate analysis**

Multivariate COX proportional hazard analysis model including the tumour grade, LN stage, tumour size and the systemic therapy groups has shown that Bcl-2 was an independent prognostic maker of longer BCSS in the ER-positive luminal-like cohort (HR=0.473, $p$<0.001, 95% CI=0.339-0.659) **(Table 6.15)**.

**Table 6.15:** COX multivariate analysis of predictors of BCSS in luminal-like cohort

| Variable | p value | HR | 95% CI | |
|---|---|---|---|---|
| | | | Lower | Upper |
| **p27 expression** | 0.335 | 0.841 | 0.591 | 1.196 |
| **Bcl-2 expression** | <0.001 | 0.473 | 0.339 | 0.659 |
| **Tumour size** | <0.001 | 1.961 | 1.367 | 2.813 |
| **LN stage** | <0.001 | | | |
| **LN stage (2) vs. (1)** | 0.011 | 1.671 | 1.124 | 2.483 |
| **LN stage (3) vs. (1)** | <0.001 | 3.002 | 1.835 | 4.911 |
| **Tumour grade** | <0.001 | | | |
| **Tumour grade (2) vs. (1)** | 0.239 | 1.369 | 0.811 | 2.310 |
| **Tumour grade (3) vs. (1)** | <0.001 | 3.296 | 1.911 | 5.684 |
| **Endocrine therapy** | 0.165 | 0.745 | 0.492 | 1.129 |
| **Chemotherapy** | 0.084 | 0.604 | 0.341 | 1.070 |

### 6.3.7 Discussion

Many authors have repeatedly implied a strong relationship between cellular proliferation and poor prognosis in breast cancer with particular emphasis on ER-positive luminal subtypes (Cheang et al., 2009).

The effect of Bcl-2 on tumour formation is complex and its high expression may either delay or increase tumour formation (Pietenpol et al., 1994). The oncogenic activity is due to its anti-apoptotic function while the growth inhibitory function has been attributed to its inhibition of cellular proliferation. In the ER-positive subgroup, we found that Bcl-2 was significantly associated with LN negativity, low grade, low mitotic index, good NPI group and absence of tumour recurrence. In addition, Bcl-2 was positively associated with markers of good prognosis including steroid receptors and CK18 and was inversely associated with proliferation as assessed by mitotic counts. Supporting this, *in vitro* experiments have revealed that high levels of Bcl-2 can result in dramatic growth inhibition in different cell types (Pietenpol et al., 1994) (Oreilly et al., 1996). These findings are supported also by the association of Bcl-2 with longer breast cancer specific survival as demonstrated by other investigators (Callagy et al., 2006).

To further support its growth inhibitory role in breast cancer, we have found a significant association between Bcl-2 and the cell cycle inhibitor p27. These results collectively, support that the dominant role of Bcl-2 in breast cancer is related to growth inhibition via decreasing cellular proliferation and inducing cell cycle delay.

In this study, we have shown that Bcl-2 and p27 are positively associated with luminal phenotype and inversely associated with mutated p53, basal CKs and EGF family members which provide more evidences for their biological role is characterising the luminal-like breast cancer subclass.

p27 (kip1) is an important negative regulator of cell cycle progression and plays an important role in the pathogenesis of many tumours including breast cancer. In breast cancer cells, the level of p27 (kip1) expression usually decreases during tumour development and progression. Low p27 expression appears to be associated with poor prognosis, especially among patients with steroid receptor positive tumours (Porter et al., 2006, Traub et al., 2006) in agreement with our findings.

Recent studies demonstrated that p27 may mediate the effects of Bcl-2 on cellular proliferation in tumour development (Cheng et al., 2008). We have found that combined positive expression of Bcl-2 and p27 in the Bcl-2+p27+ cancer phenotype is associated with significant longer breast cancer specific survival when compared to their single phenotype. So, we propose that the luminal phenotype expressing double markers is highly characterised with lower rate of proliferation and in a state of growth arrest which may explain the good prognosis associated with their combined expression.

In conclusion, Bcl-2 is associated with luminal phenotype and has a growth inhibitory function in breast cancer possibly via its effect on p27 expression. Bcl-2+p27+ phenotype is associated with good prognosis in luminal-like breast cancer.

# 7 The use of cluster analysis of protein expression to identify prognostic and biological ER positive breast cancer subclasses

## 7.1 Introduction

In common with other cancers, the development of breast cancer is a complex process involving genetic, epigenetic and environmental factors. There is now general acceptance that the histological criteria used for classifying breast cancer reflect molecular events occurring only at the cellular level. For this reason, recent studies have focused on the development of a 'molecular classification' system to profile the genetic and protein expression of an individuals' tumour (Perou et al., 2000, Sorlie et al., 2001). Subsequently, a number of different patient groups have been identified, with group membership reflecting similarities in the tumour biological characteristics.

Oestrogen receptor positive breast cancers form a large proportion of breast cancer patients and within this group; much effort is being applied to discover reliable markers that can be used in patient prognosis. Our study aimed at subclassifying ER-positive luminal-like cancers into prognostic patho-biological subgroups using their protein expression characteristics to identify the biological and behavioural characteristics seen in this heterogeneous group of tumours.

This might lead to a sub-classification of luminal breast cancer patients into discrete entities that represent clinically relevant groups and when possible, Identification of unique sets of genes whose expression best identify subgroups of luminal ER positive tumours.

An alternative approach to gene expression profiling is to use established laboratory method, such as immunohistochemistry on formalin fixed paraffin embedded clinical patient tumour samples with follow-up data. We have applied protein biomarker panels with known relevance to breast cancer, to large numbers of cases using tissue microarrays, exploring the existence and clinical significance of distinct ER-positive breast cancer classes. We studied these markers in patients with breast cancer treated by a uniform drug regimen so that treatment was not associated with variability in outcome.

## 7.2 Material and methods

### 7.2.1 Patient selections

In this analysis, we have used different clustering methods to analyze the protein expression data for identification of biological classes in the ER-positive patient subgroup. 583 immunohistochemically confirmed ER-positive cases with data available on the selected biomarkers (FOXA1, RERG, GATA3, TFF1, TFF3, XBP1, BEX1, CARM1, PELP1, CD71, FOXO3a, AGTR1, p27, Bcl-2, TK1, and cyclin B1) in addition to other 9 important markers that are available from the database (ER, PgR, MIB1, HER2, CK5/6, C-MYC, EGFR, CK18 and p53) and have been used previously in immunohistochemical definition of the breast cancer subtypes or has a strong relevance to breast cancer development, were used in the analysis.

Levels of immunohistochemical reactivity were determined by microscopic analysis using the modified H-score (values between 0 and 300), giving a semi quantitative assessment of both the intensity of staining and the percentage of

positive cells. For the intensity, a score of 0–3, corresponding to negative, weak, moderate and strong positivity, was recorded. For MIB1, TK1, FOXO3a and Bcl-2, we used the percentage of the positive cells normalised to a scale of 0-300 to prevent the bias that may result from including different scales in the same analysis. For RERG and XBP1, we rescored the available TMAs slides using the H-score.

K-nearest neighbor algorithm (KNN) was used to adapt for missing values (Troyanskaya et al., 2001) which provides a more robust and sensitive method for missing value estimation in comparison to other methods over the range of 1-20% missing values (Mehra et al., 2005). **Table 7.1** shows the biomarkers used in the analysis.

**Table 7.1:** Markers used in the clustering analysis

| Antibody | supplier | Cat number/clone | Dilution | Pretreatment |
|---|---|---|---|---|
| FOXA1 | Abcam | Ab40868/2F83 | 1:2000 | Microwave |
| TFF1 | Abcam | Ab17829 | 1:2000 | Microwave |
| CD71 | Abcam | Ab49517/10F11 | 1:30 | Microwave |
| PELP1 | Novus | NB100-1749 | 1:100 | No |
| CARM1 | Novus | NB100-1817 | 1:300 | Microwave |
| Bcl-2 | Dako | M0887/124 | 1:100 | Microwave |
| BEX1 | Abcam | Ab69032 | 1:3500 | Microwave |
| TK1 | Abcam | Ab57757 | 5µg/ml | Microwave |
| AGTR1 | Abcam | Ab9391 (1E10-1A9) | 1:100 | No |
| XBP1 | Novus | NB100-80861 | 0.5µg/ml | Microwave |
| Cyclin B1 | Abcam | Ab72 | 0.3µg/ml | Microwave |
| TFF3 | Abcam | Ab57752 | 3 µg/ml | Microwave |
| FOXO3a | Cell Signalling | 9467 | 1:50 | Microwave |
| RERG | Proteintech | 10687-1-AP | 1:20 | Microwave |
| p27 | Dako | SX53G8 | 1:40 | Microwave |
| GATA3 | Santa Cruz | sc-268/HG3-31 | 1:80 | Microwave |
| C-Myc | Abcam | Ab32/9A10 | 1:100 | Microwave |
| ER | Dako | 1D5 | 1:80 | Microwave |
| PgR | Dako | PgR636 | 1:100 | Microwave |
| EGFR | Novocastra | EGFR.113 | 1:10 | Microwave |
| p53 | Dako | DO7 | 1:50 | Microwave |
| Ki67 | Dako | MIB1 | 1:100 | Microwave |
| HER2 | Dako | cerbB-2 | 1:250 | No |
| CK5/6 | Boehringer | D5/16134 | 1:100 | Microwave |
| CK18 | Dako | DC10 | 1:50 | Microwave |

### 7.2.2 Clustering algorithms

#### 7.2.2.1 K-means clustering of the protien expression data

The K-means (KM) technique aims to partition the data into $K$ clusters so that the sum of squares from points to the assigned cluster centres is minimised. The algorithm repeatedly moves all cluster centres to the mean of their Voronoi sets which is the set of data points which are nearest to the cluster centre. K-means clustering is dependent on the initial cluster centres setting which, in turn, determines the initial cluster assignment. For this study, we used a fixed initialization obtained with hierarchical clustering. The number of clusters is an explicit input parameter to the K-means algorithm (Jain et al., 1999, Soria et al., 2010).

#### 7.2.2.2 Partitioning around medoids clustring of the protien expression data

The partitioning around medoids (PAM) algorithm is a technique which attempts to minimise the distance between points labelled to be in a cluster and a point designated as the centre of that cluster. In contrast to the K-means algorithm, PAM chooses data points as centres (medoids) and then assigns each point to its nearest medoid. A medoid is defined as the object within a cluster for which the average dissimilarity to all other objects in the cluster is minimal, i.e. it is the most centrally located data point in the given cluster. Dissimilarities are nonnegative numbers that are small and close to zero when two data points are near to each other and become large when the points are

very different usually, an Euclidean metric is used for calculating dissimilarities between observations.

The algorithm consists of two phases: the build phase in which an initial set of k representative medoids is selected and the swap phase in which a search is carried out to improve the choice of medoids (and hence the cluster allocations) (Jain et al., 1999, Soria et al., 2010).

Biplots are generated by firstly transforming the original data space using principal component analysis and then plotting the points at their projected position on axes of the first and second principal components.

All clustering statistical analysis was done in collaboration with Dr Daniele Soria (University of Nottingham-School of Computer Science) using R, a free software environment for statistical computing and graphics. The methodology was applied to a 583 ER-positive cases of breast cancer patients in order to obtain core classes in the ER-positive positve disease. Once these core classes were obtained, the clinical relevance of the corresponding patient groups were investigated by means of associations with related patient data.

**Consensus clustering**

The idea of combining and comparing the results of different clustering algorithms is particularly important in order to evaluate the stability of a proposed classification. Monti and colleagues (Monti et al., 2003) used a new methodology of class discovery and clustering validation tailored to the task of analysing gene expression data. The new methodology, termed 'consensus clustering', provides a method to represent the consensus across multiple

analyses of a clustering algorithm and to assess the stability of the discovered clusters (Monti et al., 2003).

#### 7.2.2.3 Validity indices

Two different algorithms were used for cluster analysis: the K-means and the partitioning around medoids methods. They were both run for between 2 and 20 clusters. Six validity indices were calculated and recorded to determine the best number of clusters. The indices are Calinski and Harabasz, Hartigan, Scott and Symons, Marriot, Trace W, and Trace W-1B. For each index, the number of groups to be considered was chosen according to the rules reported by (Dimitriadou et al., 2002).

## 7.3 Results

### 7.3.1 Optimal number of clusters

After application of the six validity indices of the kmeans **(Fig 7.1)** and PAM **(Fig 7.2)** clustering algorithms, three clusters were identified as the most appropriate number which could represent the true core classes of the data.

