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An Investigation of Metastatic Colorectal Cancer

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Declaration

This work entitled 'An investigation of Metastatic Colorectal Cancer' has been composed by Abutaleb Fitnan Asiri, and I confirm that the result presented in this thesis is my own work during my period of study at the University of Nottingham, unless otherwise mentioned. No part of this thesis has been submitted for any degree, diploma or any other type of qualification at any other institution.

Abutaleb Fitnan Asiri

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"In memory to my Brothers 'Mohammad and Amer' who passed away last year"

I have never needed anyone when you both were there; you are the most I needed to see me make you proud

Abbreviation

5-FU	Fluorouracil
AJCC	America Joint Committee on Cancer
AKT	PKB Protein kinase B aka AKT
ANOVA	Analysis of Variance
APC	Adenomatous Polyposis Coli
BAX	BCL2-Associated X Protein
BCA	Bicinchoninic acid
Bp	Base pair
BRAF	v-Raf murine sarcoma viral oncogenes homolog B
BSA	Bovine Serum Albumin
cAMP	Cyclic adenosine monophosphate
CDC4	Cell Division Control Protein 4
CDK	Cyclin-Dependent kinases
CDKN2A	Cyclin-dependant kinase inhibitor 2A
cDNA	Complementary DNA
CENP-E	centromere-associated protein
cfDNA	Circulating Free DNA
cfmiRNA	Circulating Free MicroRNA
CIMP	CpG island methylator phenotype
CIN	Chromosomal Instability
CNV	Copy number variations
COLD-PCR	Co-amplification at Lower Denaturation Temperature PCR
COSMIC	Catalogue of somatic mutations in cancer
COX-2	Cyclooxygenase-2

CRC	Colorectal Cancer
Ct	Cycle Threshold
DCC	Deleted in Colorectal Carcinoma
DFS	Disease Free Survival
DMEM	Dulbecco's Modified Eagle's Medium
dMMR	Deficient Mismatch Repair
DNA	Deoxyribonucleic Acid
DNMT	DNA methyltransferase
dPCR	Digital PCR
dsDNA	Double Stranded DNA
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EMAST	Elevated Microsatellite Alterations at Selected Tetranucleotides
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal-regulated Kinase
FAK	Focal adhesion kinase
FAP	Familial Adenomatous Polyposis
FBS	Fetal Bovine Serum
FFPE	Formalin-Fixed Paraffin-Embedded
FOLFOX	Folinic acid (Leucovorin) Flourouracil (5-FU) and Oxaliplatin (Eloxatin)
GAPDH	GlycerAdehyde-3-Phosphate DeHydrogenase
gDNA	Genomic DNA
GNAS	Guanine Nucleotide Binding Protein
GPCR	G-Protein Coupled Receptor

Gsα	alpha G protein subunit
GTP	guanosine 5’ triphosphate
HER	Human Epidermal Growth Factor Receptor
HIF1	Hypoxia Inducible Factor-1
HNPCC	Hereditary non-polyposis colorectal cancer
HRM	High Resolution Melting
IHC	Immunohistochemistry
ILGF	Insulin-like Growth Factor
IPMN	Intraductal Papillary Mucinous Neoplasm
KRAS	Kirsten Rat Sarcoma Viral oncogene Homolog
LEF	Lymphoid Enhancer Factor
LOH	Loss of Heterozygosity
LS	Lynch Syndrome
MAPK	Mitogen Activated Protein Kinase
mCRC	Metastatic Colorectal Cancer
MDM2	Murine/Human Double Minute 2
MGMT	O-6-Methylgaunine DNA Methyltransferase
MINT	Mutant in Tumour
miRNA	MicroRNA
MLH1	MutL Homolog 1
MMR	Mismatch Repair
MSI	Microsatellite Instability
MSI-H	Microsatellite Instability High
MSI-L	Microsatellite Instability Low
MSS	Microsatellite Stable
mTOR	Mammalian Target of Rapamycin
NGS	Next Generation Sequencing

NSAID	non-steroidal anti-inflammatory drugs
PAP	pyrophosphorolysis-activated polymerisation
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factors
PDGH	15-prostaglandin dehydrogenase
PDM	Pre-diagnostic Multiplex
PGF	Placental Growth Factors
PI3K	phosphatidylinositol-3-kinase
PIK3CA	Phosphatidylinositol-3-kinase Catalytic Alpha
PIP	phosphatidylinositol 4,5 biphosphate
pMMR	Proficient Mismatch Repair
PTEN	Phosphatase and tensin homolog
PVDF	PolyVinyliDene Fluoride
QMC	quick-multiplex consensus
qPCR	Quantitative Polymerase Chain Reaction
RAF	Rapid Accelerated Fibrosarcoma
RAS	Rat Sarrcoma
RIPA	Radio Immunoprecipitation Assay
RNA	Ribonucleic Acid
RT-PCR	Real-time Polymerase Chain Reaction
SEPT9	Septin 9
siRNA	Small Interfering RNA
SMAD4	Mother Against Decapentaplegic homolog 4
SSAs	Sessile Serrated Adenomas
SSD	Single Specific Diagnostic
STAT	Signal Transducer and Activator of

	Transcription Factor
Ta	Annealing Temperature
TBST	Tris Buffered Saline-Tween
Tc	Critical Temperature
TCF	T-cell Factor
TGF- β	Transforming Growth Factor beta
TGF-a	Transforming Growth Factor alpha
TGFβRII	Transforming Growth factor-beta Receptor 2
Tm	Melting Temperature
TP53	Tumour Protein 53
VEGF	Vascular Endothelial Growth Factor
V*EGFR*	Vascular Endothelial Growth Factor Receptor
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
WT	Wild-Type
XELOX	Xeloda (capecitabine) and Eloxatin (oxaliplatin)
ZEB1	Zinc-finger E-box binding homeobox

Abstract

Colorectal cancer (CRC) is the third most common cancer worldwide and the fourth most common cause of death. This complex disease process starts in the colon or rectum, as a non-cancerous polyp which can become malignant over time. In the later stages of CRC, tumour cells become detached from the primary tumour, migrate and enter the blood or lymphatic vessels and ultimately form a secondary tumour at another site (distant metastasis). CRC metastasis may also arise from residual tumour cells that persist after treatment.

Identifying biomarkers for potentially metastatic disease or residual disease may provide novel tools for early detection and therapy monitoring patients prior to metastasis and tumour recurrence. Genetic and epigenetic changes are required during every step in metastatic spread and these may have use as biomarkers as well as providing information about the mechanisms behind these changes. In addition, tumour cells release cellular contents into the bloodstream as a consequence of cell death during the metastatic process. These circulating free (cf) contents have potential to be cancer biomarkers for treatment monitoring and residual diseases detection. This thesis investigated the molecular events associated with metastatic CRCs in order to improve diagnosis and prognosis in this disease.

DNA was extracted from 82 cases of formalin-fixed paraffin-embedded (FFPE) human CRCs. Mutation and methylation analysis was performed by QMC-PCR followed by high resolution melting (HRM) analysis. Using statistical methods, we analysed the association between the targeted mutations and lymph node involvement, local recurrence, and distant metastasis. The findings showed significant association of *KRAS, PIK3CA, PTEN, SMAD4* mutations and *P16* promoter methylation with lymph node involvement, advanced disease and local/metastatic recurrences.

The study also confirmed that CRCs with microsatellite instability (MSI) were significantly associated with mutant *BRAF*. MSI occurs in sporadic tumours and tumours arising in Lynch Syndrome and *BRAF* is commonly found in sporadic tumours but almost never in Lynch Syndrome tumours. However not all sporadic tumours with MSI have mutation in *BRAF* and therefore a new assay was developed to discriminate sporadic tumours with MSI from tumours arising due to Lynch Syndrome.

In order to develop tests to test for residual disease, blood samples of 25 CRC patients were collected pre-operation and daily post-operation (until discharge) and plasma was extracted for the analysis of *cfDNA*/*ctmiRNA* following operation. The matched primary tumours were also collected. A protocol for COLD-HRM (a combination of COLD-PCR and HRM designed for detection of low frequency of mutant alleles) was optimized to screen for *KRAS* and *BRAF* mutation. This protocol was subsequently used to screen *cfDNA* for mutations. *ctmiRNA* expression was quantified by Q-PCR. Findings in this study showed that patients can be divided into a group which either loses or retains mutant *cfDNA*/*ctmiRNA* following operation. Detection of mutations in *cfDNA* is a good means of non-invasive screening for CRC and may provide a novel method of assessing surgical clearance and testing for recurrence.

The activation of *GNAS1* by mutation leads to several biological possibly metastasis promoting events including cell proliferation, survival and motility. *GNAS1* was found to be mutated in CRCs and therefore investigated for its activity in CRC cell lines. *GNAS1* was knocked-down in two CRC cell lines (RKO, and SW620). Gene knockdown was undertaken by transfecting small interfering RNAs into the cells and this was followed by an evaluation of cell proliferation and motility. The findings of this study revealed that inhibition of *GNAS1* expression does not show any effects on cell proliferation or migration in the CRC cell lines, RKO and SW620.

In conclusion, this study identified specific targets, such as *KRAS, PIK3CA, PTEN, SMAD4* mutations and *P16* promoter methylation, in correlation with lymph node involvement, advance stage CRCs and local/distant recurrences. Further analysis and investigation for their functional role in CRC progression is required to further identify their exact impact on CRC cell proliferation and motility. This study also confirmed that *cfDNA/cfmiRNA* is detectable in plasma of CRC patients and may provide potential biomarker for surgical clearance and residual disease. In addition, it was shown that *GNAS1* knock-down did not increase both cell proliferation and migration in the CRC cell lines, RKO and SW620. However, further validation for these findings may enhance the understanding of these molecular markers in invasion-metastatic transformation in CRC.

Thesis-related Publications and Conference Communications.

Henry O. Ebili, James C. Hassall, **Abutaleb Asiri**, Hersh Ham-Karim, Wakkas Fadhil, Ayodeji Johnson Agboola, Mohammad Ilyas. (2017) 'QMC-PCRx: A novel method for rapid mutation detection', Journal of Clinical Pathology, 70(8), pp. 702–711. doi: 10.1136/jclinpath-2016-204264.

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Hersh Ham-Karim, Henry Okuchukwu Ebili, Wakkas Fadhil, **Abutaleb Asiri**, James Hassall, Mohammad Ilyas. cold-hrm: A combination of methods to infer the nature of somatic mutations. Adv Cytol Pathol 2017; 2(2): 1-9.

Susanti Susanti*, Wakkas Fadhil*, Henry Okuchukwu Ebili*, **Abutaleb Asiri,** Ausrine Nestarenkaite, Efthymios Hadjimichael, Hersh A Ham-Karim, Joanne Field6, Katherine Stafford6, Balwir Matharoo-Ball7, James C Hassall1,3, Abid Sharif6, Anca Oniscu8, Mohammad Ilyas (2018). 'N_LyST: a simple and rapid screening test for Lynch syndrome.', Journal of clinical pathology, p. jclinpath-2018-205013. doi: 10.1136/jclinpath-2018-205013

Presentation and Conference Communication

Abutaleb Asiri , James Hassall , Hersh Ham-Karim , Henry Okuchukwu Ebil1, Wakkas Fadhil , Oliver Ng, Peter Fardouly, Austin G Acheson, and Mohammad Ilyas. Detection of mutant circulating free DNA as a biomarker of colorectal cancer. 10th Joint Meeting of the British Division of the International Academy of Pathology and the Pathological Society of Great Britain & Ireland- poster presentation.

Abutaleb Asiri, James Hassall, Hersh Ham-Karim, Henry Okuchukwu Ebili, Wakkas Fadhil, Oliver Ng, Peter Fardouly, Austin G Acheson, and Mohammad Ilyas.Circulating free DNA in colorectal cancer as a marker of surgical clearance. NCRI Cancer Conference- poster presentation.