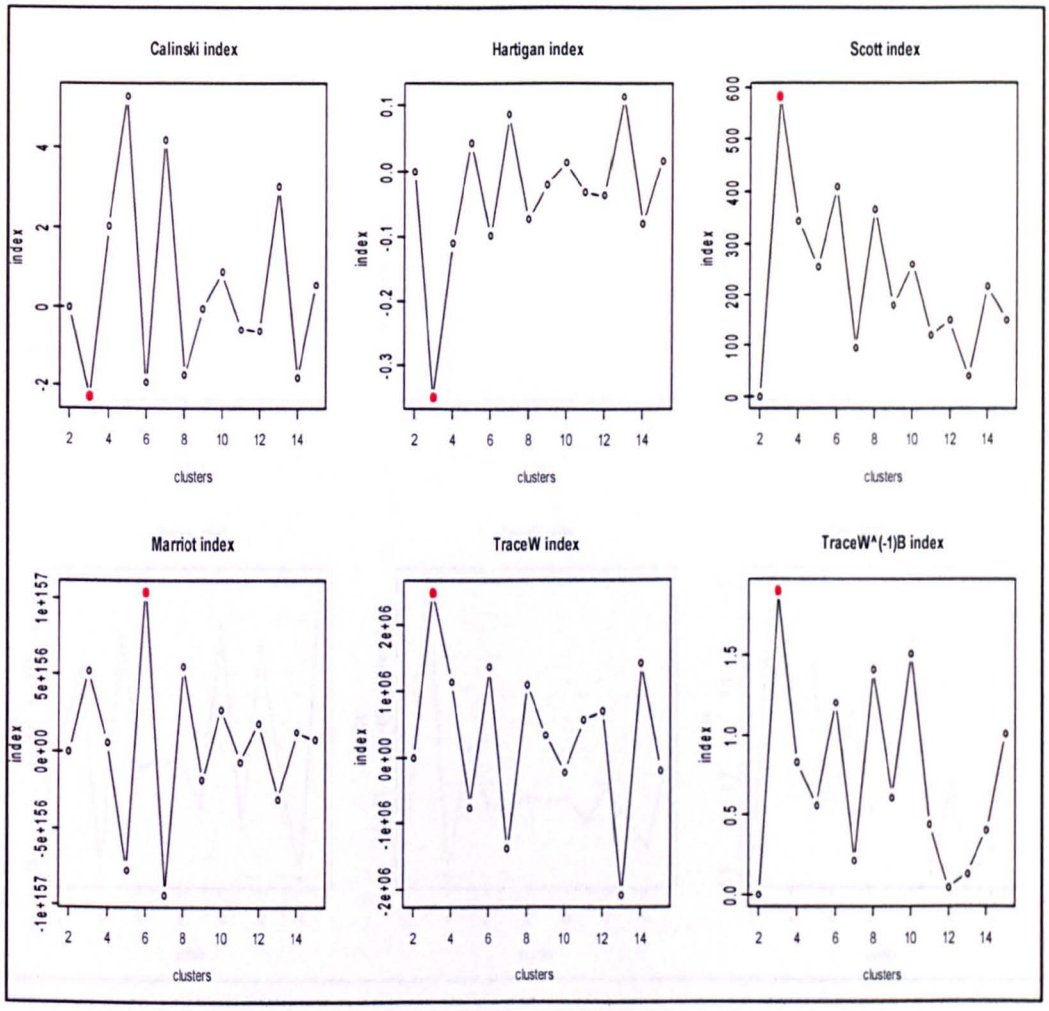


**Figure 7.1**: Kmeans validity indices

5 indices agreed on 3 clusters solution

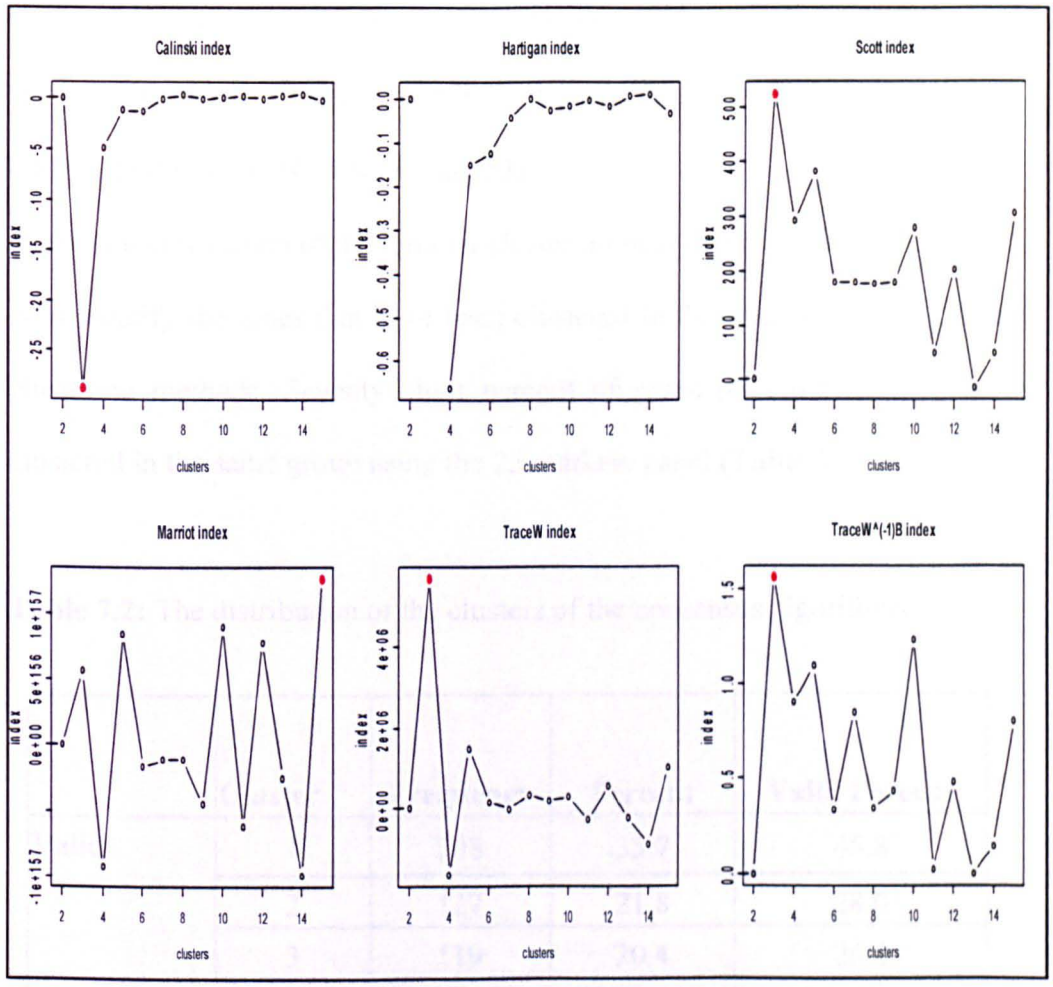


**Figure 7.2:** PAM validity indices

4 indices agreed on 3 clusters solution

### 7.3.2 Clustering the protein expression data

#### 7.3.2.1 Consensus cluster identification

Initially we have used 16 markers to cluster the 583 cases using the same methodology and we found that the optimal cluster number is three. In order to make sure that the number does not change when using a different set of biomarkers, we have added 9 important biomarkers which proved previously to

have a defining role in breast cancer subtypes characterisation and those with strong relevance to breast cancer biology including (ER, PgR, MIB1, HER2, CK5/6, EGFR, C-MYC, CK18 and p53).

Next to identification of the optimal cluster number using the different indices, is to identify the cases that have been clustered in the same group by the two clustering methods. Seventy eight percent of cases (454 out of 583) were clustered in the same group using the 25 markers panel **(Table 7.2)**.

**Table 7.2:** The distribution of the clusters of the consensus algorithms

| | **Cluster** | **Frequency** | **Percent** | **Valid Percent** |
|---|---|---|---|---|
| Valid | 1 | 208 | 35.7 | 45.8 |
| | 2 | 127 | 21.8 | 28.0 |
| | 3 | 119 | 20.4 | 26.2 |
| | Total | 454 | 77.9 | 100.0 |
| unclassified | | 129 | 22.1 | |
| **Total** | | 583 | 100.0 | |

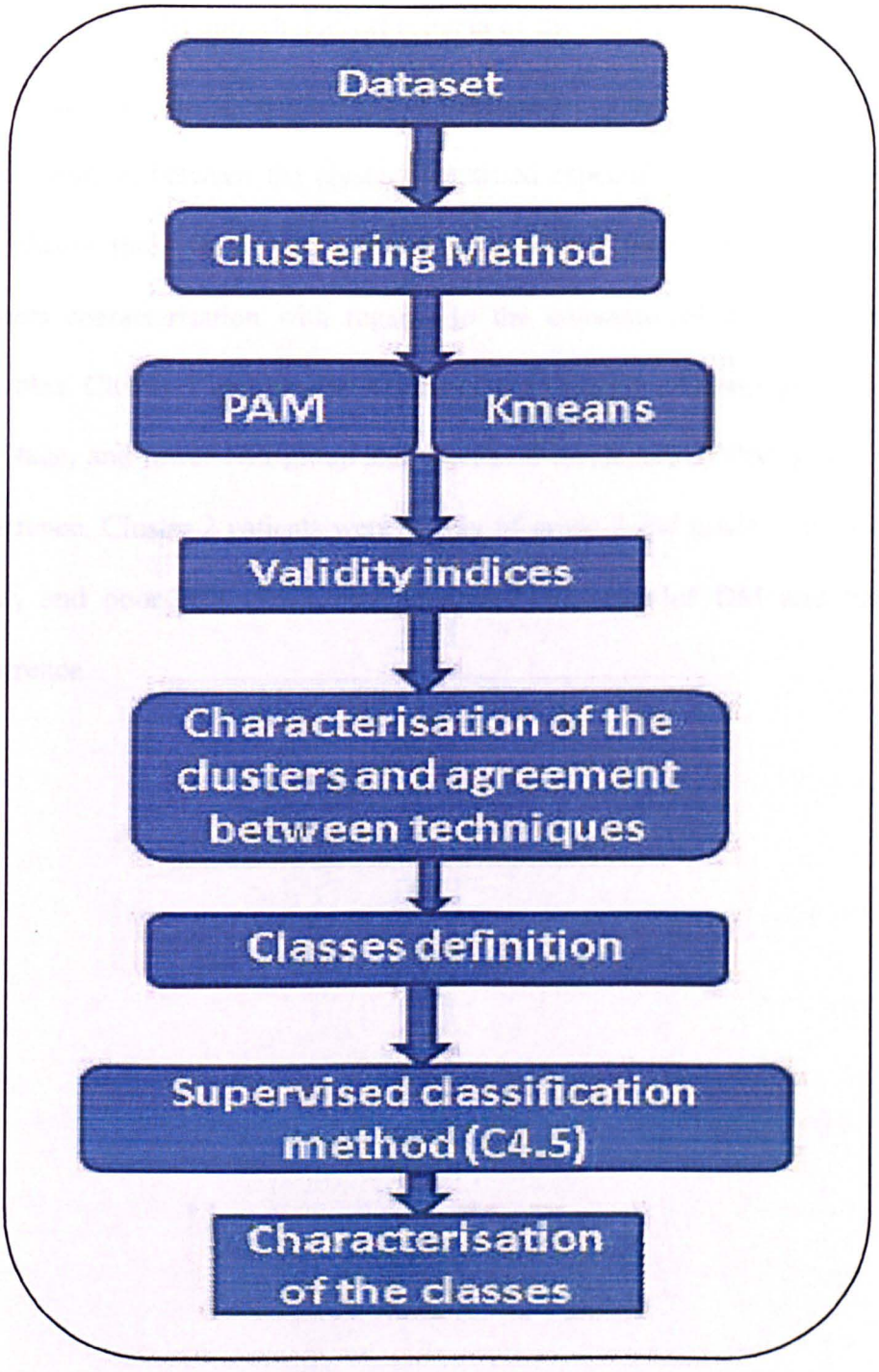


**Figure 7.3:** A diagram showing the flow of methods used in the clustering process

#### 7.3.2.2 Histopathological criteria of the clusters

Examination of the histopathologic characteristics of the clusters has shown a wide variation between the clusters identified especially cluster 2. Cluster 1 and cluster three were not significantly variable. **Table 7.3** summaries the clusters characterisation with regards to the conventional histopathological variables. Cluster 1 and cluster 3 patients were mainly of lower grade, lower LN stage, and lower NPI group and decreased incidences of DM and tumour recurrence. Cluster 2 patients were mostly of grade 2 and grade 3, higher LN stage, and poor NPI group with increased incidence of DM and tumour recurrence.