Abutaleb Asiri, Hersh Ham-Karim , Wakkas Fadhil, James Hassall and Mohammad Ilyas. The clinicopathological significance of molecular alterations in colorectal cancer patients. 9th Saudi Student Conference in the UK - poster presentation.

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1 General Introduction

1.1 Colorectal Cancer

1.1.1 Epidemiology and Aetiology

Colorectal cancer (CRC) is a major cause of morbidity and mortality worldwide. It is considered as the third most common cancer and fourth most common cause of death in the world. CRC has 5-year survival rate of 90% if detected at the localised stage, 70% detected at more advanced stage, and 10% for those diagnosed with distant metastatic CRC (Haggar and Boushey, 2009). Several risk factors have been associated with colorectal cancer categorised into modifiable and non-modifiable (Taunk and Calderwood, 2015). Modifiable risk factors include those that can be avoided such as smoking, alcohol consumption, obesity and dietary, whereas non-modifiable risk factors are uncontrollable including increasing age, history of adenomatous polyps, personal history of inflammatory bowel disease and genetic inheritance (Haggar and Boushey, 2009; Edwards *et al.*, 2010; Tarraga, Albero and Montes, 2014). Ninety percent of those diagnosed with CRC are aged 50 or older and the risk is five times higher in people aged between 60 and 79 years than in those younger than 40 years (Taunk and Calderwood, 2015). The presence of family history of CRC increases the risk of developing CRC, although only 20% of CRC patients have a family history of this disease. Approximately 5-10% of CRCs are due to hereditary conditions, most commonly a consequence of familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC), also known as Lynch syndrome (World Cancer Research Fund, 2007; DeVita VT, Lawrence TS, 2011).

Various genes have been associated with these forms of inherited conditions in CRC. Mutations in genes involved in the MMR system, such as *MLH1, PMS2, MSH6* and MSH2, are reported to be responsible for HNPCC (also called Lynch syndrome), which accounts for approximately 2-6% of all CRCs. People with a HNPCC-related mutation are found to be at a risk of 80% to develop CRC (Papadopoulos *et al.*, 1994; World Cancer Research Fund, 2007). CRCs arising from Lynch syndrome are located on the right side of the

colon and develop from adenomas that are often proximal and tend to be larger with high-grad dysplasia and/or villous histology in comparison to sporadic adenomas (Kwak and Chung, 2010; Win and Lindor, 2017). Lynch syndrome adenomas also progress more rapidly in the adenoma carcinoma sequence as compared to those evolving from sporadic adenomas (Møller *et al.*, 2017; Win and Lindor, 2017). Mutations in MMR genes are also associated with a higher risk of developing other cancers, including pancreas, stomach, small bowel, uterus and kidney cancers (Haggar and Boushey, 2009). FAP is found in only 1% or less of total CRC cases and it is a consequence of mutations in the adenomatous polyposis coli (*APC*) tumour suppressor gene (Umar *et al.*, 2004; Davies, Miller and Coleman, 2005). Many people with FAP normally develop numerous polyps and a few of these polyps usually transform into malignant tumours with age. Furthermore, sporadic CRCs are responsible for 70% of overall CRC and most individuals develop this condition aged 70 years or older. Sporadic CRCs are a result of a series of genetic mutations in various tumour suppressor genes and oncogenes (Haggar and Boushey, 2009).

1.1.2 Staging

CRC staging is a necessary process in cancer management that provides information for the extent of disease and selection of the most suitable treatment for the patients (Wu, 2007). Several staging systems have been introduced for this purpose, most commonly Tumour, Node Metastasis (TNM) and Dukes' staging systems. TNM was first introduced by the American Joint Committee on Cancer (AJCC) and it is based on the extent of the primary tumour (T), involvement of the lymph node (N), and presence of metastases (M) (Washington, 2010). The TNM staging system is shown in figure 1-1 and summarised in Table 1-1. Alternatively, Dukes' staging was proposed by Cuthbert Dukes in 1937 and it has been modified to improve prognostic classification and involve both colon and rectal cancers, as well as including the "D" stage which refers to the presence of metastases. Dukes A indicates a tumour confined in the mucosa, whilst B describes

the invasion through the bowel wall and penetration of the muscularis propria, but not involving the lymph node (Akkoca *et al.*, 2014). Stage C describes the involvement of the lymph node where C1 means the tumour is extending into muscularis propria and involving 1-3 of regional lymph node and C2 describes tumour penetrating through the muscularis propria with at least 4 regional lymph nodes involved. Stage D has been modified recently by Astler and Coller to describe the presence or absence of distant metastases (Washington, 2010; Akkoca *et al.*, 2014).

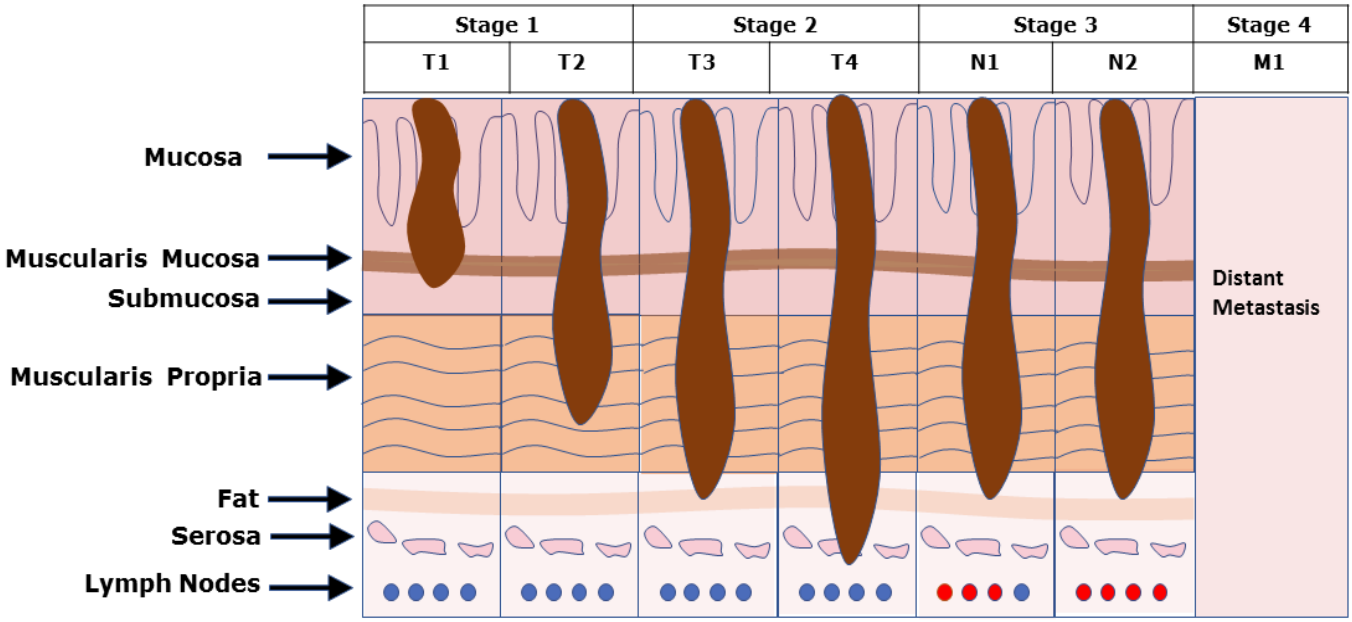

Figure 1-1: TNM staging system of CRC.
The TNM staging system is categorised into four stages which evaluates the size and depth of the tumour (T1-T4), involvement of lymph node penetration (N0-N2), and the presence and absence of distant metastasis (M0 or M1).

Table 1–1: TNM classification system

TNM Classification			
Primary Tumour (T)		**Regional Lymph Node (N)**	
T0	Tumour is not invasive	N0	No regional lymph node
T1	Tumour has grown through mucosa and invades submucosa	N1	1-3 regional lymph nodes involved
T2	Tumour has grown into the muscularis propria	N1a	One regional lymph node involved
T3	Tumour has grown through entire colon or rectum and penetrates through muscularis propria into serosa	N1b	2-3 regional lymph nodes involved
T4a	Tumour Penetrates visceral peritoneum	N1c	Tumour deposits in the subserosa, without regional lymph node metastasis
T4b	Tumour directly invades other organs or structures	N2	4 or more regional lymph nodes involved
		N2a	4-6 regional lymph nodes involved
		N2b	7 or more regional lymph nodes involved
		Distant Metastasis (M)	
		M0	No spread to distant organs
		M1	Tumour is confined in secondary site (Metastasis)
		M1a	Tumour metastasis is confined to one organ or site
		M1b	Metastasis is confined in more than one organ or site

1.1.3 Pathogenesis of Colorectal Cancer

Colorectal cancer (CRC) is a complex disease with various molecular pathways causing diverse phenotypes. The alterations, which are genetic and epigenetic in nature, act to dysregulate conserved signalling pathways involved in cellular metabolism, proliferation, differentiation, survival, and apoptosis (Markowitz and Bertagnolli, 2009). In both the prognosis and management of CRC, the consideration of the molecular basis of colorectal carcinogenesis has significant consequences. It is believed that outcome for CRC results will be enhanced by improving the screening and examination protocols, as well as the evaluation of the disease stage, and individualising treatment based on pathologic and molecular characteristics of the tumours (Markowitz and Bertagnolli, 2009). Various gene mutations have been associated with colorectal carcinogenesis, but their involvement in the progression and prognosis of the disease remains unclear. Certain genes, specifically *APC*, Kirestein rat sarcoma (*KRAS*), and p53, have been commonly found mutated at in CRC (Starr *et al.*, 2009).

A series of events causes colorectal cancer, causing the transformation of normal mucosa to adenoma and eventually, carcinoma. An essential part in this transformation process is genomic instability (Al-Sohaily *et al.*, 2012). CRCs can arise from one of the three specific molecular pathways, namely chromosomal instability (CIN) pathway, microsatellite instability (MSI) pathway, and the CpG island methylator phenotype (CIMP) pathway (Figure2-1) (Worthley *et al.*, 2010; Colussi *et al.*, 2013). The classical CIN pathway is characterised by various genomic alterations starting with the acquisition of *APC* mutations, usually followed by the activation of *KRAS* mutation, and inactivation of the tumour suppressor gene *TP53* (Pino and Chung, 2010; Colussi *et al.*, 2013) Aneuploidy and loss of heterozygosity (LOH) are prominent features involved in 85% of sporadic CRC and found to be associated with *APC* germline mutations(Pino and Chung, 2010). Aneuploidy arises from a defect in the mitotic checkpoint leading to chromosomal abnormalities. Mutational changes in many mitotic components, such hRod, mitotic

arrest deficient (MAD), and centromere-associated protein (CENP-E), are one of the most common events in CIN (Pino and Chung, 2010; Al-Sohaily *et al.*, 2012)

The MSI pathway involves the development of insertion-deletion mutations within short repeat sequences (microsatellites) and it is detected in 10–15% of sporadic colorectal cancers. This is as a result of failure of the DNA mismatch repair (MMR) system to fix errors occurring during DNA replication in one of the MMR genes, such as *MLH1*, MSH2, and M2H6 (Al-Sohaily *et al.*, 2012).

The CIMP pathway is characterised by hypermethylation in the promoter region of different tumour suppressor genes including *MLH1*, *MGMT*, *APC* and *CDKN2A*/*P16*, commonly used CIMP markers (Al-Sohaily *et al.*, 2012; Arends, 2013). Hypermethylation analysis revealed significant correlation between CIMP and different clinicopathological features, as well as MSI and *BRAF* mutations. These three pathways are not specific, because there are certain tumours that exhibit characteristics of more than one pathway (Goel *et al.*, 2007).

Recently, gene expression-based classification system of CRC known as consensus molecular subtypes (CMSs) has been introduced based on consensus between different previous classification systems (Guinney *et al.*, 2015; Roelands *et al.*, 2017; Thanki *et al.*, 2017). CMSs are divided into four subtypes: CMS1 is characterised by MSI, *BRAF* mutations, hypermethylation in CpG islands and a diffuse immune infiltrate; CMS2 features high chromosomal instability and activation of Wnt and MYC pathways; CMS3 is characterised by *KRAS* mutations and disrupted metabolic pathways; CMS4 is composed of activation of TGFβ and mesenchymal phenotype which consists of stromal infiltration and angiogenesis (Roelands *et al.*, 2017; Thanki *et al.*, 2017).