**Table 7.3:** Histopathological criteria of the three clusters identified

| Variable | Clusters | | | $\chi^2$ | *p*-value |
|---|---|---|---|---|---|
| | Cluster 1 | Cluster 2 | Cluster 3 | | |
| **Patients' age** | | | | 23.134 | 0.001 |
| <40 | 8(38.1) | 7(33.3) | 6(28.6) | | |
| 40-50 | 52(37.7) | 30(21.7) | 56(40.6) | | |
| 51-60 | 73(48) | 48(31.6) | 31(20.4) | | |
| >60 | 75(52.4) | 42(29.4) | 26(18.2) | | |
| **Tumour size** | | | | 7.998 | 0.018 |
| **≤1.5 cm** | 104(46.8) | 50(22.5) | 68(30.6) | | |
| >1.5 cm | 103(44.6) | 77(33.3) | 51(22.1) | | |
| **Lymph node stage** | | | | 12.144 | 0.016 |
| 1(Negative) | 122(46.9) | 63(24.2) | 75(28.8) | | |
| 2(1-3 LN) | 71(47) | 44(29.1) | 36(23.8) | | |
| 3(>3 LN) | 12(30) | 20(50) | 8(20) | | |
| **Tumour Grade** | | | | 14.635 | 0.006 |
| 1 | 44(48.9) | 13(14.4) | 33(36.7) | | |
| 2 | 81(43.8) | 54(29.2) | 50(27) | | |
| 3 | 82(46.1) | 60(33.7) | 36(20.2) | | |
| **NPI** | | | | 19.585 | 0.001 |
| Good | 71(49.3) | 23(16) | 50(34.7) | | |
| Moderate | 105(43.8) | 77(32.1) | 58(24.2) | | |
| Poor | 32(45.7) | 27(38.6) | 11(15.7) | | |
| **DM** | | | | 21.627 | <0.001 |
| No | 158(50.6) | 67(21.5) | 87(27.9) | | |
| Positive | 49(35.5) | 59(42.8) | 30(21.7) | | |
| **Recurrence** | | | | 13.517 | 0.001 |
| No | 131(52) | 54(21.4) | 67(26.6) | | |
| Positive | 74(37.6) | 71(36) | 52(26.4) | | |
| **VI** | | | | 9.896 | 0.042 |
| No | 114(48.5) | 56(23.8) | 65(27.7) | | |
| Probable | 32(56.1) | 15(26.3) | 10(17.5) | | |
| Definite | 61(38.6) | 56(35.4) | 41(25.9) | | |
| **Histologic tumour type** | | | | 22.954 | 0.011 |
| Ductal/NST | 115(47.1) | 77(31.6) | 52(21.3) | | |
| Lobular | 24(39.3) | 23(37.7) | 14(23) | | |
| Tubular and Tubular mixed | 46(45.1) | 18(17.6) | 38(37.3) | | |
| Medullary | 1(50) | 1(50) | 0(0) | | |
| Other special types* | 2(33.3) | 0 | 4(66.7) | | |
| Mixed** | 20(55.6) | 8(22.2) | 8(22.2) | | |
| **Menopausal status** | | | | 28.242 | <0.001 |
| Premenopausal | 60(37.3) | 35(21.7) | 66(41) | | |
| Postmenopausal | 148(50.5) | 92(31.4) | 53(18.1) | | |

*Includes Mucoid, invasive cribriform and invasive papillary carcinoma, ** Include ductal/NST mixed with lobular or special type

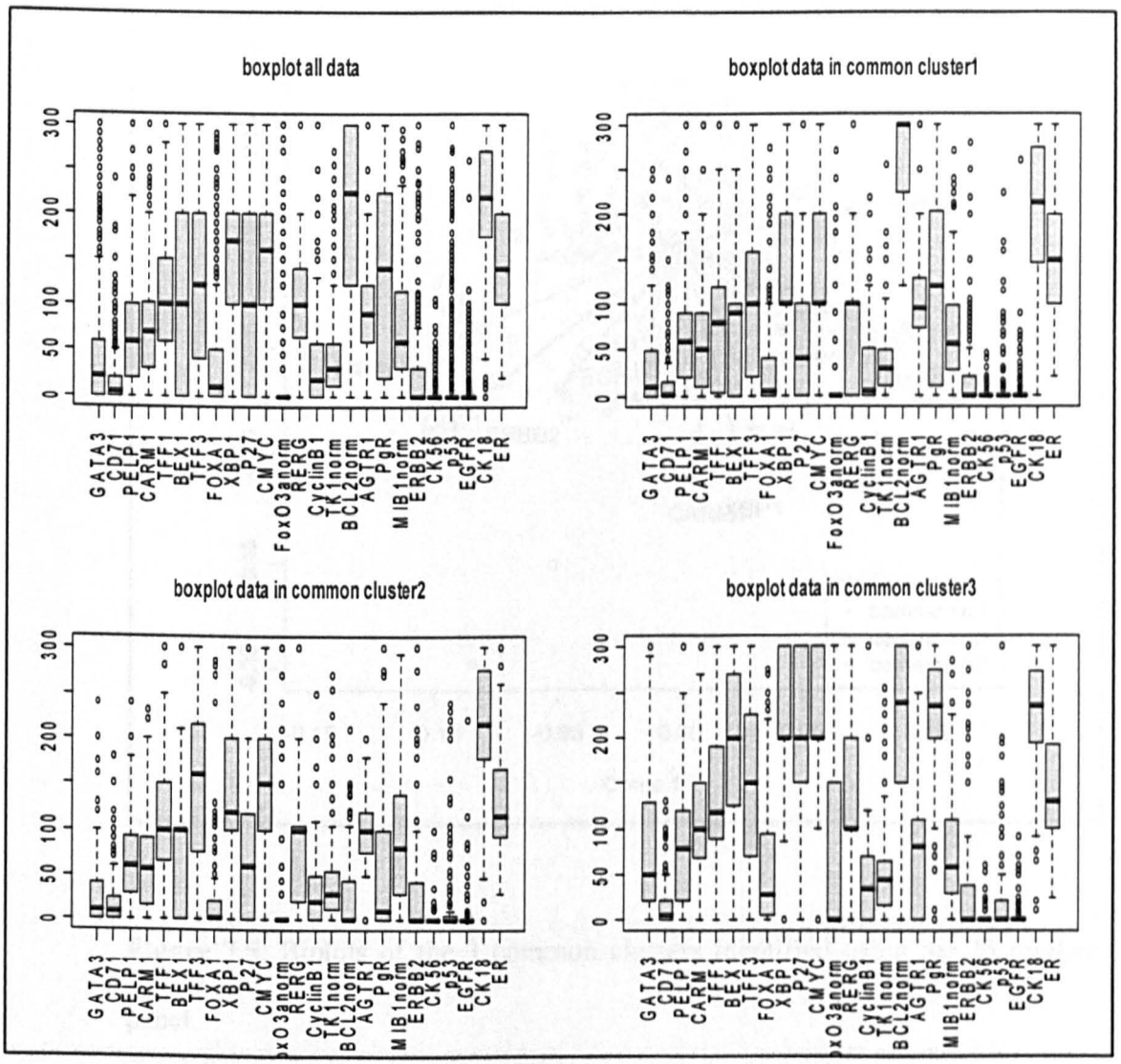


**Figure 7.4:** Boxplots of the biomarkers expression of the consensus clusters using the two clustering methods

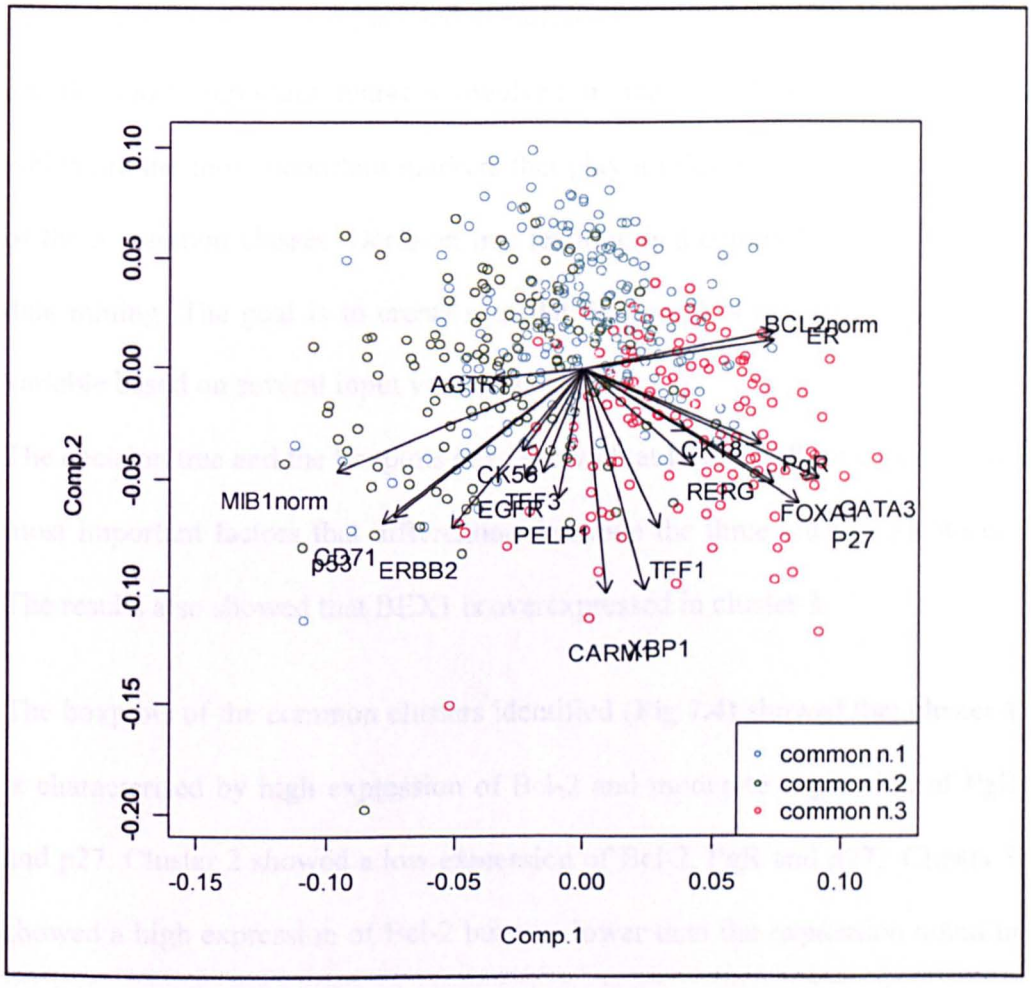


**Figure 7.5**: Biplots of the 3 common clusters identified using the 25 marker panel.

Biplots are generated by transforming the original data space using principal component analysis and then plotting the points at their projected position on axes of the first and second principal components.

### 7.3.3 Decision tree analysis of the common clusters

'C4.5 decision tree' is a supervised classifier to get a set of rules to see which are the most important markers involved in the classification process and which are the most important markers that play a relevant role in the decision of the 3 common classes. Decision tree learning is a common method used in data mining. The goal is to create a model that predicts the value of a target variable based on several input variables.

The decision tree and the boxplots have shown that Bcl-2, PgR, and p27 are the most important factors that differentiate between the three clusters identified. The results also showed that BEX1 is overexpressed in cluster 3.

The boxplots of the common clusters identified **(Fig 7.4)** showed that cluster 1 is characterised by high expression of Bcl-2 and moderate expression of PgR and p27. Cluster 2 showed a low expression of Bcl-2, PgR and p27. Cluster 3 showed a high expression of Bcl-2 but was lower than the expression noted in cluster 1, a high expression of PgR, p27 and BEX1 with relative increase of CARM1 and TFF3 expression in comparison to the other clusters.

### 7.3.4 Univariate analysis

Univariate analysis of the common clusters identified has shown a significant association with survival. Cluster two was associated with shorter breast cancer specific survival (LR= 28.185 & $p<0.001$) **(Fig 7.6)** and DFS (LR=14.900 & $p=0.001$) **(Fig 7.7)**. This relation was also maintained in the untreated patient group (n=161) where cluster two showed the worth BCSS (LR= 10.776 & $p=0.005$) **(Fig 7.8)**.