However this thesis focused on the three well-known pathways (CIN. MSI, and CIMP pathways) figure 1-2.

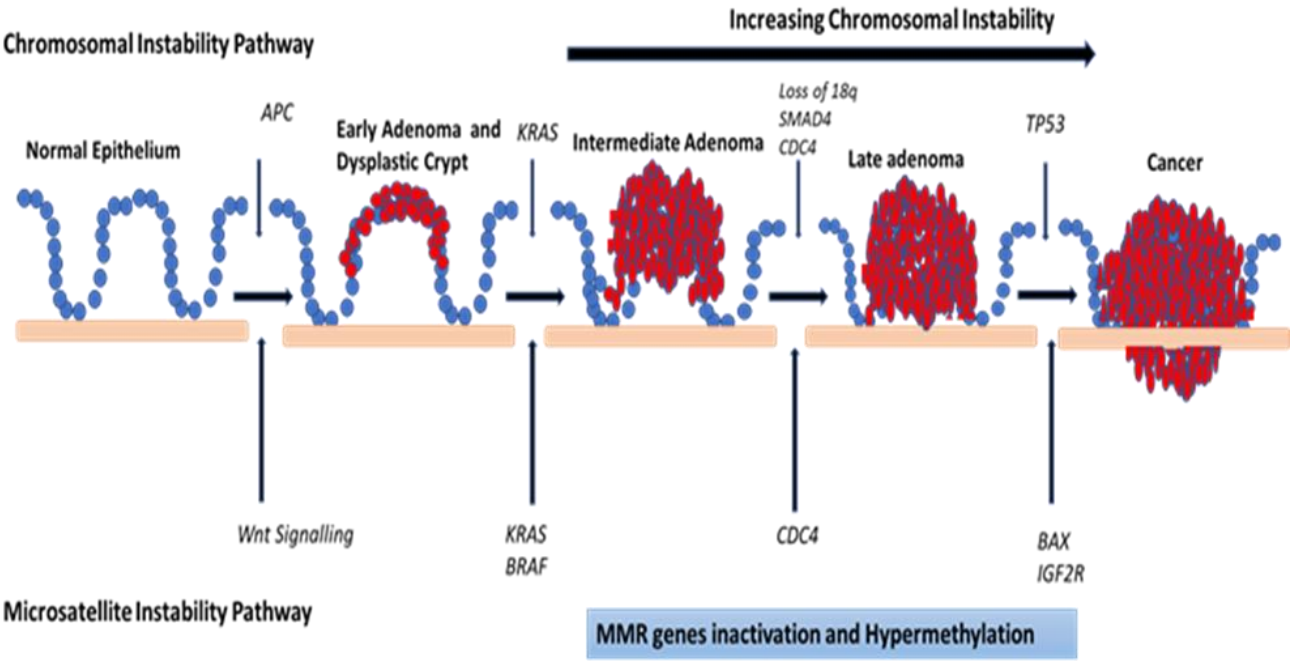

Figure 1-2: Molecular pathways driving the progression of CRC
The initiation and progression of colorectal adenocarcinoma from normal epithelium to carcinoma are driven by various molecular alterations. These genetic events including mutations in APC, wnt signalling, KRAS/BRAF, Loss of 18q, SMAD4, CDC4, TP53, BAX, and IGF2R as well as MMR genes inactivation and Hypermethylation occurred during the three classical pathways; CIN, MSI and CIMP pathways.

1.1.3.1 Chromosomal Instability (CIN)

The first model for colorectal carcinogenesis was initially proposed by Vogelstein and colleagues in 1980s. The most familiar cause of genomic instability in CRC is chromosomal instability, as it is responsible for 65–70% of sporadic CRC (Vogelstein *et al.*, 1988). It features either the gain or loss of whole chromosomes or chromosomal regions harbouring genes essential for the process of colorectal carcinogenesis (Jemal *et al.*, 2008). The first step in the adenoma–carcinoma sequence involves this transition from normal epithelium to a malignant tumour. This sequence includes a series of genetic alterations of various genes leading to the formation of an adenocarcinoma from a benign colorectal adenoma (Al-Sohaily *et al.*, 2012). Mutation in the *APC* tumour suppressor gene is considered to be one of the early events in initiating carcinogenesis (Fodde, 2002; Smith *et al.*, 2002). This process is usually combined with a *KRAS* mutation to promote tumour progression (Smith *et al.*, 2002). Carcinogenesis promoting mutations in other genes, such as *BRAF*, *TP53*, *PIK3CA* and TGF-β, are also acquired. These mutational events are further described in the sections below.

Genetic alterations in the Wnt signalling pathway have been observed in both sporadic and hereditary (FAP) CRC tumours defining abnormalities at chromosome 5q (Colussi *et al.*, 2013; Novellasdemunt, Antas and Li, 2015). The inactivation of the Wnt pathway is a result of frequent mutational events in various oncogenes and tumour suppressors such as *APC*, *B-catenin* and *AXIN2* (Fodde, 2002; Arends, 2013). The *APC* gene encodes a protein that possesses different functional domains in the Wnt signalling pathway and regulates the concentration of a variety of intracellular proteins including B-catenin (Al-Sohaily *et al.*, 2012). In sporadic tumours, most *APC* mutations are frameshift and nonsense mutations which lead to premature protein degradation (Arends, 2013; Novellasdemunt, Antas and Li, 2015). Mutations in the *APC* gene are one of the earliest events which lead to chromosomal instability and are detected in 90% of all CRCs. Accumulation of cytoplasmic *β-catenin* occurs on the loss of the *APC* function (through

mutation, LOH, or promoter methylation), which leads to nuclear translocation and β-catenin binding to T-cell factor (TCF)/lymphoid enhancer factor (LEF) (Arends, 2013; Colussi *et al.*, 2013). This then results in the activation of *Wnt* signalling pathway. Multiple cellular functions are affected by the Wnt target genes such as regulators of cell cycle progression (c-myc and cyclin D1), cell proliferation, angiogenesis, and apoptosis (Behrens, 2005). There are various triggers for Wnt activation other than the loss of *APC* function, such as activating β-catenin mutations, AXIN1 and AXIN2 mutations (which are important for β-catenin degradation), or mutations being activated in the transcription factor TCF-4 (Al-Sohaily *et al.*, 2012; Colussi *et al.*, 2013)

The epidermal growth factor receptor (*EGFR*) is a 170-kDa transmembrane tyrosine kinase receptor that belongs to the HER-erbB family of receptor tyrosine kinases, which is a cell surface receptor. In normal tissues, *EGFR* on activated ligand initiates the signalling cascade by binding to ErbB family receptors such as Epidermal Growth factor (EGF), transforming growth factor α (*TGF-α*), amphiregulin, betacellulin, and epiregulin (Barton, Starling and Swanton, 2010; Winder and Lenz, 2010). Ligand binding induces the phosphorylation of various intracellular tyrosine kinase components which activate two main downstream signalling pathways; mitogen activated protein kinase (MAPK) pathway and the phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT) pathway (Figure 1-3) (Al-Sohaily *et al.*, 2012; Colussi *et al.*, 2013). The activation of these intracellular pathways induces multiple transcription factors crucial for the regulation of cell proliferation, migration, survival and motility. *EGFR* overexpression has been detected in many types of cancers, such as lung, neck, breast or CRC tumours, and is reported to be strongly associated with tumorigenesis and poor survival. In CRC, enhanced activity of *EGFR* has been found in 25–82% of all cases and more frequently found in advanced stage tumours suggesting that *EGFR* may predict a potential metastatic risk; although there is ambiguity regarding its impact on survival (Kanthan, Senger and Kanthan, 2012)..

Phosphatidylinositide-3-kinases (PI3K) are lipid kinases that are responsible for many biological events including cellular proliferation and survival. PI3K is characterised into different classes depending on the structural and functional differences (Papadatos-Pastos *et al.*, 2015). Mutation in the *PIK3CA* (p110 subunit) is detected in 10–20% of CRC tumours, mostly involving hotspots on exons 9 and 20 (Cantley, 2002; Yuan and Cantley, 2008). Activating mutations in *PIK3CA* induce the activation of the PI3K pathway in cancer cells and are strongly associated with mucinous differentiation, tumour location as well as *KRAS* mutation (Papadatos-Pastos *et al.*, 2015). Activated PI3K causes phosphorylation of phosphatidylinositol 4,5 biphosphate (PIP2) to phosphatidylinositol 3, 4, 5 biphosphate (PIP3), leading to a subsequent accumulation of PIP3 at the membrane(Alessi *et al.*, 1997; Cantley, 2002) This process results in phosphorylation of AKT by PKD1 and mTORC2, promoting cell growth and survival (Alessi *et al.*, 1997). AKT is an anti-apoptotic kinase within the cytoplasm which activates various targets, such as IkB kinase, in cell growth, proliferation and survival through regulating the transcription of anti-apoptotic genes (Papadatos-Pastos *et al.*, 2015). It also plays a critical role in angiogenesis by activating endothelial nitric synthase as well as activation of telomerase. Thus, AKT activation involved in cell growth and promotion may promote tumour invasion and metastasis by stimulating the matrix metalloproteinase protein (Papadatos-Pastos *et al.*, 2015). The activation of PI3K and AKT pathways has been shown to be associated with the anti-apoptotic effects in some cell cycle types affecting ErbB receptor signalling. Major proteins involved in cell cycle regulation are overexpressed as a consequence of MAPK activation (Cathomas, 2014; Papadatos-Pastos *et al.*, 2015). Consequently, the identification of *EGFR* signalling inhibitors may provide promising therapeutic strategies to minimise tumour growth during tumour progression by controlling the two main mechanisms, apoptosis and cell proliferation (Yu *et al.*, 2008; Shimizu *et al.*, 2012). *PTEN* is a tumour suppressor gene encoding a protein tyrosine phosphatase enzyme (*PTEN*) that acts as a down-regulator for PI3K by dephosphorylating PIP3 (Chalhoub and Baker, 2009). Loss of *PTEN*

expression is a common event in many cancers, which is believed to be a consequence of either somatic mutations or epigenetic silencing by promoter hypermethylation (Goel *et al.*, 2004; Sood *et al.*, 2012) Mutations and LOH of *PTEN* have been detected in 13–18% and 17–19%, respectively, of all CRC cases. In CRC, *PTEN* alteration results in the activation of PI3K-AKT pathway, suggesting that this valuable element can be employed in therapeutic research (Chalhoub and Baker, 2009; Papadatos-Pastos *et al.*, 2015)

KRAS is a vital activator within the *EGFR* pathway, which plays a critical role in tumour formation by regulating essential proteins involved in many mechanisms including cell proliferation, survival and metastasis (Starr *et al.*, 2009). The *KRAS* proto-oncogene is a member of the RAS family and encodes a 21-kDa guanosine 5' triphosphate- (GTP-) binding protein downstream of *EGFR* in the PI3K/*PTEN*/AKT and RAF/MEK/ERK signalling pathways. *KRAS* mutations have been detected in approximately 40% of CRC , mostly found in intermediate adenoma sequence, and is one of most common proto-oncogenes involved in early colorectal carcinogenesis (Bos, 1989; Adjei, 2001; Leslie *et al.*, 2002; Malumbres and Barbacid, 2003). *KRAS* mutations occurred following the inactivation of *APC* tumour suppressor gene in the CIN pathway and appear to be associated with advanced adenomatous lesions (Kinzler and Vogelstein, 1996; Pino and Chung, 2010; Armaghany *et al.*, 2012). This might provide evidence of mutant *KRAS's* role in promoting colon cancer formation as an early event in the adenoma carcinoma sequence by mediating adenoma growth (Kinzler and Vogelstein, 1996). Mutations in *KRAS* are a predictive marker of the therapeutic responses in CRC patients, due to the association with clinical resistance to different chemotherapies such as cetuximab and panitumumab (Lièvre *et al.*, 2008; Douillard *et al.*, 2010; Van Cutsem *et al.*, 2011). *BRAF* (B-raf murine sarcoma viral oncogenes homolog) is a common proto-oncogene member of the RAF gene family encoding a downstream effector of activated RAS, the so called serine-threonine protein kinase (Barras, 2015; Clarke and Kopetz, 2015). *BRAF* somatic mutation is one of the oncogenic events involved in the of 10% of CRC patients (Joneson

and Bar-Sagi, 1997; Davies *et al.*, 2002; Michaloglou *et al.*, 2008) The V600E mutation within the kinase activation domain of the *BRAF* protein is the most frequent, accounting for 80% of all *BRAF* mutations which lead to resistance to targeted therapies (Corcoran et al., 2015; Lea et al., 2010). This allows the *BRAF* mutation to develop in cancer cells without functional RAS, therefore *BRAF* and *KRAS* mutations are found to be exclusive to CRC. Many studies have reported a significant correlation of *BRAF* mutations with different clinicopathological features, such as high grade, gender and age, as well as associated with sporadic MSI-high CRC tumours (French *et al.*, 2008; Chen *et al.*, 2014; Sideris *et al.*, 2015) In addition, *BRAF* mutations have been observed in metastatic tumours and associated with poor survival in many types of cancer (Michaloglou *et al.*, 2008; Tol, Nagtegaal and Punt, 2009; Tran *et al.*, 2011; Yaeger *et al.*, 2014)

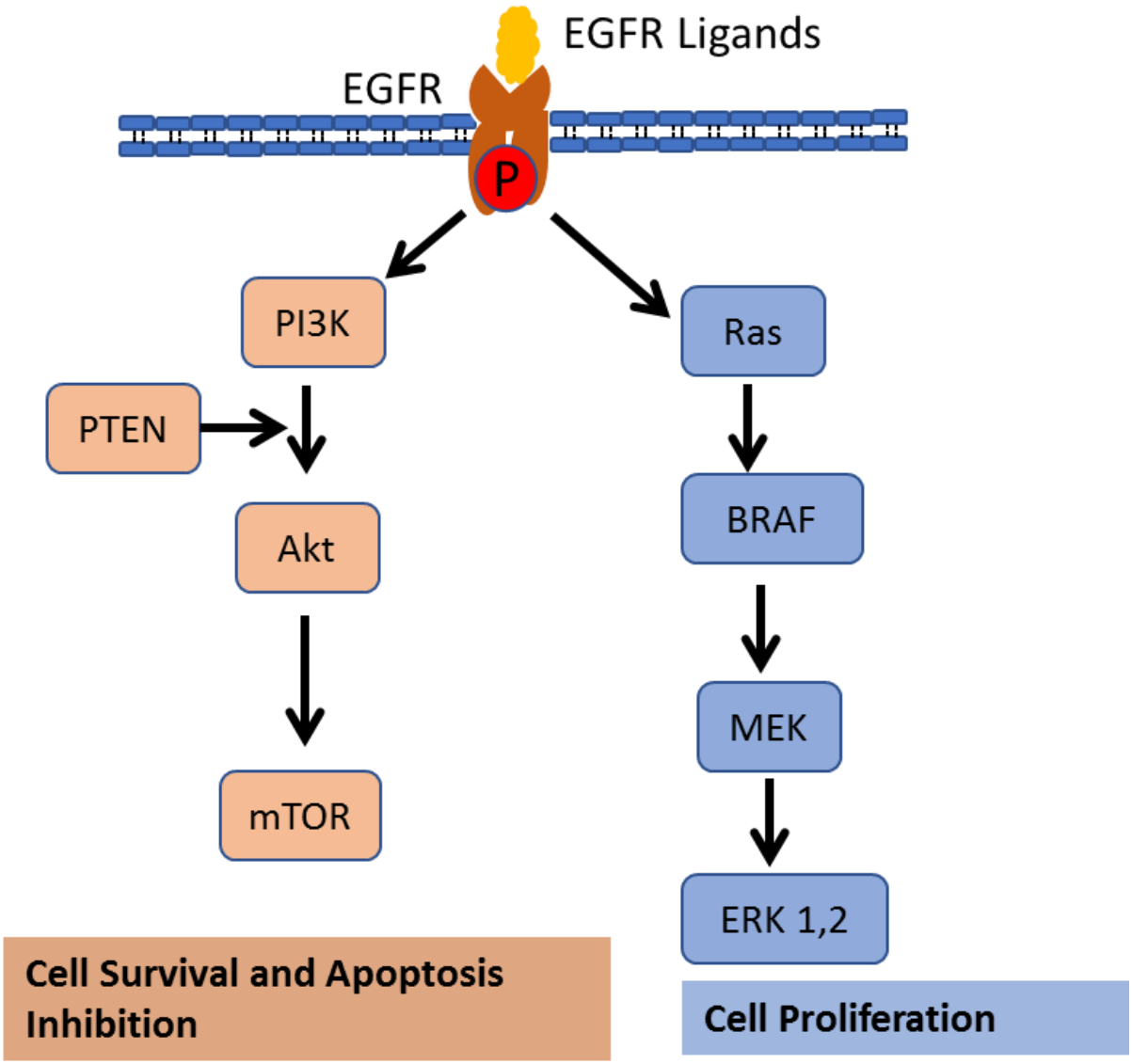

Figure 1-3: The EGFR pathway.
EGFR on ligand activates different pathways including RAS/RAF/MEK and PI3K/PTEN/AKT pathways causing cell survival, apoptosis inhibition, and cell proliferation.

LOH at chromosome 18q is a major step in the adenoma carcinoma sequence and has been identified in 70% of primary CRC tumours. Various tumour suppressor genes, such as Cables, Deleted in Colorectal Carcinoma (DCC), Smad2, and *SMAD4* map to 18q with chromosomal abnormalities in this region causing loss of expression of these genes (Rowan *et al.*, 2005; Pino and Chung, 2010). *TP53* (tumour protein 53) is a tumour suppressor gene located on the short arm of chromosome 17 and is induced by several oncogene proteins such as c-myc, RAS and adenovirus E1A(Levine, 1997; Rowan *et al.*, 2005; Pino and Chung, 2010; Saha, Qiu and Chang, 2013; X. L. Li *et al.*, 2015). It is known as a genomic guardian that maintains the genomic stability by regulating different transcriptional genes involved in cell cycle, DNA metabolism, apoptosis, motility, cell differentiation and migration (Pino and Chung, 2010). *TP53* is a well-studied gene in the genetic pathways of many cancers in relation to its loss of function or LOH. *TP53* negatively regulates MDM2 (murine/human double minute 2) which functions as an E3 ubiquitin-ligase that targets *TP53* ubiquitination and degradation. This process maintains a low level of *TP53* in normal cells (Pino and Chung, 2010). During the presence of excessive levels of cellular stress, *TP53* plays a vital role in induction of genes involved in cell division, growth arrest and apoptosis following DNA damage. Inactivation of *TP53* plays a pivotal role in the development of CRC, including the transition for adenoma to carcinoma (López *et al.*, 2012). Mutation in the *TP53* tumour suppressor gene has been reported in 4–26% of early adenoma, 50% of late adenomas and 50–75% of CRC. The majority of *TP53* mutations occur in exons 5–8 (Leslie *et al.*, 2002; Béroud and Soussi, 2003). Mutations in *TP53* have been found to be associated with various pathological events of CRC including invasion and metastasis as well as having prognostic value. It also has been reported that there is a significant correlation of mutant *TP53* with lymphatic and vascular invasion in CRC (Antonio Russo *et al.*, 2005). Findings in different CRC studies observed that patients with a p53 mutation show resistance to chemotherapy and the mutation commonly occurs in advanced stage tumours and is related to poor survival in proximal colon (Iacopetta, 2003; Antonio Russo *et al.*, 2005).

Cables is a cell cycle protein that regulates the tyrosine phosphorylation levels of cyclin-independent kinases (cdk2, cdk3, and cdk5) (Wu *et al.*, 2001; Park *et al.*, 2007). In CRC, loss of expression of Cables is found in 60–70% of sporadic tumours and is suggested to be associated to CpG island hypermethylation in the promoter region coupled with LOH at 18q (Park *et al.*, 2007). *DCC* is a cell surface receptor for neuronal protein netrin-1 and it plays an important role in the regulation of cell adhesion and apoptosis (Mehlen and Fearon, 2004). *DCC* mutations are rare in CRC (6%), though they have been reported to be correlated with CRC development (Pino and Chung, 2010). SMAD proteins are transcription factors acting as intracellular mediators of the *transforming growth factor β* (*TGF-β*) signalling pathway (Pino and Chung, 2010). They are involved in regulation of cell growth, differentiation and apoptosis. TGF-β dimer binding to its receptors phosphorylates SMAD (R-SMAD). This phosphorylation has a transcriptional effect resulting from R-SMAD family binding to *SMAD4* forming a complex in the nucleus (Pino and Chung, 2010). Thus, many studies suggested that the transcription of the main target genes, such as c-myc, CBFA1, FLRF, and furin are regulated by Smad proteins (Mcdermott, Longley and Johnston, 2002). Other studies observed that claudin-1, a potential metastatic modulator, is down regulated by *SMAD4* in a *TGF-β* independent manner (Shiou *et al.*, 2007). *SMAD4* is mutated in approximately 15% of CRCs and associated with poor prognosis and advanced stage CRC (Takagi *et al.*, 1996, 1998)

1.1.3.2 Microsatellite Instability (MSI)

Microsatellites are small repeat nucleotide sequences found throughout the human genome. During DNA replication, the base pair mismatch errors are identified and conserved by the DNA mismatch repair (MMR) system (Al-Sohaily *et al.*, 2012). MSH2, *MLH1*, MSH6, PMS2, MLH3, MSH3 and PMS1 are the main members of MMR system with interact and form heterodimers (Boland and Goel, 2073). DNA damage is a result of cellular exposure of various endogenous and exogenous reactions (Schärer, 2003; Altieri *et al.*, 2008; Morita *et al.*, 2010). With regard to exogenous source, DNA damage is caused by induction of chemical reactive and physical agents, such as ultra violet (UV) radiation and alkylating compounds (Morita *et al.*, 2010; Ambekar, Hattur and Bule, 2017). Endogenous damage can be induced spontaneously by the deamination of cytosine, guanine, and adenine produce uracil, xanthine, and hypoxanthine, respectively (Ambekar, Hattur and Bule, 2017). These damages lead to biological consequences depending on the chemical nature of the lesion which affects the DNA replication leading to mutations (Morita *et al.*, 2010). The mechanism to fix these errors during DNA replication by MMR proteins includes three steps; first recognition of errors, excision step which includes the removal of errors containing strand, and finally gap repair by replacing it with resynthesized DNA (Matson and Robertson, 2006; Ambekar, Hattur and Bule, 2017). These three steps are guided by mutator genes conducting proteins such as Mut S, Mut L and Mut H (Morita *et al.*, 2010; Ambekar, Hattur and Bule, 2017). Mismatch in DNA sequence base is recognised by Mut S which binds the respective region, this process requires ATP (Matson and Robertson, 2006; Ambekar, Hattur and Bule, 2017). The formation of Mut S DNA complex is followed by Mut L complex which couple the recognition of sequence mismatch and brings the mispaired regions closer to form a look like structure around that region (Ambekar, Hattur and Bule, 2017). The DNA sequence surrounding the mismatch base is then cleaved by Mut H protein with the presence of endonuclease enzyme (Ambekar, Hattur and Bule, 2017). This process is followed by recruiting another protein UVr-D which functions as helicase to release the