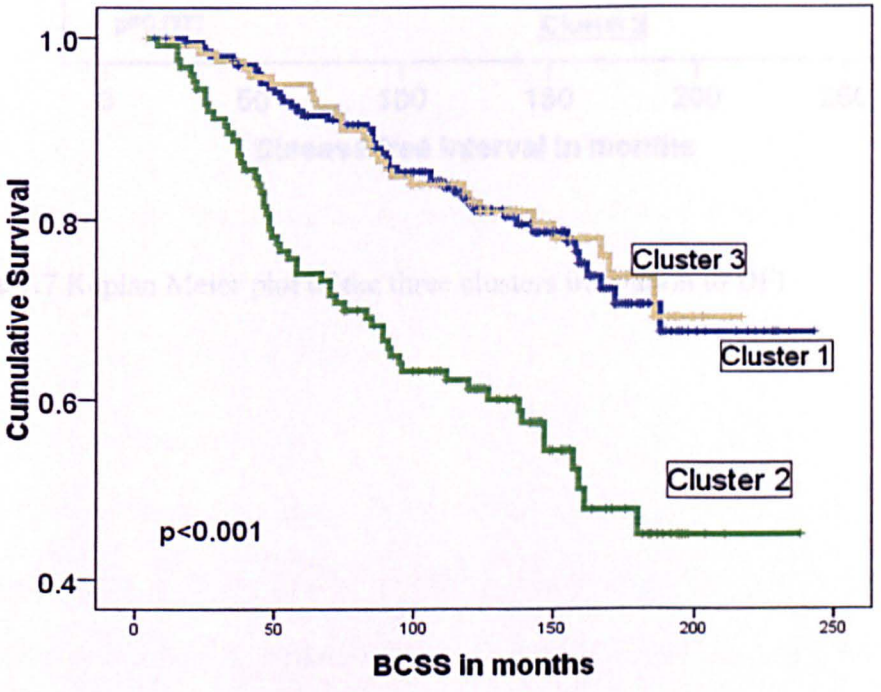


**Figure 7.6**: Kaplan Meier plot of the three clusters in relation to BCSS

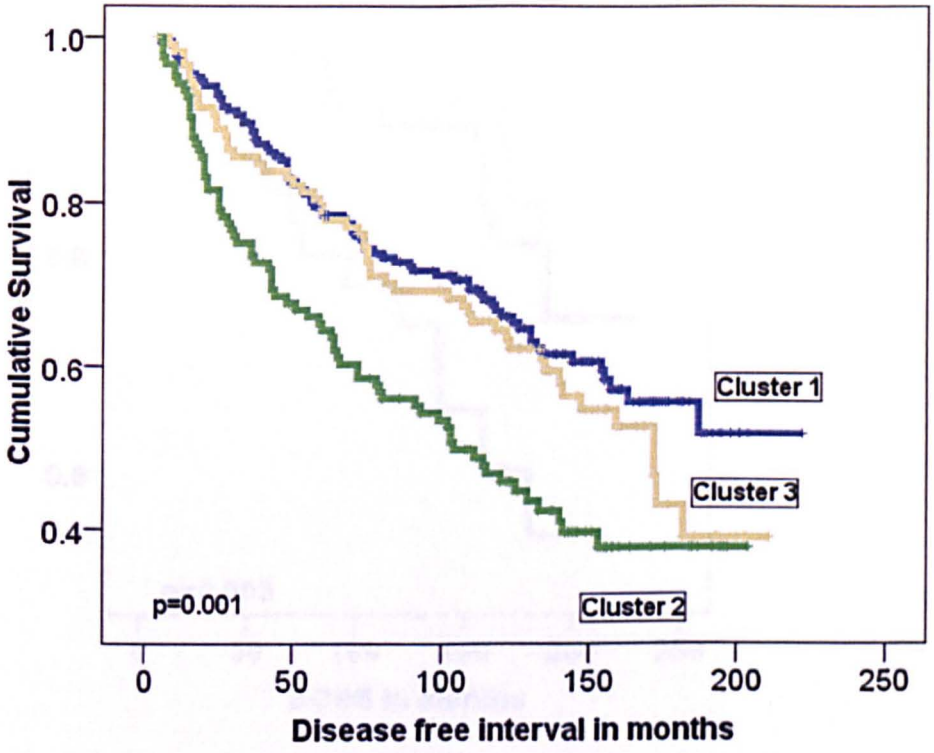


**Figure 7.7** Kaplan Meier plot of the three clusters in relation to DFI

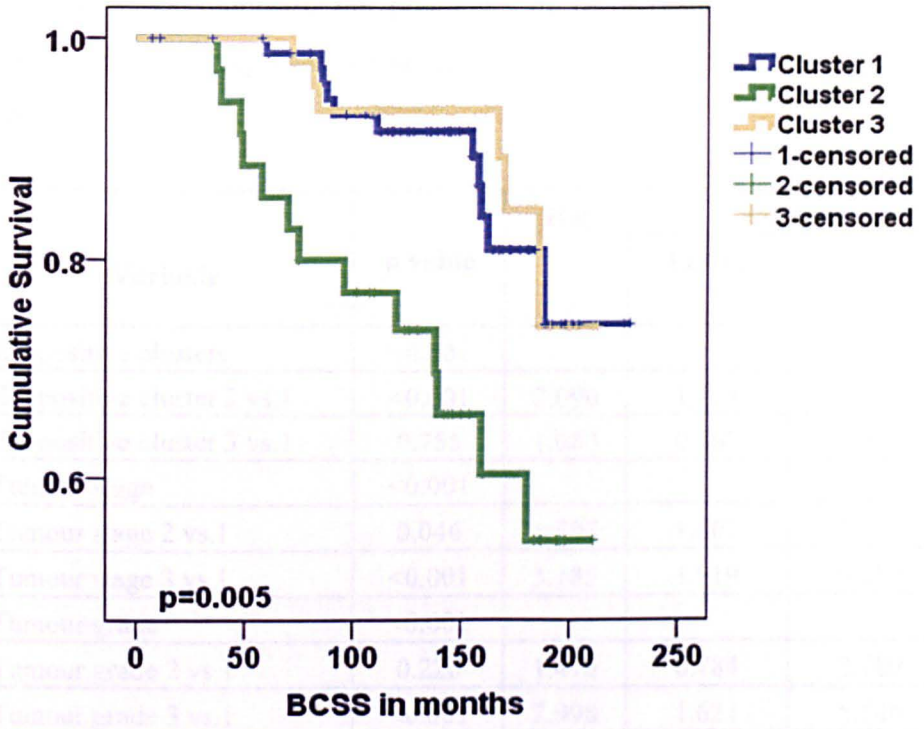


**Figure 7.8:** Kaplan Meier plot of the three clusters in relation to BCSS in the untreated patient group.

### 7.3.5 Multivariate analysis

To further test the prognostic significance of the luminal clusters identified, we have developed a COX model including the clusters and the standards prognostic factors such as tumour size, grade and LN stage. Our results showed that the ER-positive clusters identified is independent on the other factors in the model ($p<0.001$) **(Table 7.4)**.

**Table 7.4:** COX model of the predictors of breast cancer specific survival using the cluster assignment identified by the K-means and PAM clustering methods

| Variable | p value | HR | 95% CI | |
|---|---|---|---|---|
| | | | Lower | upper |
| ER-positive clusters | <0.001 | | | |
| ER-positive cluster 2 vs.1 | <0.001 | 2.090 | 1.404 | 3.111 |
| ER-positive cluster 3 vs.1 | 0.755 | 1.080 | 0.667 | 1.748 |
| Tumour stage | <0.001 | | | |
| Tumour stage 2 vs.1 | 0.046 | 1.507 | 1.007 | 2.254 |
| Tumour stage 3 vs.1 | <0.001 | 3.185 | 1.919 | 5.284 |
| Tumour grade | <0.001 | | | |
| Tumour grade 2 vs.1 | 0.226 | 1.479 | 0.784 | 2.789 |
| Tumour grade 3 vs.1 | <0.001 | 2.998 | 1.621 | 5.546 |
| Tumour size | 0.004 | 1.808 | 1.215 | 2.691 |

## 7.4 Discussion

Tumour formation is widely accepted as an evolution that incorporates the harmonised action of a set of genes, instead of a single one. The alteration in biomarker expression patterns may provide a unique molecular feature of each tumour. In order to study the coordinated action of a group of biomarkers rather than studying each biomarker separately, we need a powerful and reliable statistical method for this purpose. Single clustering methods have often been used to interpret clusters in high dimensional datasets, however depending on a single algorithm is known to be questionable (Soria et al., 2010) . The principle of combining the results of different clustering methods is particularly important in order to evaluate the robustness of a clustering classification. The two clustering methods that we have used here have been successfully used before to identify biological subclasses within certain groups of breast cancer patients (Elsheikh et al., 2009).

In this study, we have shown that a classification of ER-positive breast cancer can be done based on clustering analysis of the immunohistochemical profiles of selected important biomarkers on tissue microarray slides. The methodology we have used for clusters identification from our complex dataset was done using a variety of clustering algorithms and the most appropriate number of clusters was investigated by means of cluster validity indices to ensure that the identified optimal cluster number is based on a statistical base and not randomly selected.

Our data showed that the expression of certain biomarkers was associated with specific tumour groups when used in combination. Furthermore, our data also suggest that correlations can be made with immunohistochemical profiles of highly discriminatory panel of related biomarkers in breast tumours as we have shown in the previous chapters. The clusters we have identified showed a wide variation in the histopathological criteria. Cluster 2 patients were mostly of grade 2 and grade 3, higher LN stage, and poor NPI group with increased incidence of DM and tumour recurrence.

Our panel of markers as identified by the boxplots and the decision tree analysis indicates that Bcl-2, PgR, p27 are the most important biomarkers for ER-positive biological class identification in this study. BEX1, also showed a defining role in characterisation between cluster 1 and cluster 3 where cluster three showed the highest level of BEX1 expression. We would propose that within the ER-positive breast cancer, three distinct biological classes that show a wide variation in their biological features with emphasis on cell cycle regulators and apoptosis related genes as evidenced in our study by the strong differentiating role of p27 as a cell cycle inhibitor or Bcl-2 as an antiapoptotic factors with a cell cycle regulatory role (Vairo et al., 2000).

Many studies have previously used HER2 to characterise luminal subclasses with poor prognosis. The clustering approach that we have used here did not show any association with HER2 in agreement with others (Natrajan et al., 2009, Bhargava and Dabbs, 2008). Regarding HER2 expression, although GEP studies have shown that some luminal tumours express HER2 and some authors include HER2 positivity as a feature of Luminal B tumours , others

argue against that and include HER2-positive tumours, regardless of the expression of ER, with the HER2-positive subgroup and argue against this definition of Luminal B tumours (Bhargava and Dabbs, 2008). In addition, it has been reported that prognostic factors, to be of clinical use, must show a wide separation in the outcome of the groups identified and select adequate numbers in each group. However, most studies have shown that the number of HER2-positive ER-positive tumours is usually too small to constitute a meaningful prognostic subgroup. ER-positive HER2 positive tumours are currently receive specific systemic therapy which may be different to those of other ER-positive HER2 negative tumours.

Cheang and colleagues have used MIB1 to characterise poor luminal subclasses within the luminal group (Cheang et al., 2009). We found a strong role of cellular proliferation in characterising luminal subclasses, although MIB1 was not a strong characterising marker of the three clusters identified here, p27 loss was seen in the poor prognosis cluster identified in our study supported by the Bcl-2 growth inhibitory role. Estimation of cell proliferation pathways could have significant clinical benefit in predicting behaviour and subclassification of the luminal-like subclasses, and their potential for response to systemic therapy.

ER+PgR+ phenotype has previously shown to be associated with better outcome in comparison to ER+PgR- group (Rakha et al., 2007). Our clustering result have identified that PgR has a powerful characterising role in ER-positive breast cancer subclassification and its loss proved to be more reliable

than HER2 and MIB1 in identification of poor prognostic subclasses in ER-positive breast cancer.

In conclusion, the value to study the proteomics of hundreds of tumours by TMA makes it a powerful tool for the molecular classification of breast cancer. Furthermore, the results of this study indicate that a powerful clustering algorithm using protein expression TMA data could be used as a simple method for better characterization and refinement of large tumour series for identification of biological subgroups with homogenous distribution. Molecular profiling of breast cancer using protein biomarkers on TMAs can sub-classify ER-positive tumours into clinically and biologically relevant subgroups.