strand out and form a gap within the DNA sequence (Matson and Robertson, 2006; Ambekar, Hattur and Bule, 2017). The corrected sequence is added by DNA polymerase and joined by ligase (Ambekar, Hattur and Bule, 2017). MSI refers to the failure of MMR system to fix these errors during DNA replication. This leads to genetic alteration and base pair mismatch alongside the microsatellite sequences. MSI was first introduced in 1990s and the majority of genetic defects were observed in HNPCC tumours (60%), with a low rate in sporadic tumours (15%) (Boland and Goel, 2010; Geiersbach and Samowitz, 2011; Al-Sohaily *et al.*, 2012). It has been reported that MSI sporadic tumours are caused by somatic mutation or hypermethylation of MMR genes. Hypermethylation silencing of *MLH1* is the common mechanism for MMR inactivation and responsible for the sporadic CRC tumours. MSI can be detected by polymerase chain reaction (PCR) amplification of specific MSI markers and categorised into MSI-H (high) or MSI-L (low) depending on the degree of instability at different loci. A board of five microsatellite loci containing two mononucleotide repeats (BAT25 and BAT26) and three dinucleotide repeats (D5S346, D2S123, and D17S250) are assessed to identify MSI, although other markers were introduced later (Murphy *et al.*, 2006; Boland and Goel, 2010). Instability of two or more markers identifies tumours with high frequency microsatellite instability (MSI-H), whereas instability of one marker refers to low frequency microsatellite instability (MSI-low). Tumours with no detected instability at any marker are considered as microsatellite stable (MSS)(Murphy *et al.*, 2006). Elevated microsatellite alterations at selected tetranucleotides (EMAST) is another form of MSI which has been observed in 60% of CRC (Carethers, Koi and Tseng-Rogenski, 2015). Dysfunction of MSH3 is associated with MSI-low and EMAST which causes dinucleotide and tetra nucleotide instability (Boland and Goel, 2010). When compared with MSS tumours, MSI-low tumours are linked with poorer patient survival (Kohonen-Corish *et al.*, 2005). MSI-H tumours are likely to be diploid, with less LOH and unlikely to be associated with *KRAS* and *P53* mutations (Söreide *et al.*, 2006). Mutations in *BRAF* are commonly found in sporadic MSI-high CRC, but rarely observed in HNPCC tumours

(Deng *et al.*, 2004). A variety of genes with coding repeats are affected in the presence of defective MMR function, such as genes associated with the DNA repair (*RAD50, MSH3, MSH6, BLM, MBD4, and MLH3*), signal transduction (*TGFβRII, ACTRII, IGFIIR, and WISP-3*), the cell cycle and apoptosis (*APAF1, BAX, BCL-10, and caspase 5, PTEN and RIZ*), and the transcription factor TCF-4 (Iacopetta et al., 2010). Although, MSI testing is a more favourable and sensitive method for Lynch Syndrome diagnosis, immunohistochemistry (IHC) is an alternative test for the expression of MMR proteins to identify loss of protein product of the affected MMR gene (Vilar and Gruber, 2010). Originally, the sensitivity and specificity of IHC analysis for *MLH1* and MSH2 were estimated as 92.3% and 100%, respectively (Ogino, Nosho, Irahara, *et al.*, 2009; Boland and Goel, 2010). Subsequent meta-analysis compared MSI with IHC analysis for *MLH1* and *MSH2* expression showed less sensitivity of IHC in the overall findings (Boland and Goel, 2010). The sensitivity of the test increased when *PMS2* and *MSH6* proteins were included in the analysis, but IHC staining still limited for such analysis by causing heterogeneity throughout the tumour section affecting the sensitivity of the test (Shia, 2008; Sinicrope and Yang, 2011). Employing antibodies against MMR proteins can provide information for any deficiency within the MMR system (Lindor *et al.*, 2002; Sinicrope and Yang, 2011). Loss of expression of one or more MMR proteins or MSI-H is collectively referred to as deficient MMR and can be indicative of gene inactivation or promoter hypermethylation (Boland *et al.*, 1998; Thibodeau *et al.*, 1998; Gafa *et al.*, 2000). However, testing the expression of specific heterodimers, such as *MLH1* expression, with IHC should be coupled with methylation analysis and somatic testing for *BRAF* V600E mutation to differentiate between sporadic CRC and Lynch syndrome (Sinicrope and Yang, 2011). Clinicopathological studies of MSI CRC showed that MSI cases are diagnosed at a lower disease stage, with longer survival times compared with MSS tumours. The findings showed that sporadic MSI are detected in older patients (>70 years old), whereas familial MSI are found in younger cases (<50 years old) (Gryfe *et al.*, 2000; Ward *et al.*, 2001; Jass *et al.*, 2002). Initially, Gryfe et al. (2000) explored the

prognostic value of MSI in CRC, demonstrating that MSI tumours had prognostic advantage and minor metastatic potential compared to MSS cases (Gryfe *et al.*, 2000). These results were validated in meta-analysis studies involving a large number of CRC patients confirming the prognostic importance of MSI-H in CRC tumours (Benatti *et al.*, 2005; Kets *et al.*, 2006; Lubbe *et al.*, 2009; Sinicrope and Yang, 2011). Conversely, the importance of MSI as predictive marker is still unclear due to the conflicting results published over the last decade. A small study by Elsaleh et al. (2001) demonstrated the effective role of MSI as a predictive factor in response to 5-FU-based chemotherapy in patients with MSI CRC stage III (Elsaleh and Iacopetta, 2001). In contrast, several studies compared MSI and MSS status in CRC patients receiving 5-FU showing a lack of predictive value of MSI (Ribic *et al.*, 2003; Müller *et al.*, 2008; Des Guetz, Uzzan, *et al.*, 2009). These discrepancies of the predictive value of MSI for the response to chemotherapy could be influenced by other molecular mechanisms involved in CRC carcinogenesis, such as *BRAF* mutations and *MGMT* hypermethylation, which may require further analysis.

1.1.3.3 CpG Island Methylator Phenotype (CIMP)

CpG islands are regions found within the genome promoter and up to 50% of human genes are thought to be regulated by methylation of these islands. CIMP was first recognised by Toyota and colleagues in 1999 in colorectal carcinogenesis (Minoru Toyota *et al.*, 1999). Alterations in gene expression or function without altering the DNA sequence of a particular gene are called epigenetic alterations, which take place at the 5'-CG-3' (CpG) dinucleotide(Coppedè, Lopomo, *et al.*, 2014). Other methods of epigenetic gene regulation include histone modifications, chromatin looping as well as non-coding RNAs (S Ogino *et al.*, 2006). It is essential that gene promoter is accessible to transcription factors prior proceeding with gene transcription (Watt and Molloy, 1988; Lim and Maher, 2010). DNA methylation changes chromatin structure which prevents transcription factors from binding to gene promoter (Lim and Maher, 2010). This process results in histone modification through the attraction of methylated CpGs with methyl-CpG-binding proteins which recruit repressor complexes (Lim and Maher, 2010). Histones are important proteins component of chromatin that play critical role in wrapping DNA which can be altered (Lim and Maher, 2010). Chromatin structure is then more condensed and restricted to transcription factor binding as a result from the modification of histones by repressor complexes (Jones *et al.*, 1998; Nan *et al.*, 1998; Bird and Wolffe, 1999; Lim and Maher, 2010). DNA methylation within the CpG region influences transcriptional silencing of various genes involved in several mechanisms such as cell cycle control, DNA repair, tumour suppression, apoptosis, and invasion (Coppedè, Lopomo, *et al.*, 2014). This phenomenon has been detected in various cancers including ovarian, lung, liver, breast and CRCs (Esteller *et al.*, 1999; Silva *et al.*, 1999; Wong *et al.*, 1999; Zou *et al.*, 2002). The insight of CIMP has led to the proposal of the third significant path in CRC pathogenesis driven by promoter hypermethylation. Most sporadic MSI and CIMP-positive are found exclusively in colon tumours, whereas CIMP is not associated with Lynch syndrome cases which exhibit MSI, providing further evidence of distinctive molecular process in colon carcinogenesis (McGivern *et al.*, 2004;

Weisenberger *et al.*, 2006). Approximately 15–20% of sporadic CRC are due to the CIMP-high CRC with distinctive features and are commonly found in older patients and proximal-site colon cancers. Large prospective cohort studies reported around 30-40% of sporadic CIMP are detected in the proximal colon, with approximately 3-12% in distal colon and rectal cancers (Hawkins *et al.*, 2002; Van Rijnsoever *et al.*, 2002; Barault *et al.*, 2008; English *et al.*, 2008; Slattery *et al.*, 2009). CIMP is also observed in sessile serrated adenomas (SSAs), which are usually the precursor lesions of CIMP-high tumours, accounting for 9% of colorectal polyps and also harbour *BRAF* mutations (Fernando *et al.*, 2014; East *et al.*, 2017). DNA methylation is an enzymatic process whereby a methyl group is added to the pyrimidine ring of cytosine within CpG dinucleotide sequences in CpG islands. This process is initiated by DNA methyltransferase (DNMT) to 5-methylcytosine at the 5-position of cytosine (Jin, Li and Robertson, 2011). DNA hypermethylation silences the genes concerned with colorectal carcinogenesis, such as *APC*, *MLH1*, *MGMT*, and *CDKN2A*. *MLH1*, a central MMR gene, has been reported to be hypermethylated in sporadic CRC tumours and strongly associated with MSI. Studies observed *MLH1* hypermethylation in sporadic MSI colon cancer, suggesting that aberrant methylation is an initiating process in CRC rather than a result of colon carcinogenesis (M Toyota *et al.*, 1999; Toyota, Issa and U, 1999; Samowitz *et al.*, 2006). Meta-analyses showed that the *MLH1* methylation at the CpG promoter region is regulated by the transcriptional status of the gene after exploration of the methylation status of specific CpGs in the *MLH1* promoter. Hakins and Ward confirmed the presence of *MLH1* hypermethylation in hyperplastic (serrated) polyps in MSI CRC tumours, indicating the significant role of such methylation in tumour progression to carcinoma. Conversely, recent reports showed the association of *MLH1* hypermethylation in normal mucosa and the development of MSI-high colon tumours. Other studies explored the frequency of *MLH1* hypermethylation in MSI-high tumours, reporting that approximately 80% of those hypermethylated were MSI-positive, with loss of expression and no aberrant mutation detected in the MMR genes (Poynter *et al.*,

2008; Tariq and Ghias, 2016). The *BRAF* mutation (V600E) is considered as an early step in CIMP tumours, which were found to be strongly associated with hypermethylation in the *MLH1* promoter, with a frequency of 20% in unselected and 18% in sporadic CRC cases (Lapeyre and Becker, 1979; Poynter *et al.*, 2008). *CDKN2A*/*P16* is also a hypermethylation key target within the CIMP pathway, , with hypermethylation detected in 12-50% of CRCs and reported to be significant factor in colon adenoma (Shima *et al.*, 2011). Furthermore, hypermethylation in the *CDKN2A*/*P16* promoter is more common in early adenoma (adenoma with tubulovillous histology)(Rashid *et al.*, 2001; Zitt, Zitt and Müller, 2007; Bihl *et al.*, 2012). Defining the DNA methylation status of the most common CIMP markers (*MLH1*, *CDKN2A* and *MGMT*) in colon adenoma and hyperplastic polyps has given potential targets to analyse these methylated genes in adenoma carcinoma progression, suggesting the significant advantage of these markers in adenoma and hyperplastic polyps (Zitt, Zitt and Müller, 2007). It has been reported that methylated *MLH1*, *CDKN2A* and *MGMT* was detected 7%, 34% and 49% of adenoma and 7%, 10% and 5%, respectively, in hyperplastic (serrated) polyps, respectively (Petko *et al.*, 2005; Zitt, Zitt and Müller, 2007). It has been also reported that specific methylation markers in CRCs are different from those found in other cancer types, suggesting a unique process causing the occurrence of methylated genes(M Toyota *et al.*, 1999; Lind *et al.*, 2004). Bai et al. (2004) observed the methylation status of specific genes more common in the adenoma stage of the adenoma carcinoma sequence compared to advanced tumours, suggesting the potential role of these events in the initiation of colon neoplasms, rather than the progression of colon cancer (Bai *et al.*, 2004). In contrast, DNA methylation of a subset of genes, including *MLH1*, *CDKN2A*, RASSF1 and GSTP1, was observed more frequently in CRCs than in adenoma, indicating the role of some genes in the transformation step in CRC formation(Minoru Toyota *et al.*, 1999). Furthermore, recent reports showed that epigenetic alteration in colorectal carcinogenesis induces the silencing of potential genes involved in the Wnt signalling pathway, such as *APC* and AXIN2 (Lind *et al.*, 2004; Koinuma *et al.*, 2006).

A classic panel has been utilised in the majority of studies to assess CIMP status in CpG sites including *MLH1*, *P16*, and methylated in tumours (*MINT*1, 2, and 31) (Mojarad *et al.*, 2013). Using MethyLight technology, Weisenberger et al. (2006) categorised CRC into two CIMP subclasses: CIMP-positive and CIMP-negative, showing strong association of CIMP-positive and *BRAF* V600E mutations (Weisenberger *et al.*, 2006). Using real time quantitative PCR, Ogino et al. introduced eight CMP markers (*MLH1*, CDKNA/*P16*, SOCS1, CRABP1, RUNX3¸ NEUROG1, CRABP1, and IGF2) to classify CIMP CRC into three subtypes; CIMP-high for 6-8 methylated markers, CIMP-low when 1-5 markers are methylated, and CIMP-negative when no methylation is detected (Shuji Ogino *et al.*, 2006). Recently, Hinoue et al. (2009) proposed three CIMP subtypes depending on distinct epigenetic and genetic association; 1) CIMP-high defines tumours exhibiting DNA hypermethylation of *MLH1* associated with MSI and *BRAF* mutations, 2) CIMP-low refers to CIMP specific marker hypermethylation and *KRAS* mutation and 3) CIMP-negative indicative to tumours with *TP53* mutations frequently occurring in the distal colon (Hinoue *et al.*, 2009). These findings indicate that these epigenetic alterations in CRC could be used as biomarkers for diagnosis, progression, prognosis, metastasis, and treatment response prediction (Mojarad *et al.*, 2013). Several reports have explored the role of epigenetic modifications as biomarkers for CRC using DNA based screening assays. Most of these methylation events of specific genes are found in the polyp-cancer sequence, which can be indicative for early detective markers (Mojarad *et al.*, 2013). Currently, hypermethylation of the *MLH1* promoter is being considered as a marker for sporadic CRCs other than hereditary MSI tumours due to the lack of methylated *MLH1* in Lynch syndrome. Therefore, the methylated *MLH1* status was investigated in the diagnosis of Lynch syndrome in conjunction with genetic testing (Bouzourene *et al.*, 2010; Mojarad *et al.*, 2013) It has also been reported that the presence of CIMP is a potential predictive marker of 5FU-based treatment in colon cancer stage II/III. In addition, a panel of methylated genes could be used to predict chemoresistance or chemosensitivity (Curtin, Slattery and Samowitz, 2011; Mojarad *et al.*, 2013).

1.1.3.4 MicroRNA Biomarkers in CRC

Short RNAs 18–25 nucleotides in length are known as microRNA (miRNA). They bind to the mRNA to regulate translation of complementary genes (Slaby *et al.*, 2009). To date, a large number of miRNA sequences have been identified, each regulating hundreds of genes amounting to approximately 30% of the human genome (Dawson *et al.*, 2011). These miRNAs also play a regulatory role in growth, cellular differentiation, proliferation, and apoptosis. In cancer, miRNA dysregulation plays a significant role by silencing or over-expressing different tumour suppressor genes or oncogenes, respectively (Slaby *et al.*, 2009). It has been reported that half of the miRNAs have a higher risk of dysregulation as they are present at the breakpoints of chromosomes (Dawson *et al.*, 2011). There is still ambiguity regarding the direct microenvironment affecting miRNA dysregulation (Chen *et al.*, 2009). Transcriptional activation and/or amplification of the miRNA encoding gene upregulate mature miRNAs which favour of tumour growth, whereas those adverse to growth may be silenced by either deletion or epigenetic modifications (Slaby *et al.*, 2009). MiRNAs have been shown to regulate proteins involved in potential pathways contributing to colorectal carcinogenesis. Indeed, Mir-143, miR-18a, miR-145 and let7 downregulate RAS protein, a member of the MAP pathway, and act as tumour suppressive miRNAs in CRC (Johnson *et al.*, 2005; Chen *et al.*, 2009). Other miRNAs, such as miR-194, have been found to suppress *AKT2*, one of main key proteins in the PI3K pathway, indicating a potential role of miRNAs in colorectal tumorigenesis (Zhao *et al.*, 2014; Thomas *et al.*, 2015). In contrast, some dysregulated miRNAs, including miR-195 and miR-491, have been found to be involved in apoptotic regulation for CRC by targeting B-Cell CLL/lymphoma 2 (BCL2) and BCL2-like 1 (Bcl-xL) in CRC cells(Liu *et al.*, 2010; Nakano *et al.*, 2010). Furthermore, miRNAs have effects on *p53* activity, which plays important roles in cell cycle and apoptosis. Previously, miR-96 was reported to be upregulated in CRC and shown to negatively regulate *p53* through targeting *p53* inducible nuclear protein 1 (*TP53*INP1)(Gao and Wang, 2015). Conversely, miR-43 has been found to inhibit sirtuin-1 (SIRT1), which in turn increases *p53* activity

(Yamakuchi, Ferlito and Lowenstein, 2008). Other miRNAs, such as miR-155, are associated with the MSI pathway, regulating genes involved in the MMR system which is responsible for correcting errors during DNA replication(Valeri *et al.*, 2010; Colling *et al.*, 2015; Thomas *et al.*, 2015). In addition, miR-21 and miR-224 play a key role in the development of CRC to metastasis by regulating several genes involved in controlling migration and invasion, including *SMAD4*, *PTEN*, TIAM1, SPRY and PCDC4(Asangani *et al.*, 2008; Sayed *et al.*, 2008; Cottonham, Kaneko and Xu, 2010; Xiong *et al.*, 2013; Ling *et al.*, 2015)

MiRs may affect Epithelial-mesenchymal transition (EMT). This is a mechanism that assists invasion and metastases, in which epithelial cells are dedifferentiated and acquire features of mesenchymal cells categorised by reduced E-cadherin, which is suppressed by miR-92a(Zhang *et al.*, 2014; Thomas *et al.*, 2015). Furthermore, miR-27b has been shown to be involved in angiogenesis, in the development to metastasis by targeting an angiogenic factor known as vascular endothelial growth factor C (VEGFC)(Ye *et al.*, 2013). Bandres and colleagues detected 13 miRNAs with altered expression in CRC patients, which correlated with some mutations in the RAS-RAF pathways, such as *BRAF* and *KRAS* mutations (Bandrés *et al.*, 2006). Downregulation of miR-143 and miR-145 was identified in the precancerous adenomatous polyp stage, indicative to their role in the early development of CRC(Michael *et al.*, 2003; Cummins *et al.*, 2006; Motoyama *et al.*, 2009). Conversely, Lanza et al. (2007) found the upregulation of miR-17-5p, miR-17-92, miR-20, miR-25, miR-92-1, miR-92-2, miR-93-1 and miR-106a more common in MSS CRC cases than in MSI tumours(Lanza *et al.*, 2007). Moreover, a panel of miRNAs, including miR17-5, miR-20a, miR-31, miR-92, miR-183, were overexpressed in tumoural tissue when compared to normal colorectal mucosa, some of which were associated with poor prognosis in CRC, such as miR-183 (Motoyama *et al.*, 2009) and overexpression of mir-31 was associated with stage IV CRC (Bovell *et al.*, 2013; Thomas *et al.*, 2015).

Circulating miRNAs can be detected in blood serum or plasma, providing a powerful method for monitoring tumour progression in CRC patients. MiRNAs were first detected in the serum of CRC patients by Chen et al. in 2008, followed by further analysis of various miRNAs in many studies (Chen *et al.*, 2008). Ng et al. performed a large scale analysis of miRNAs, identifying upregulation of five miRNAs (miR-17-3p, miR-92, miR-95, miR-135b, and miR-222) in plasma and matched tissue CRC samples, with a significant decrease in miR-17-3p and miR-92 levels, suggesting the potential role of miRNAs as markers for the therapeutic response(Ng *et al.*, 2009). Huang et al. demonstrated the upregulation of miR-92a and-29a in the plasma of CRC patients and observed an association with advanced adenomas, indicating the possible role of miRNAs in diagnosis(Huang *et al.*, 2010). Recently, several miRNAs including miR-10a, miR-21, miR-92a, miR-141, miR-192, miR-221, and miR-224, have been shown to be dysregulated in CRC plasma samples, some of which, such as miR-21, were highly differentiated when compared to healthy individuals (Guo *et al.*, 2010; Kanaan *et al.*, 2012; Wang and Zhang, 2012). Providing more suitable and accurate diagnostic methods, circulating miRNAs have the potential to be used as biomarkers for early detection, progression and treatment of CRC, contributing to the improvement of patients' survival rate (Mazeh *et al.*, 2013).

1.2 Molecular Features in Metastatic CRC

In the later stages of CRC, the tumour cells detach from the primary tumour, migrate into the blood or lymphatic vessels, ultimately forming a secondary tumour. Either type of dissemination can lead to blood circulation as lymphatics can also flow into the systemic circulation sharing same route with haematogenous dissemination to distant sites (Wong and Hynes, 2006; Kawada and Taketo, 2011). The disseminated tumour cells in lymph node or peripheral blood are used as prognostic biomarkers to detect

metastatic cancer cells. In the absence of distant metastasis, tumour cells spreading through haematogenous route can be detected in the peripheral blood of the patients. Tumours cells detected in blood circulation are called circulating tumour cells (CTCs) and some are found migrating from primary sites to distant metastatic, indicating that metastasis is in progress (Kamiyama *et al.*, 2014). Furthermore, the detected CTCs may reflect high metastatic levels or aggressive disease and these CTCs are found to be associated with poor outcome in patients with metastatic CRC (Groot Koerkamp *et al.*, 2013; Kamiyama *et al.*, 2014). Lymphatic metastatic is one of the most prognostic and key factor that identifies the tumour extend in regional lymph node and potential of spreading to distant sites (Ong and Schofield, 2016). The significance of lymphatic metastatic in prognosis is dependent on the classification systems which reflect the stage and extend of the tumour, in particular the widely used tumour node metastasis (TNM) staging system (Ong and Schofield, 2016). It has been observed that the detection of lymph node involvement has reduced the 5-year survival rate from90% to 60%, suggesting strong prognostic impact in CRC (Sundlisæter *et al.*, 2007; Ren *et al.*, 2012) . However, identifying biomarkers in lymph node metastasis may help to categorised patients into lymph node-negative and lymph node positive, to detect tumour recurrences and those are benefiting from adjuvant therapy (Rahbari *et al.*, 2012; Kamiyama *et al.*, 2014). Certain genetic and epigenetic changes are required during every step in metastatic spread, however, the underlying mechanisms have not been fully elucidated (Kanthan, Senger and Kanthan, 2012). The potentiators of metastatic spread are the growth factors including prostaglandin E2, EGF, and vascular endothelial growth factor (VEGF), along with molecular mediators of EMT (Figure 1-4). The median survival time of patients with mCRC has increased to 23.5 months owing to the presence of biological agents particularly targeting these markers (Asghar, Hawkes and Cunningham, 2010). Approximately 50% of CRC patients have a poorer survival rate due to the complications of metastases; hence, early detection of these molecular changes

involved in CRC progression is vital to develop appropriate therapeutic strategies (Dawson *et al.*, 2011).