# 8 General Discussion

Human breast cancer (BC) represents a heterogeneous group of tumours differing in their morphology, biologic behaviour, outcome, and response to therapy (Gusterson et al., 2005). Currently, pathological diagnosis and classification of BC is mainly based on well-established traditional histopathologic morphological features. However, morphological features alone do not adequately reveal the molecular heterogeneity and complexity of BC.

Immunohistochemistry (IHC) was developed prior to gene expression profiling and currently remains the preferred technique for biomarker profiling in routine pathology laboratories. In situ hybridization was introduced after that and is now also routinely used to classify breast cancer into HER-2 gene amplified or non-amplified groups. Recently, high throughput proteomic and gene-expression profiling methods are being studied as diagnostic tools (Pusztai et al., 2006).

The identification of subgroups within ER-positive breast cancer based on biological characterisation of tumours could be used to better predict therapy of clinical outcome.

## 8.1 The ER-positive/luminal-like subtype

ER plays a crucial role in the progression of breast cancer by regulating genes and signalling pathways mostly related to cellular proliferation. Regulation of gene transcription by ER requires the activity of ligand binding and other protein interactions. However, the intracellular signalling pathways that control

these events and regulate ER alpha transcriptional activity are not fully understood and need further refinement.

The ER-positive luminal-like breast cancer subtype has recently attracted a lot of attention due to its association with more than 70% of breast cancer patients Seminal studies have identified more than one luminal class with different prognoses implying heterogeneous biological variation within the ER-positive breast cancer subtype (West et al., 2001). Despite its importance, there is no standard definition of this important class in routine clinical practice. Moreover, there is an immediate need for well-defined biomarker panels for clinical diagnostic use as currently there is no gold standard for what defines these tumours.

Three luminal-like subclasses (Luminal A, B and C) have been proposed but differ in terms of their prognosis. Luminal B and C have been described as having a worse prognosis in comparison to Luminal A cancers (Sorlie et al., 2001). The reasons for this difference in prognosis are still unknown but a possible explanation relates to ER function and signalling differences, which could be attributed to the influence of transcription factors, coactivators, and corepressors that modulate ERα activity. In addition, overexpression of proliferation and cell cycle genes in breast cancer is well recognised to be associated with poor outcome suggesting that these genes may contribute to the poor prognosis in some luminal cancers. Also, it has been proposed that abnormal apoptosis function, DNA damage response and PI3K/AKT pathways may be additional factors influencing prognosis (Bertucci et al., 2009).

Subclassification of ER-positive luminal-like cancer using gene expression studies and microarray analysis can be expensive and time-consuming and generally requires fresh frozen tissue. Our study aimed to subclassifying ER-positive luminal-like cancers into prognostic and biological subgroups using their protein expression with routinely processed FFPE tissue. Furthermore, the biological phenotype characteristics and the associations between these subgroups and survival outcome were investigated.

## 8.2 The value of TMAs in subclassification of ER-positive breast cancer

We have used protein expression profiling using a large panel of biomarkers with strong relevance to breast cancer, by immunohistochemistry on tissue microarrays for refining the classification and prognostication in ER-positive breast cancer.

We proposed that an alternative approach to gene expression profiling is to use established robust laboratory technology, such as immunohistochemistry on formalin fixed paraffin embedded patient tumour samples on a high throughput proteomic platform such as TMAs to explore the existence and clinical significance of distinct breast cancer classes. In this study, we have studied the protein expression of 25 biomarkers to investigate if it was possible to classify the luminal cancer into biological subgroup.

TMAs allow large populations of patients' tumours to be rapidly screened to detect overall protein expression in large patient groups, overcoming the weakness of IHC results when using smaller cohorts. The validity of TMA

analysis has been shown by comparisons with full section examination in breast (Camp et al., 2000, Gillett et al., 2000), prostate (Mucci et al., 2000), and bone marrow (Zimpfer et al., 2007). All studies reported >90% concordance for common breast cancer biomarkers such as oestrogen and progesterone receptors and the HER-2 oncoprotein. Moreover, prognostic associations for these markers could be reproduced with the TMAs.

It is arguable that whether these specimens are representative of their donor tumours. Some alterations may not be detected if the analysis of heterogeneous tumours is restricted to samples measuring 0.6 mm in diameter. However, it is important to mention that the TMA technique has been developed to examine large tumour populations and not to study individual tumours. This suggests that TMA studies will provide consequential data, even if only one sample is analyzed per tumour (Moch et al., 2001). The assessment of TMA sections is, when possible, to be carried out by more than one assessor to overcome inter-observer variability, to achieve the maximum level of concordance and to strengthen the accuracy of the study. Alternatively, the assessment could be done by one observer on two separate occasions and the results could be compared by kappa statistics.

The value of studying the proteomics of hundreds of tumours by TMA makes it a powerful tool for the molecular classification of breast cancer. Furthermore, the results of this study indicate that a powerful clustering algorithm using protein expression TMA data could be used as a simple method for better characterization and refinement of large tumour series for identification of

biological subgroups with homogenous distribution as reported previously (Abd El-Rehim et al., 2005).

By this high throughput approach, we have been able to identify three biological clusters with unique characteristics and variable biological and prognostic features.

## 8.3 The prognostic and biological roles of the studied markers

We aimed to identify potential candidate markers for inclusion in the study by applying novel bioinformatics methods including artificial neural networks, Ensemble cross validation analysis to our gene expression data. In addition to a literature search for genes with strong relevance in ER-positive breast cancer or have been the subject of recently published studies and strongly suggest an important role in the biology and molecular classification of ER-positive breast cancer. The selection criteria was based on the published literature concentrating mainly on ER related pathways such as ER coregulators, cellular proliferation, apoptosis, AKT/PIK3 pathway and endocrine resistance.

We have characterised a number of biomarkers that have not been characterised before in breast cancer and luminal-like subtype using a large number of patients including RERG, CARM1, PELP1, CD71, BEX1, XBP1, AGTR1 and TFF3 producing novel data. Some of these markers were previously identified as characteristics markers of the Luminal A subclass in the seminal gene expression studies. GATA3, BEX1 and RERG were able to differentiate between luminal-like tumours associated with poor and good

prognosis and as such they could be useful markers for the definition of the luminal phenotype as reported by others (Sorlie et al., 2001).

Although TFF3 and XBP1 are associated with the good prognosis Luminal A subtype in the published gene expression studies, they showed associations with shorter BCSS while TFF1 and FOXA1 were not associated with survival in the ER-positive subtype in this study.

This could be attributed to the difference in the downstream technique used (RNA in expression profiling as opposed to protein in immunohistochemistry studies) and sensitivity of the detection system, or a post-translation modification of the protein product of the gene. These results may support the view that translation of gene expression profiling studies into clinical practice should be interpreted with care and individual markers may not show the same significance when studied in isolation rather than as part of an expression signature.

Apart from ligand binding to ER, the biologic functions of nuclear receptors, including ER, are also regulated by a group of proteins known as transcriptional coactivators, as well as by another group of proteins known as transcriptional corepressors (Nair and Vadlamudi, 2007). Subsequently, we aimed to study PELP1 and CARM1 as two of the novel ER coactivators to characterise their biological associations within ER-positive breast cancer. This study showed that CARM1 and PELP1 protein expression is associated with features of poor prognosis in breast cancer particularly in the ER-positive luminal class implying a potential role in their biological stratifications.

In this study, in collaboration with Dr Julia Gee and Prof R Nicholson (Cardiff School of Pharmacy), it was proposed that assessment of CD71 expression might equally be used to stratify ER-positive patients to define subgroups with poor prognosis, high proliferation and resistance to hormonal therapy. We demonstrated that prominent expression of CD71 protein is a feature of breast cancers with poor prognosis and as such, we proposed that transferrin receptor expression may have implications for diagnosis and prognosis. CD71 protein expression could be of value in characterizing a subset of ER-positive/luminal-like tumours with poor prognosis in clinical practice, as well as defining patients less likely to respond to endocrine therapy. Therapies targeting iron delivery or CD71 itself, may have therapeutic benefits in treating ER+ CD71+ breast cancers in the clinic.

Previous studies have highlighted the important role of PTEN/Akt/PI3K pathway and its upstream and downstream targets in the biology and prognosis of breast cancer (Zou et al., 2008). PI3K dependent Akt activation can be regulated PTEN, which works essentially as the opposite of PI3K. PTEN is a tumour suppressor gene involved in the biology of breast cancer and its loss is associated with high grade tumour and inversely correlated with the pAkt activation (Bose et al., 2005).

Upregulation of Akt was found to inhibit cell cycle arrest in G1 and G2 phases. The activated Aktmay enhance proliferation and survival of cells which may lead to mutations in other genes (Ramaswamy et al., 1999). Akt1 has also been shown to play a role in angiogenesis and tumour development. AKT1 deficient mice showed enhanced pathological angiogenesis and tumour growth (Chen et

al., 2005). Given these facts, it would be of interest to study the biological correlation of PTEN and AKT to the other biomarkers included in this thesis.

Our results demonstrated the biological and prognostic role of FOXO3a protein expression and its subcellular localization in breast cancer. Loss of nuclear FOXO3a expression could tilt the growth balance in favour of proliferation and poor outcome in luminal-like breast cancers through active Akt/PI3K pathway, highlighting the importance of cellular proliferation in their biological stratification as reported by other investigators (Zou et al., 2008).

In the future, it is important to study the relation between PTEN as an upstream target to Akt/PI3K pathway to the FOXO3a expression.

Recently, it has been shown that AGTR1 overexpression defines a subset of ER-positive breast cancer that can benefit from AGTR1 antagonists. Specifically, AGTR1 was overexpressed only in tumours that were ERBB2-negative and ER-positive (Rhodes et al., 2009). In this study, AGTR1 expression in the luminal-like breast cancer characterised an aggressive phenotype with high proliferation and shorter survival which provides further evidence of its importance in the biology of ER-positive breast cancer. A finding that warrants the evaluation of its potential application as a novel targeted therapy in breast cancer.

Many studies have demonstrated a strong relationship between cellular proliferation and poor prognosis in breast cancer particularly in the oestrogen receptor (ER)-positive/luminal-like molecular subtype (Cheang et al., 2009). This prompted us to study the biological and prognostic implication of proliferation in the ER-positive breast cancer using cell cycle phase specific

proteins. Our findings showed that overexpression of the proliferation related markers cyclin B1 and TK1 is involved in the progression of breast cancer. This is also confirmed when using both markers in combination to produce better stratification in terms of outcome. Increased cellular proliferation occurs in some luminal cancers and appears to form a biological and clinically distinct subclass of ER-positive breast cancer patient as previously reported by others (Sotiriou et al., 2003).

The cell cycle is regulated by many mechanisms and is affected by the interaction of multiple pathways that either enhance or delay its progression. In addition to its control by cyclins and cyclin dependant kinases and cell cycle regulators, many studies have highlighted the role of Bcl-2 in controlling the cell cycle independent of its antiapoptotic function by causing retarded entry into the cell cycle (Vairo et al., 2000). Our finding has shown that Bcl-2 is associated with the luminal phenotype and has a growth inhibitory function in breast cancer possibly via its effect on p27 expression. In this study, the Bcl-2+p27+ phenotype was associated with good prognosis in luminal-like breast cancer due to cell cycle arrest as reported by others (Vairo et al., 2000). Estimation of subcellular localisation of p27 has been shown to correlate with patient's prognosis in other cancers. Cytoplasmic localisation of p27 indicates an inactive form. As expected, Rosen and co-workers have found that cytoplasmic localization of p27 predicts poorer prognosis in advanced ovarian carcinomas (Rosen et al., 2005) due to its loss of cell cycle inhibitory function.

## 8.4 Biological classes within the ER-positive breast cancer

Combining all the studied markers in a clustering analysis has revealed the presence of three biological clusters. The characteristics of the luminal-like clusters identified here reflect the biological heterogeneity of the ER-positive breast cancer. For instance, Cluster 1 and Cluster 3 patients were mainly of lower grade, lower LN stage, and lower NPI group and showed decreased incidence of DM and tumour recurrence. Although Cluster 3 was not prognostically different from cluster 1 it showed a lower Bcl-2 expression than Cluster 1, a high expression of PgR, p27 and BEX1 which reflects different biological characteristics.

Although Cluster 1 and Cluster 3 have different biological features, their prognostic characteristics were similar. Together they may represent a large luminal-like subclass with close similarity to the Luminal A subclass (Sorlie et al., 2006). In contrast, Cluster 2 patients were mostly of high grade, higher LN stage, poor NPI group and increased incidence of DM and tumour recurrence. Those patients are more likely to be resistant to hormonal therapy due to low expression of PgR and Bcl-2 and this may explain their poor prognosis. Cluster 1 is characterised by high expression of Bcl-2 and moderate expression of PgR and p27. These criteria could explain the longer BCSS seen in this group. The high expression of Bcl-2 in good prognosis luminal subclasses was also reported by other investigators (Ihemelandu et al., 2009).

The survival analyses revealed significant differences in BCSS among clusters. Cluster 2 represents a distinct group with poor prognosis and was associated

with loss of Bcl-2, PgR and p27. All of these variables are known to be associated with good prognosis in breast cancer. The significant prognostic independence of the three clusters identified in multivariate analysis further supports the relevance of the clustering methods used in this study. This indicates that the classification of ER-positive breast cancer using such methods is of a great value in the evaluation of outcome in patients with ER positive disease.

We would propose that within ER-positive breast cancer three distinct biological classes exist that show a wide variation in their biological features with emphasis on PgR, cell cycle regulators and apoptosis related genes. This is evidenced in our study by the strong differentiating role of p27 as a cell cycle inhibitor or Bcl-2 as an antiapoptotic factors with a cell cycle regulatory role. We recommend the use of an external validation cohort to confirm the results of the current study as used previously in other studies of the Nottingham Breast Cancer Research Group (Abdel-Fatah et al., 2010).

## 8.5 Summary and Conclusions

It appears increasingly evident that the ER-positive subclasses with good prognosis, as shown here in Cluster 1 and Cluster 3, have distinct characteristics, defined by high expression of ER, PgR, and Bcl-2 and cell cycle inhibitors. The remaining poor prognostic cases in Cluster 2 comprise a heterogeneous collection that can be recognised by additional genetic lesions particularly those which result in clinical behavioural characteristics of poor

outcome and lack of dominant ER related pathway especially those related to PgR, apoptosis and cell cycle regulation.

In conclusion, our results emphasised the biological and behavioural heterogeneity of ER-positive luminal–like breast cancer. More importantly, we have identified a novel panel for ER-positive luminal-like breast cancer and the existence of luminal subclasses that differ with respect to patient outcome. Identification of biologically and clinically distinct breast cancer subtypes could improve prognostic assessment of the ER-positive breast cancer.

The use of novel bioinformatic approaches to analyse high dimension datasets is of value in identifying candidate genes to characterise ER-positive/luminal like breast cancer. Subsequently, these can be used to subclassify these cancers in terms of biology and prognosis. Molecular profiling of breast cancer using protein biomarkers on TMAs can sub-classify ER-positive tumours into clinically and biologically relevant subgroups.

## 8.6 Future directions

1-We have been able to identify novel genes that showed expression differences between ER-positive luminal-like and ER negative breast cancer; further validation of more genes using either QPCR or TMAs and the IHC protein expression platform might strengthen the current findings that we have shown in this study. Also it would be of interest to perform *in vitro* functional studies on some of these novel genes identified **(Summarised in tables 5.10, 3.6, 3.7)** using breast cancer cell lines that exhibit luminal ER+ features. These would include apoptosis, proliferation assays in cells that overexpress certain genes and their siRNA knocked out subsets.

2-Our results support the prognostic and biological importance of Bcl-2, RERG and GATA3 protein expression which could be used in routine clinical diagnosis of luminal-like breast cancer and patient follow-up.

3-Evaluation of the potential application of AGTR1 blockade as a novel targeted therapy in ER-positive breast cancer is warranted in clinical trial settings.

4-This study highlighted the prognostic role of CARM1 and PELP1 as novel ER coregulators in breast cancer. Improved understanding of the functional role and mechanism of action of ER coregulators in breast cancer may reveal new therapeutic targets.

5- We would need to explore the nature of CD71 interplay with growth factor signalling and ER in the future (e.g. using immunioprecipitation studies), although the data in the paper (Habashy et al., 2010) with Faslodex or PI3K

blockade already suggests there can be productive interplay with transferrin/CD71 signalling.

6- We recommend the use of external validation cohort to confirm the results of the current study.

7- Study the amplification of certain genes related to oestrogen receptor pathways such as ESR1 and Progesterone by Chromogenic in Situ Hybridization (CISH) or Fluorescent in situ Hybridization (FISH) using the available probes.

8- Study chromosome 1q gain and 16q loss which might associate with steroid receptors in breast tumours. Gains of the long arm of chromosome 1 and losses of chromosome 16q are often the result of unbalanced translocations between these two chromosomes. These genetic changes and the resulting chromosome imbalances have been thought to play a pathogenic role in breast carcinoma development and steroid hormone pathway.