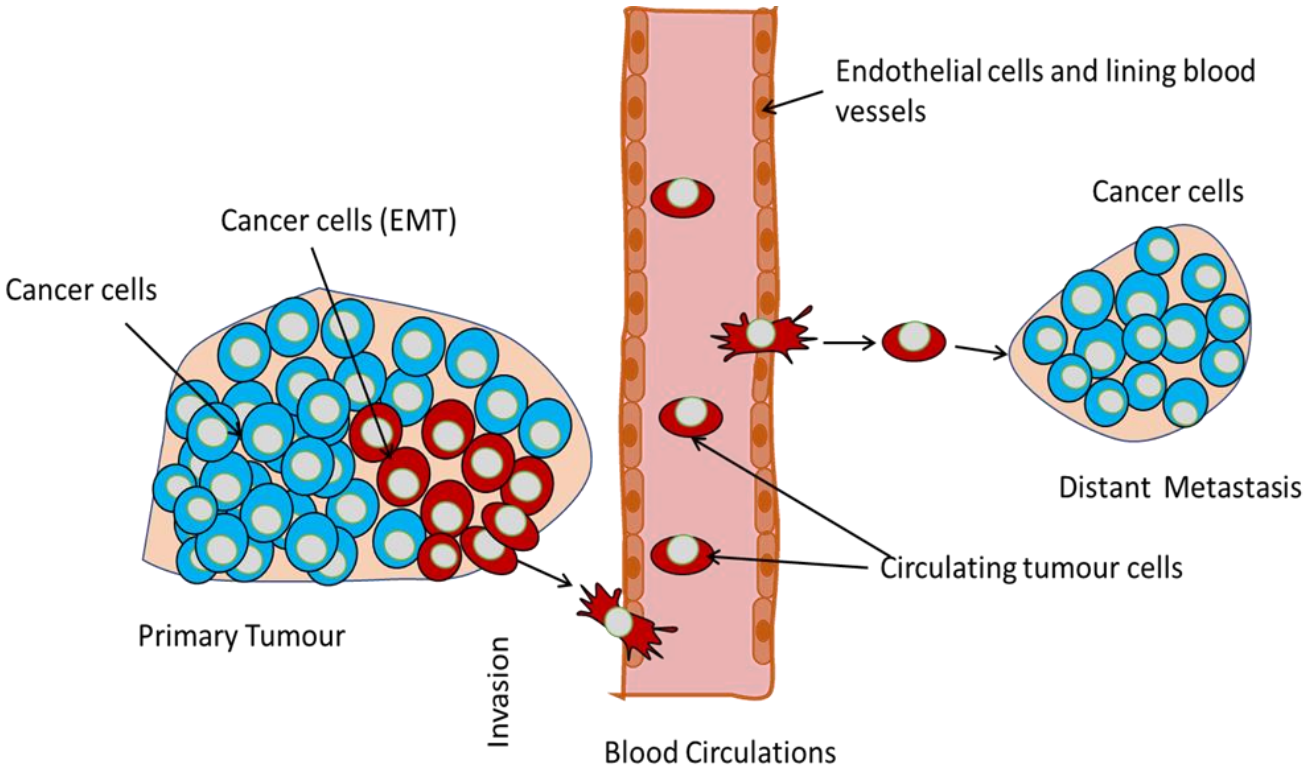

Figure 1-4: Metastatic process of CRC.
Primary tumours will eventually have cells undergone EMT to metastasize to distant organs. The cells are detached from primary tumours and gained excess to blood circulation. These cells undergo further reverse EMT and form secondary tumour at distant organs.

1.2.1 Vascular Endothelial Growth Factor (VEGF)

Angiogenesis is a fundamental process during development and wound healing, where new blood vessels are formed from preceding vessels. It is also essential to transfer oxygen and nutrients into an expanding neoplasm during carcinogenesis(Kanthan, Senger and Kanthan, 2012). These two processes differ depending on equilibrium between the signal of both pro- and antiangiogenic factors. VEGF, fibroblast-growth factor (FGF), platelet-derived growth factors (PDGFs), insulin-like growth factor (ILGF), and transforming growth factor (TGF) are proangiogenic, whereas thrombospondin-1, angiostatin, and endostatin are antiangiogenic (Winder and Lenz, 2010). In carcinogenesis, the proangiogenic factors are predominant, leading to an imbalance between these two factors. In both normal and pathologic tissues, VEGF is a key component and prognostic factor involved in stimulating endothelial cell growth, migration, differentiation, and vascular permeability (Pohl *et al.*, 2008). In addition, hypoxia stimulating the manufacture of angiogenic factors, such as VEGF, drives neovascularisation in CRC (Kanthan, Senger and Kanthan, 2012). Hypoxia plays a key role in the upregulation of VEGF gene expression by the activation of hypoxia inducible factor-1 (HIF1), which eventually promotes new blood vessel formation. Five VEGF glycoproteins (A-E) and placental growth factors (PGFs) 1 and 2 are included in two families of angiogenic and lymphangiogenic factors (Winder and Lenz, 2010). Elevated VEGF signalling has been reported in CRC. VEGF ligands signal through the binding of three tyrosine kinases receptors (V*EGFR*1 and 2 in angiogenesis and V*EGFR*3 in lymphangiogenesis) activating different pathways in CRC including RAF/MEK, ERK, AKT, mTOR, and PI3K pathways (Armaghany *et al.*, 2012). VEGF1 and 2 are implicated in angiogenesis, where V*EGFR*1 binds to VEGF-B and PGF to stimulate haematopoiesis, endothelial progenitor recruitment, and growth factor induction. The receptor of VEGF-A and -F is V*EGFR*2, which is involved in enhancing microvascular permeability, proliferation of endothelial cells, migration, and invasion. Furthermore, V*EGFR*1 is a

receptor for VEGF-C and -D glycoproteins, and its binding mediates embryonic cardiovascular development (Winder and Lenz, 2010). Poor prognosis is often associated with high serum levels of VEGF and V*EGFR*1 gene expression has been reported as a predictive biomarker for tumour recurrence (Pohl *et al.*, 2008; Winder and Lenz, 2010). Regarding its significant role in angiogenesis and increased V*EGFR* signalling in advanced CRC, monoclonal antibody therapy has been shown to inhibit this process and improve progression free survival in metastatic CRC. Angiogenesis inhibition by targeting VEGF has been shown to inhibit the formation of new tumour vasculature, subsequently repressing tumour development (Rosen, 2005). However, anti-VEGFR therapy targets VEGF-A isoforms to inhibit its binding with V*EGFR*2, resulting in regression of microvessels, inhibition of the formation of new blood vessels, thereby impacting on vascular flow in tumours (Armaghany *et al.*, 2012). Bevacizumab is a humanized monoclonal antibody that targets VEGF by binding directly to all VEGF-A isoforms to form a protein complex to prevent any further binding with VEGF receptors (Ferrara *et al.*, 2004; Moriarity *et al.*, 2016). This mechanism is found to neutralise VEGF signal transduction through both VEGFR-1 and -2 and prevents vascular endothelial cells proliferation and angiogenesis (Ellis, 2006). Combining anti-VEGF agents with chemotherapy such as 5-FU have improved the efficacy of these regimens by enhancing the suppressive impact on tumour-cell growth and inducing apoptosis in an additive manner (Ellis, 2006; Moriarity *et al.*, 2016). This also stabilizes tumour vasculature resulting in potential decrease in vascular flow and fluid pressure within the tumour allowing enhanced delivery of oxygen and improves systemic delivery of the chemotherapy agents (Ellis, 2006; Moriarity *et al.*, 2016).

1.2.2 Prostaglandin and Cyclooxygenase-2

Prostaglandin E2 is found and secreted in the stroma and epithelial cells by fibroblasts, where endoprostanoid receptors interact together to transduce the signalling. A cascade is started by the stimulation of these endoprostanoids that activates *EGFR* and PI3K/AKT

signalling pathways, resulting into β-catenin translocation to the nucleus (Early, Fontana and Davidson, 2008). The growth of the adenoma is strongly correlated with the signalling of prostanoids via regulation of proliferation, survival, migration and invasion, thereby strongly associated with CRC (Pino and Chung, 2010). Increased prostanoid signalling may be induced by various processes including inflammation or mitogen-associated upregulation of cyclooxygenase-2 (COX-2), the mediator of prostaglandin E2, or loss of 15-prostaglandin dehydrogenase (PDGH), the rate-limiting enzyme catalysing prostaglandin E2 breakdown (Kanthan, Senger and Kanthan, 2012). Loss of PDGH has been reported in around 80% of colorectal adenomas and carcinomas (Markowitz and Bertagnolli, 2009). COX-2 is overexpressed in approximately 43% of adenomas and 86% of carcinomas, and upregulation is commonly induced by growth factors, cytokines, inflammatory mediators, and tumour promoters (Pino and Chung, 2010). COX-2 is often associated with angiogenesis, as proangiogenic factors, including VEGF and FGF, are produced by the overexpression of this enzyme (Pino and Chung, 2010). Indeed, anti-*EGFR* therapy inhibits the tumorigenic effects of COX-2 overexpression (Kumar, 2005). Furthermore, epidemiologic data demonstrated the positive preventative role of non-steroidal anti-inflammatory drugs (NSAIDs) in COX inhibition in CRC patients (Mallion *et al.*, 2011).

1.2.3 Epithelial-Mesenchymal Transition (EMT)

Epithelial-mesenchymal transition (EMT) was initially described as the mechanism that assists invasion and metastases, in which epithelial cells are altered into dedifferentiated mesenchymal cells categorised by reduced E-cadherin, loss of cell adhesion, and enhanced cell motility (Kevans *et al.*, 2011). Zinc-finger E-box binding homeobox (ZEB1) is a transcriptional repressor which plays a role in inducing the transition of epithelial-mesenchymal cells, causing repression of E-cadherin transcription (Slaby *et al.*, 2009; Dawson *et al.*, 2011). E-cadherin mediates the loss of intercellular connections of these cells during EMT, which allows them to associate with extracellular matrix (ECM) to form

an anchor, facilitating further movement(Kevans *et al.*, 2011). In this regard, the remodelling of the ECM by proteinases, e.g. urokinase plasminogen activator cascade and matrix metalloproteinase, is essential for growth of the tumour, cell survival, invasiveness, and metastases (Slaby *et al.*, 2009). Furthermore, β-catenin is stabilised and translocated to the nucleus due to EMT signalling. When the basement membrane is degraded, EMT signalling is concluded and the mesenchymal cell is formed as evidenced by loss of membranous expression of both β-catenin and E-cadherin (Zlobec and Lugli, 2010). Subsequently, these mesenchymal cells can metastasise via EMT to distant sites forming new colonies histopathologically resembling the primary tumour (Zlobec and Lugli, 2010). Activation of various transcription factors, including Snail, Slug, ZEB1, Twist, Goosecoid and FOXC2, has been reported to induce EMT signalling in metastasis, which results from other growth factors in the stroma, such as HGF, EGF, placental-derived growth factor, and TGF-β (Zlobec & Lugli, 2010). Phosphorylation of p68 is enhanced by EGF, TGF-β, and placental-derived growth factor after the binding to β-catenin preventing its stabilisation by GSK3β (Huang and Du, 2008). Pro-metastatic factors, including galectin-3 and Fascin, are considered as downstream targets of β-catenin, which are both expressed at the invasive border and present a poor result (Huang and Du, 2008). The histological mark of EMT is known as tumour budding, which occurs in 20–40% of the CRCs and is represented by dedifferentiated single cells or small clusters from the invasive front (Zlobec *et al.*, 2010; Zlobec and Lugli, 2010). At the invasive front, tumour budding occurs when tiny collections of cells detach and migrate through the stroma and is considered the commencement of invasion and metastases (Kevans *et al.*, 2011). As compared to MSI, this process is more widespread in the MSS CRC, which may explain the poorer prognosis of MSS (Kevans *et al.*, 2011). The presence of tumour buds is considered as a significant prognostic factor of metastases through the tumour vessels and lymphatic to the lymph nodes, often linked with local recurrence and poor survival (Zlobec and Lugli, 2010; Kevans *et al.*, 2011) Zlobec et al. (2010) proved in their study, with up to 80% accuracy, that high grade

tumour budding is likely to have no response to anti-*EGFR* therapies in patients with a *KRAS* mutation (Zlobec *et al.*, 2010).