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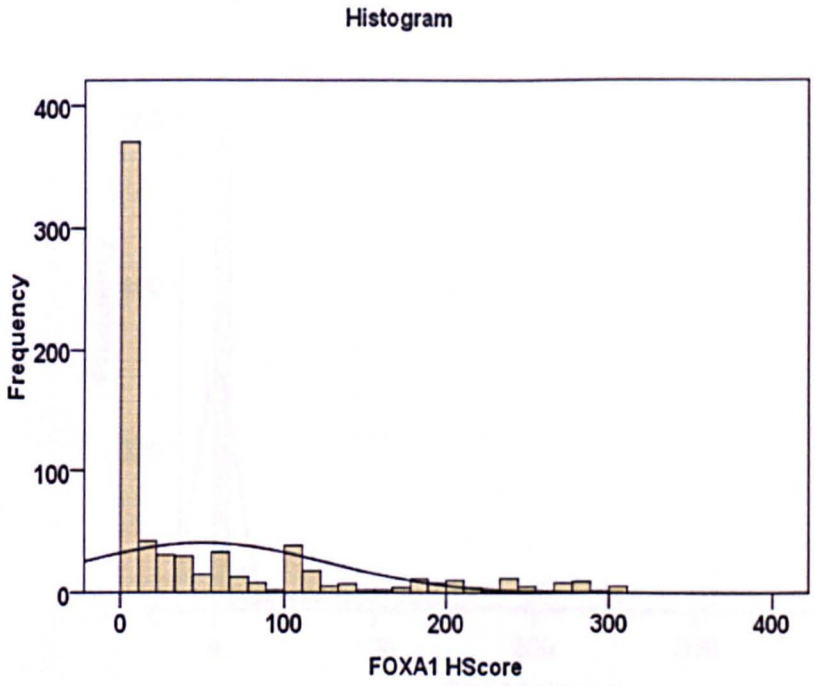
# 10 Appendix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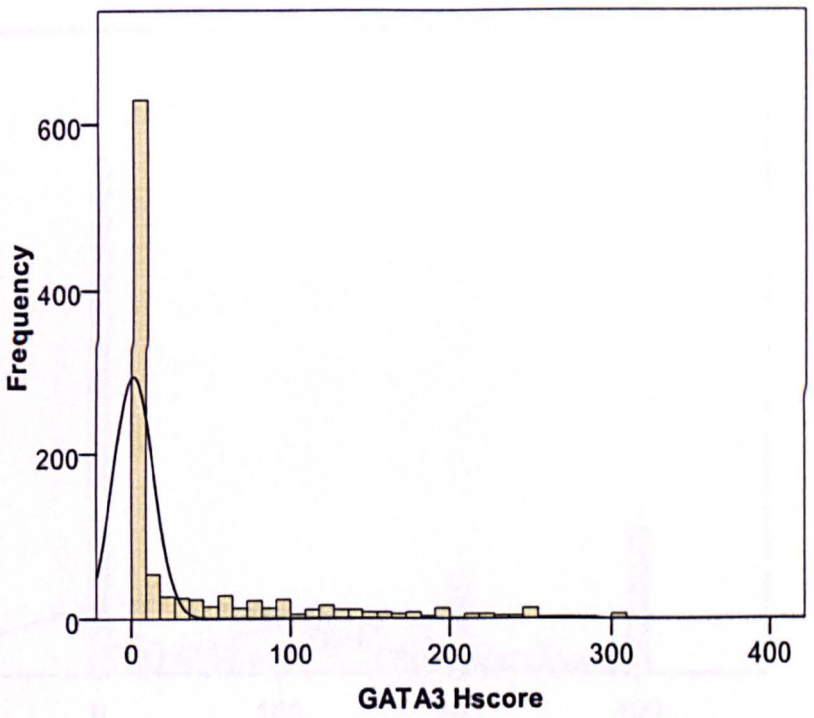
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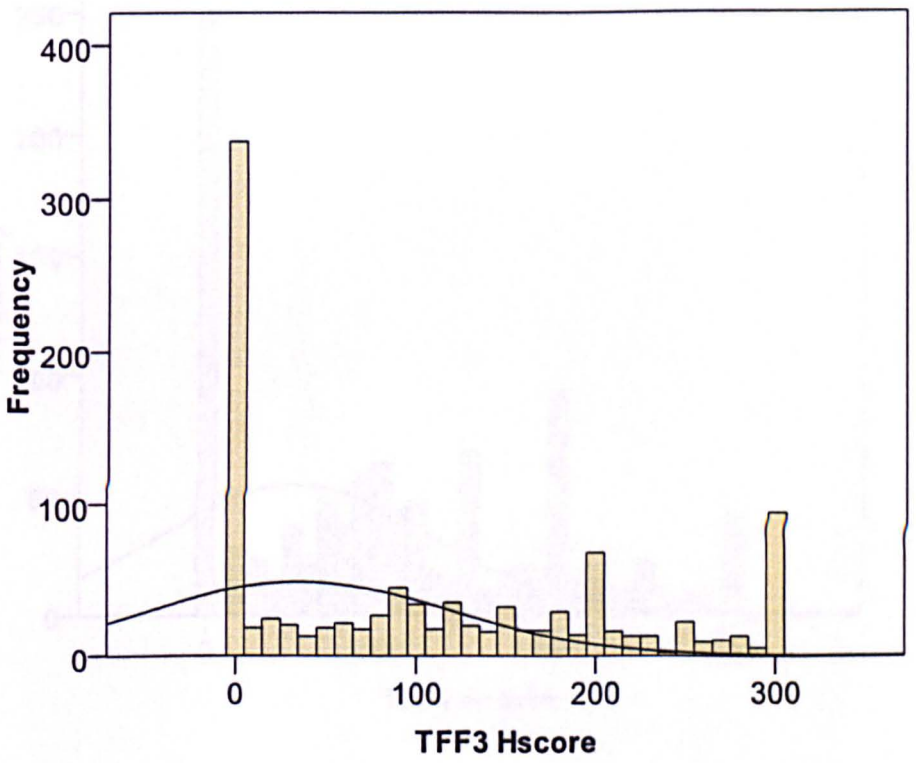
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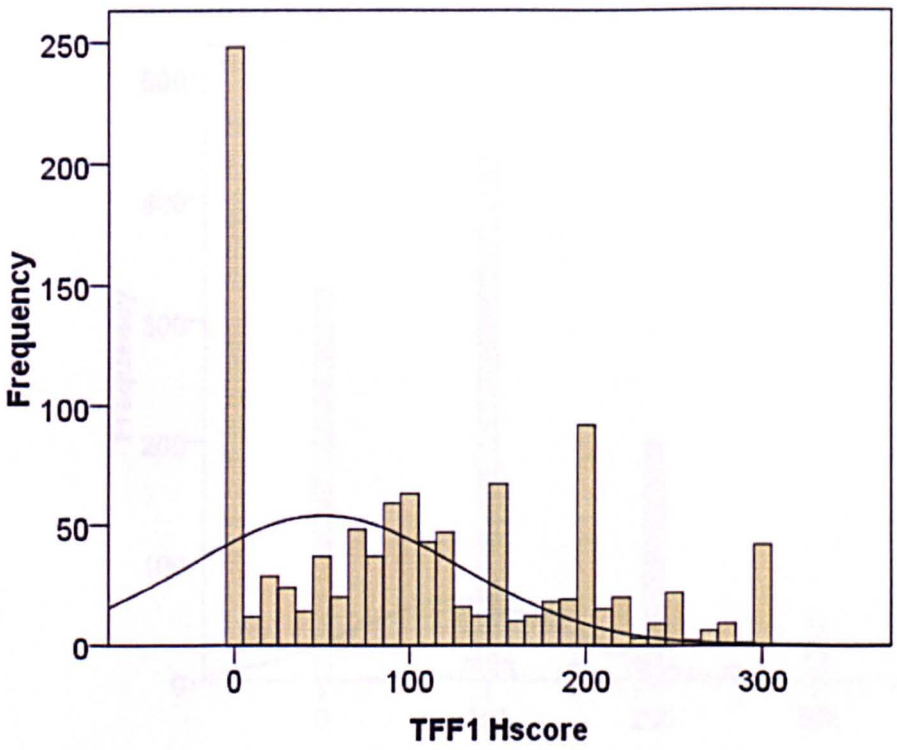
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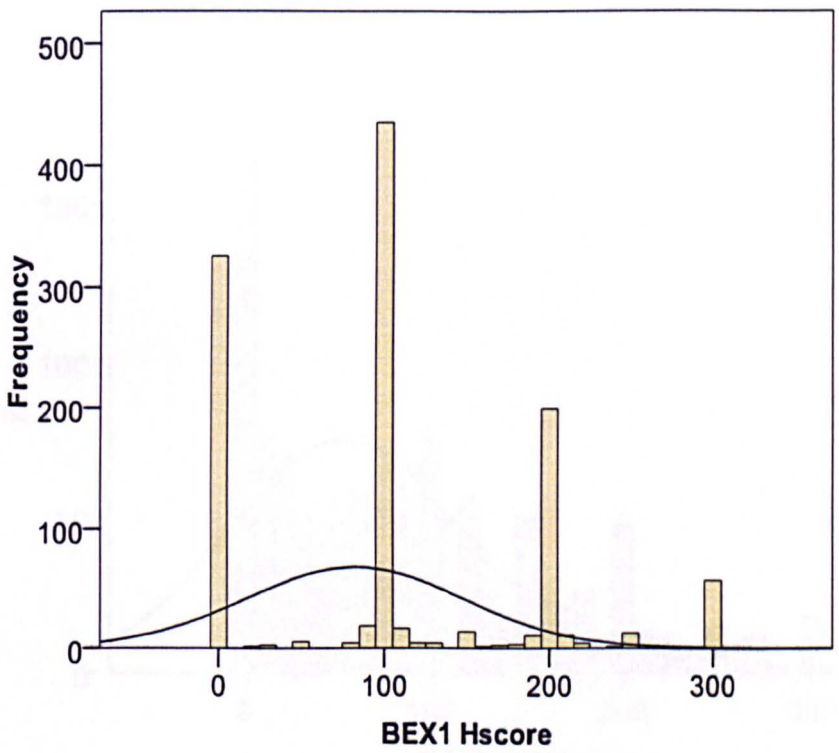
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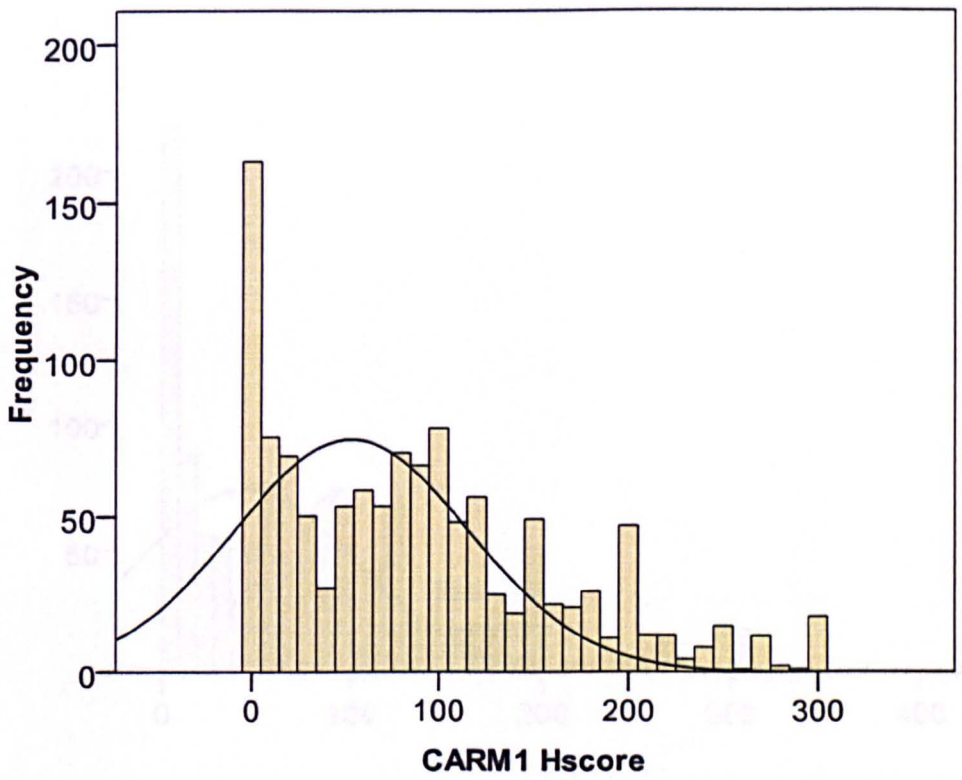
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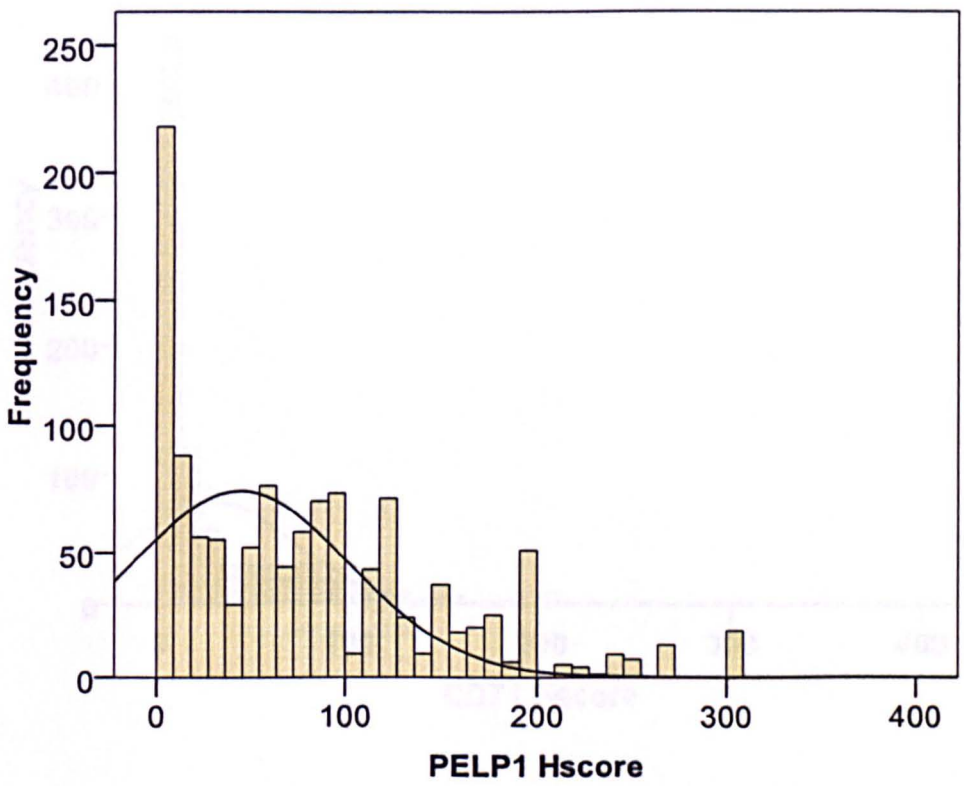
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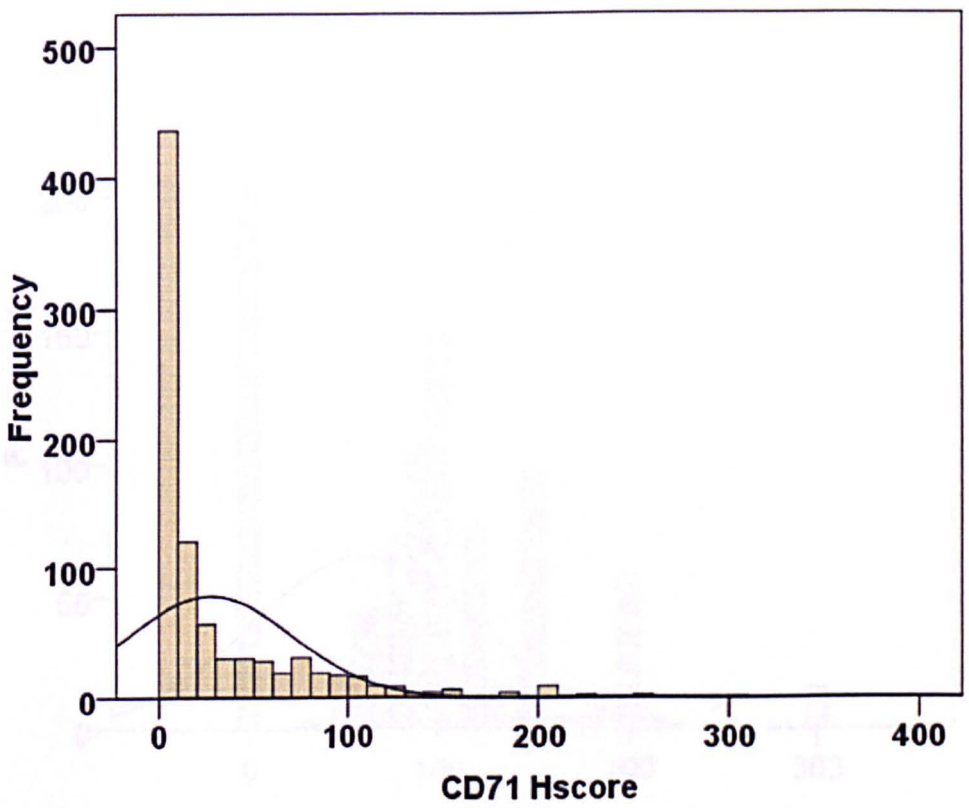
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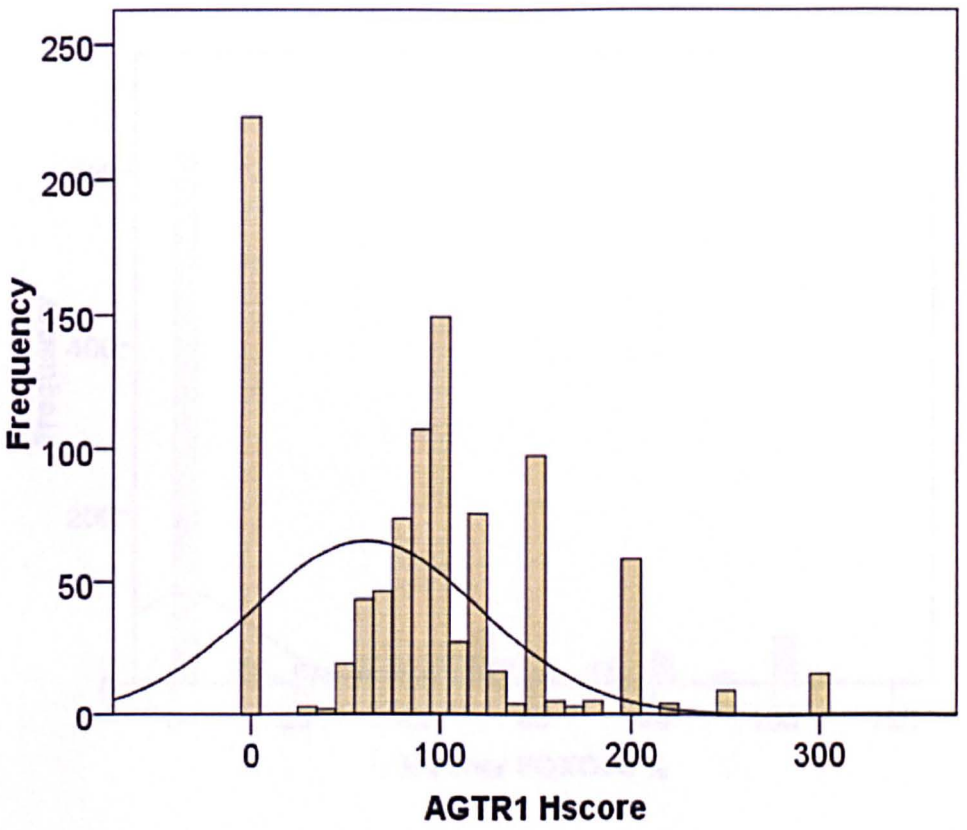
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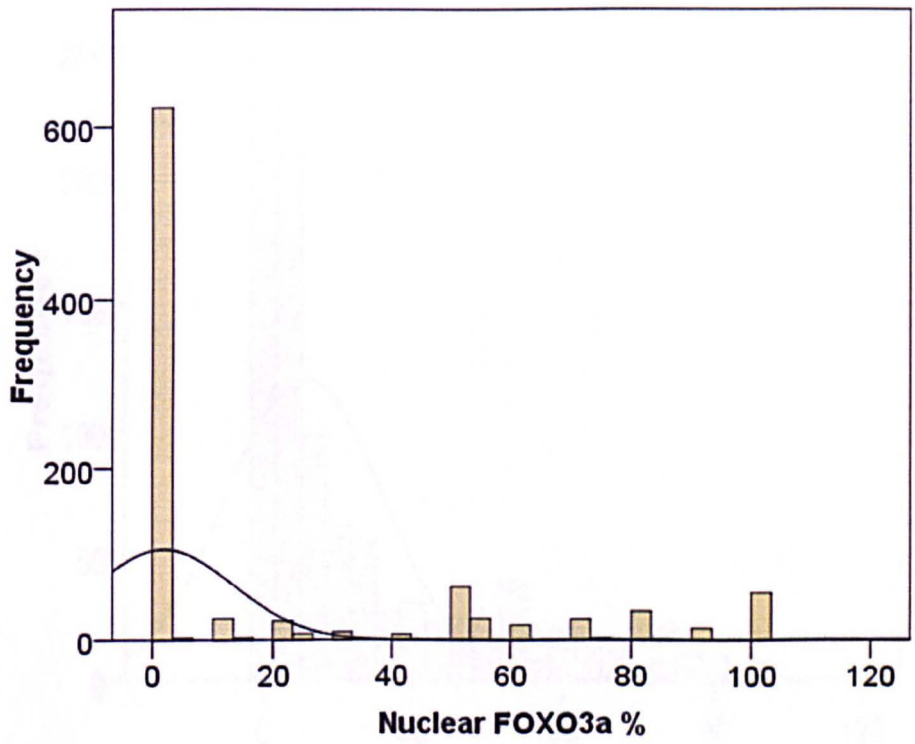
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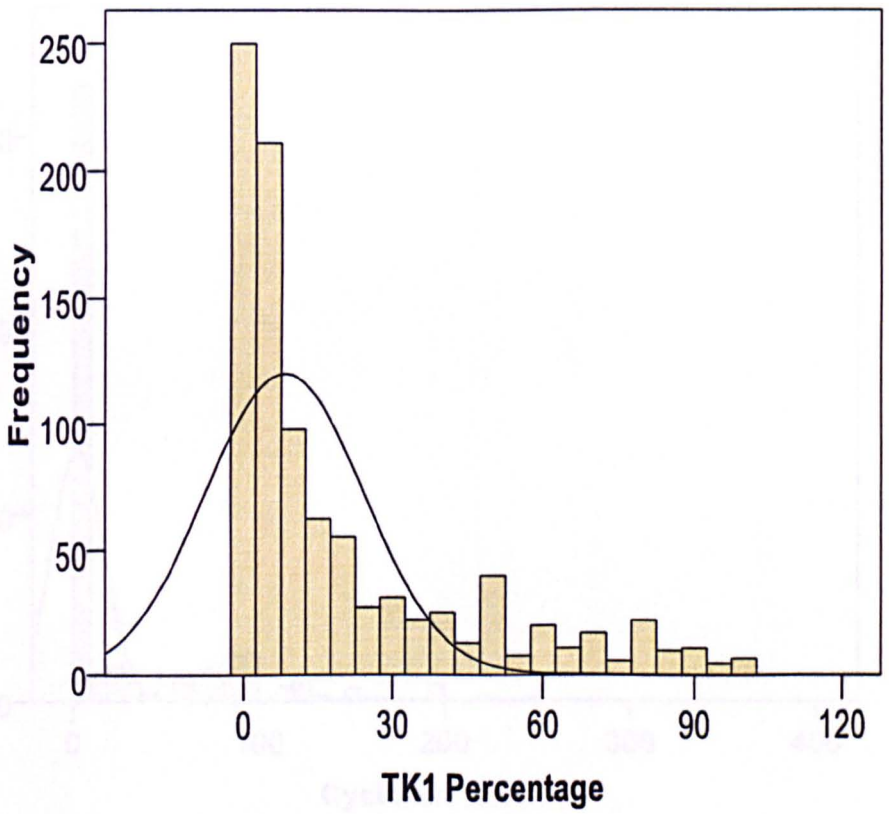
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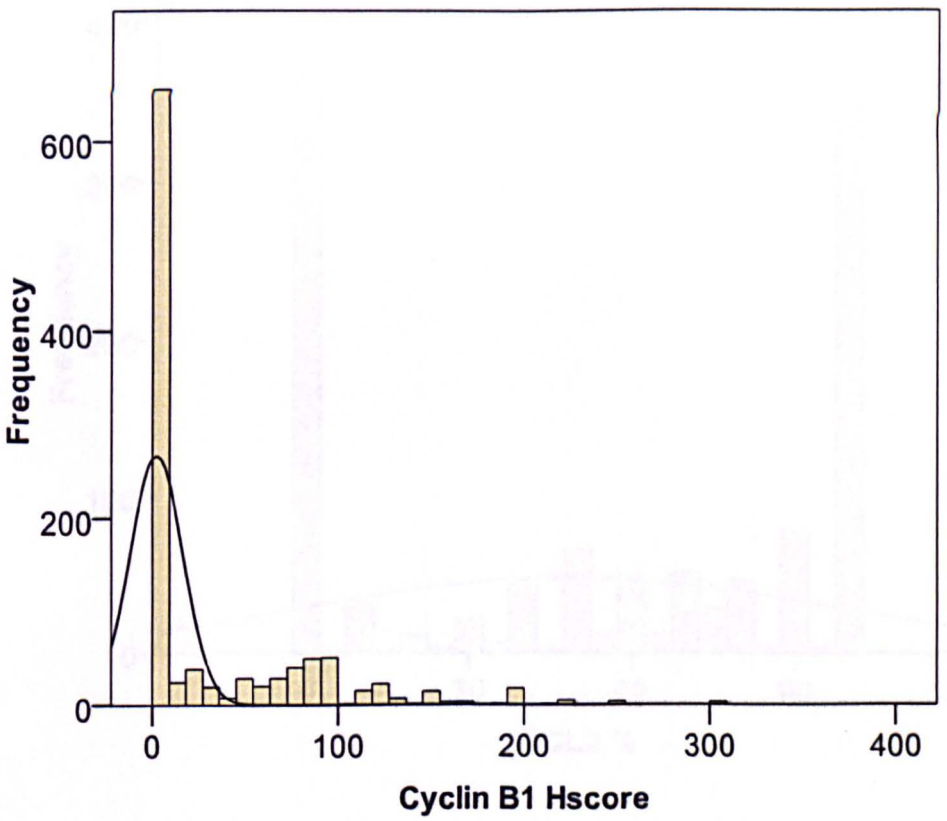
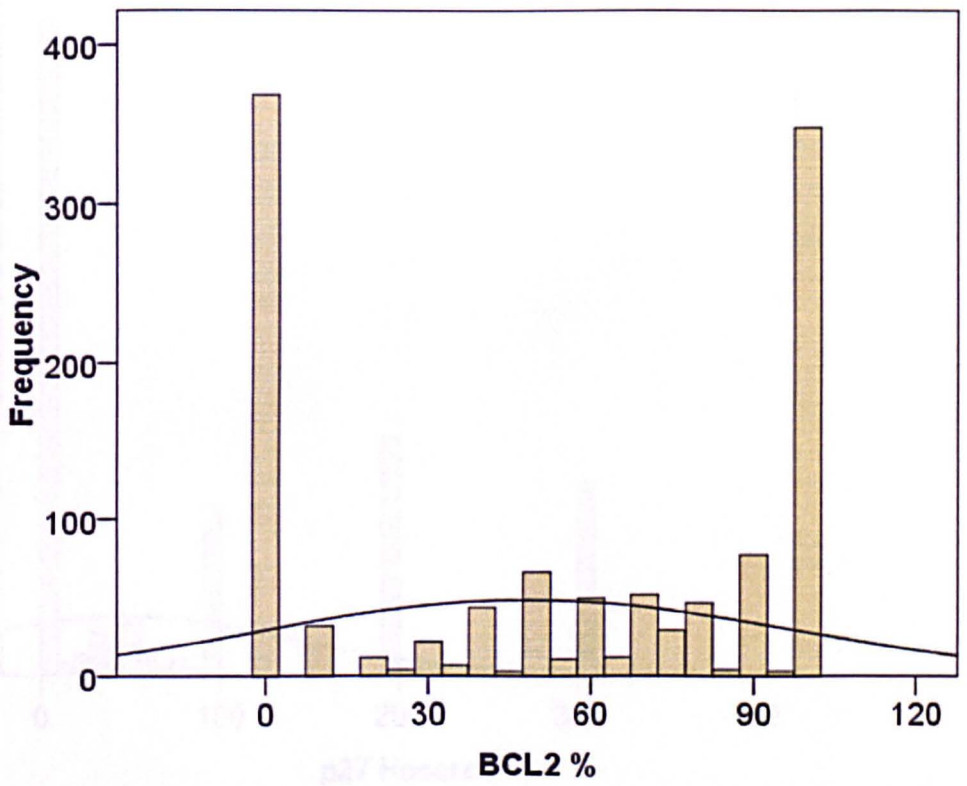
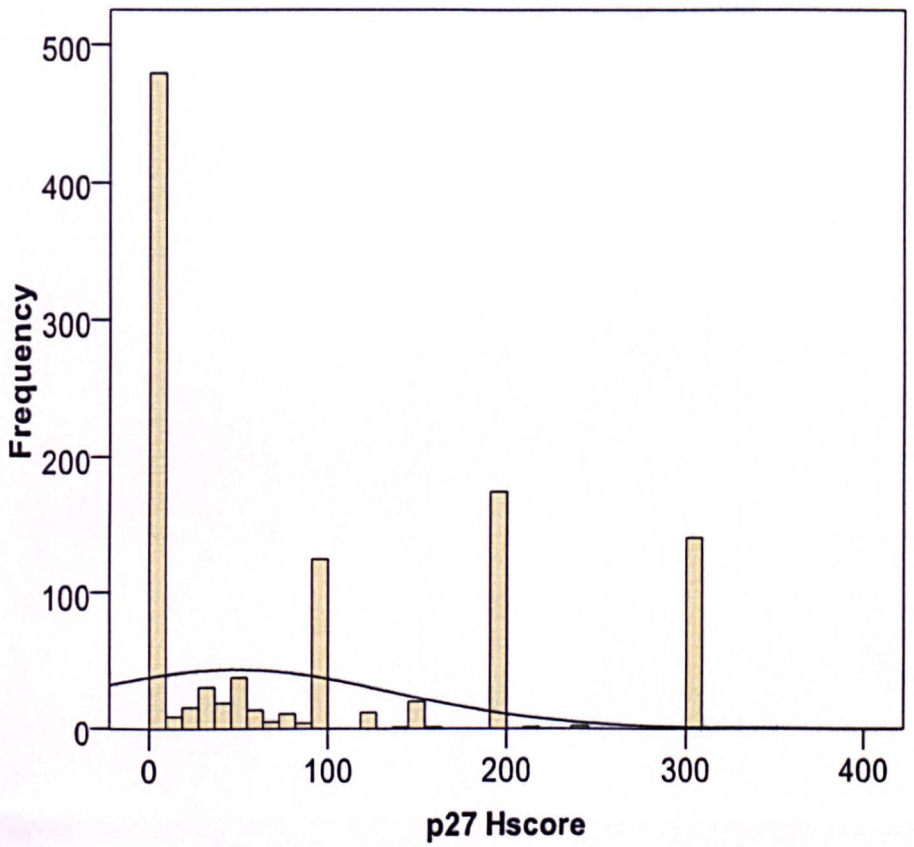
50
0
Frequency
0
30
60
90
120
TK1 Percentage


600
400
200
0
Frequency
0
100
200
300
400
Cyclin B1 Hscore


400
300
200
100
0
Frequency
0
30
60
90
120
BCL2 %


500
400
300
200
100
0
Frequency
0
100
200
300
400
p27 Hscore