1.2.4 Epidermal Growth Factor (EGF)

One of the components of the HER-erbB family of receptor tyrosine kinases is the epidermal growth factor receptor (*EGFR*), which is a cell surface receptor and binds EGF and TGF-α, (Winder and Lenz, 2010). Ligand binding induces the *EGFR* signalling cascade, stimulating the activity of intercellular tyrosine kinases and pathways, including RAS-RAF-MAPK, PI3K, STAT and SRC/FAK signalling pathways, eventually leading to the activation of different transcription factors involved in migration, proliferation, differentiation and apoptosis (Lièvre, Blons and Laurent-Puig, 2010). Dysregulation of *EGFR* signalling occurs at various points in this pathway causing different events including, (i) on receptor binding; *EGFR* overexpression, mutation and copy number change (ii) activation of different pathways, such as RAS, RAF, and PI3K, as consequence of transduction regulators (iii) and genetic and epigenetic changes in genes coding these proteins (Armaghany *et al.*, 2012; Cathomas, 2014). An interrelated network of phosphorylation reactions occurs due to the downstream targets of EGFR, including activation of RAS/MAPK and PI3K/AKT pathways in addition to STAT and SRC/FAK pathways, which are shown to be involved in tumour proliferation, angiogenesis, and cell survival (Lièvre, Blons and Laurent-Puig, 2010). These reactions can stimulate transcription factors triggering carcinogenesis through deregulation of protein synthesis, cell-cycle progression, apoptosis, angiogenesis and altered metabolism (Shaw and Cantley, 2006; Lièvre, Blons and Laurent-Puig, 2010). Molecules, such as VEGF and HIF1α, with potential role in promoting angiogenesis have been reported to be associated with the pathways stimulated by this receptor (Kanthan, Senger and Kanthan, 2012). Moreover, activating serine protease by *EGFR* enhances invasiveness and metastasis, as serine protease assists in the breakdown of the ECM (Kumar, 2005). *EGFR* is overexpressed in 65-70% of CRC patients, and more commonly detected in

advanced stage tumours, suggesting its potential as a therapeutic target in mCRC. However, the correlation between *EGFR* overexpression and poor histologic grade, advanced stage and lymph vascular invasion indicates *EGFR* as a prognostic factor in mCRC, but there is ambiguity regarding its impact on survival (Kanthan, Senger and Kanthan, 2012). The inhibition of EGFR includes monoclonal antibodies, such as cetuximab and panitumumab, which bind to EGFR to prevent any further binding with other ligands including EGF and TGF-α (Baselga, 2001). The binding of the monoclonal antibodies with EGFR extracellular domain blocks downstream signalling, which prevents any further binding leading to EGFR degradation (Moriarity *et al.*, 2016). Blocking EGFR activation and its downstream signalling pathways (RAS-RAF-MAP and PI3K-AKT pathways) results in inhibition of cell growth, induction of apoptosis, and reduces the production of VEGF and matrix metalloproteinase (MMPs) (Vincenzi *et al.*, 2010; Moriarity *et al.*, 2016).

1.2.5 Circulating Tumour Biomarkers

Early detection of the tumour is a key to improve the prognosis for patients with advanced CRC. Non-invasive methods, such as blood-based biomarkers, for early detection and have the potential to reduce morbidity and mortality linked with this disease. Cell-free DNA is a small fragmented DNA expelled into the blood circulation. Mandel and Metais (1948) first reported the clinical use of circulating cell-free DNA (*cfDNA*) in serum and plasma (Mandel and Metais, 1948). In oncology, recognition of *cfDNA* derived from tumours, also known as circulating tumour DNA (*ctDNA*), has been challenging due to the bias of *ctDNA* from normal *cfDNA* by that fact that tumour DNA is diluted in larger proportion of wild-type cfDNA, low levels of *ctDNA*, and accurate quantification methods of fragmented DNA (Diaz and Bardelli, 2014; Wang *et al.*, 2017). Tumour DNA is distinct due to mutations which differentiate *ctDNA* from normal *cfDNA*, as only the genomes of cancer cells or precancerous cells contain these somatic mutations. Thus, *ctDNA*, due to its unique biologic specificity, can be used as a

biomarker for cancer patients (Diaz and Bardelli, 2014). The excessive amount of tumour DNA fragments present in the circulation will allow accurate and easy mutation detection by sequencing technologies.

Various studies showed that *ctDNA* only represents a small portion (~1%) of total *cfDNA* in the circulation, which is challenging for standard DNA sequencing methods such as Sanger sequencing or pyrosequencing (Diehl *et al.*, 2005; F Diehl *et al.*, 2008; Holdhoff *et al.*, 2009). Owing to the development of digital genomic technologies, such as digital PCR, investigating *ctDNA* in cancer patients has allowed the detection of rare mutant variants in complex mixtures of DNA. Initially, it was believed that *ctDNA* measurement was substandard compared with other biomarkers, such as circulating tumour cells, due to the lack of advanced detection technologies of *cfDNA* derived from tumours in the past, like digit PCR, beads, emulsion, amplification, and magnetics (BEAMing) or pyrophosphorolysis-activated polymerisation (PAP) (Liu and Sommer, no date; Vogelstein and Kinzler, 1999; Dressman *et al.*, 2003; Kimura *et al.*, 2006; Daniotti *et al.*, 2007; Shinozaki *et al.*, 2007; Maheswaran *et al.*, 2008; Kuang *et al.*, 2009; Morgan *et al.*, 2012; Punnoose *et al.*, 2012). These digital genomic methods have shown high sensitivity to identify mutations in advanced tumours, the tumour tissue matches the mutation in the *ctDNA* fragments (Diehl *et al.*, 2005; F Diehl *et al.*, 2008; Higgins *et al.*, 2012).

Currently, the use of next generation sequencing (NGS) is being applied in PCR-based digital approaches to detect rare mutant variants in complex mixtures of DNA (Kinde *et al.*, 2011; Forshew *et al.*, 2012; Leary *et al.*, 2012; Taly *et al.*, 2013). Owing to these techniques, not only single point mutations, but also amplifications, rearrangements and aneuploidy can be tested (F Diehl *et al.*, 2008; Leary *et al.*, 2012; Murtaza *et al.*, 2013).

Most of the main latest advances in targeted therapies are dependent on obtaining tumour tissue via biopsy, either before commencement of therapy or after the

occurrence of resistance. Obtaining tumour through biopsies or resection is a major challenge for gold standard sequencing and clinical use. Performing biopsies or resection for patients are considered as inconvenient methods due to their invasiveness and associated pain in addition to the possibility of a poor outcome for the patients (Diaz and Bardelli, 2014; Tellez-Gabriel *et al.*, 2016). For cancer sequencing, not only are challenges faced in gaining tissue, but sample conservation and tumour heterogeneity also hinder the use of tumour tissue (Holdhoff *et al.*, 2009). These tumour tissues are normally preserved in formalin-fixed paraffin-embedded (FFPE) blocks, which have several limitations due to the amount of tumour cells in each biopsy (Diaz and Bardelli, 2014). Heterogeneity is also one of the possible limitations for biopsy tissues. This phenomenon describes the observation of different tumour sub-clones present within tumours (intratumoural heterogeneity) and differences between primary tumours and their metastatic deposits (inter-tumoural heterogeneity). Heterogeneity is in the form of variation in morphological and phenotypic features including genetic profiles (Gerlinger *et al.*, 2012; Diaz and Bardelli, 2014; Tellez-Gabriel *et al.*, 2016). Therefore, it is possible to overlook the molecular heterogeneity during a biopsy or resection tissues obtained from solid tumours (Diaz and Bardelli, 2014).

Circulating tumour DNA and tissue biopsy have the ability to provide identical genetic information for tumour diagnosis (Diaz and Bardelli, 2014). There is benefit in accessing the bloodstream, as it is a fresh source of DNA, unhindered by preservatives. Moreover, during the course of therapy, blood can be drawn at any time and allow screening of any molecular changes in the tumour in response to therapy. Detection of tumour-derived genetic and epigenetic defects in circulating tumour DNA fragments is similar to those detected in tumour tissues and it has been investigated as potential biomarker in CRC patients (Gonzalez-Pons and Cruz-Correa, 2015). Detection of point mutations, such as *KRAS*, in plasma has shown high levels of mutations (43%) and this method has achieved great specificity (93%) (Anker *et al.*, 1997; De Kok *et al.*, 1997; Kopreski *et*

al., 1997). Methylation status of specific genes including SEPT9, vimentin and NGFR have been investigated in circulating DNA as biomarkers, with specificities ranging from 69-93% and sensitivities from 48-72% (Grützmann *et al.*, 2008; Lofton-Day *et al.*, 2008; M. Li *et al.*, 2009). However, overexpression of various miRNAs has been detected in the circulating tumour miRNA, such as miR-92a which is upregulated in both plasma and matched tumour tissues (Chen *et al.*, 2008; Ng *et al.*, 2009; Gonzalez-Pons and Cruz-Correa, 2015). Other studies detected a significant decrease in the expression of mir-92a after tumour removal, implying mir-92a for tumour screening and monitoring after receiving therapy (Ng *et al.*, 2009). Mir-21 also has been reported to be associated with tumour recurrence, indicating that mir-21 can be used as prognostic marker for CRC patients (Menéndez *et al.*, 2013). Additionally, molecular heterogeneity can be accounted for during the investigation of ctDNA from patients because ctDNA fragments are expelled into the blood circulation from all tumours within the same individual's body (Tellez-Gabriel *et al.*, 2016). It has also been reported that these ctDNA fragments are more abundant in advanced stage tumours than in lower stage or pre-metastatic tumours, which further increases the value of such a test in metastatic prognosis (Diaz and Bardelli, 2014). Furthermore, additional research efforts are necessary to determine new ctDNA/miRNA biomarkers which can be implemented for tumour screening, diagnosis and prognosis as well as to improve the sensitivity and specificity of the methods.

1.3 Management of CRC

Surgery is the mainstay of curative for CRC patients and surgical type is based on the location of the tumours. Left sided tumours generally require either left hemicolectomy, sigmoidectomy, anterior or abdominoperineal resection. Right sided tumours are commonly removed as right hemicolectomy and tumours located in the rectum or transverse colon may require laparoscopic colectomy (Nakayama, Tanaka and Kodera, 2013). The management of CRC is ought to be multi-modular approach depending on

tumour site, extent, and biology. Although surgery is still most curative treatment for CRC, multi-modular treatments seem to be more effective and could maintain long-term survival (Nakayama, Tanaka and Kodera, 2013). Resectable Stage I-III tumours are generally amenable to surgery, preferably laparoscopic surgery when possible. For uresectable stage I-III tumours, patients may be offered neoadjuvant chemotherapy prior to surgical removal in order to decrease tumour size or state to maintain resectable tumour. Most common chemotherapy options include a combination of capecitabine or oxalipatin with 5-flurouracil (5-FU) and folinic acid (Nakayama, Tanaka and Kodera, 2013; NICE, 2017). Patients with stage IV tumours are usually managed by surgical removal of both primary and distant tumours or resection of primary tumour with further chemotherapy. Monoclonal antibodies including bevacizumab, cetuximab or panitumumab targeting specific molecular markers (i.e. VEGFR and EGFR) involved in metastatic CRC may be indicated to those with advanced metastatic disease (Nakayama, Tanaka and Kodera, 2013; NICE, 2017).

1.4 Molecular Impact on CRC Treatments

Defining the molecular mechanisms leading to CRC is key to a better understanding of the disease, as well as to provide effective targeted therapy (Bogaert and Prenen, 2014; Yiu and Yiu, 2016). The combination of therapies, such as 5-FU, leucovorin and oxapilatin (FOLFOX) or caspecitabine with oxalipatin (XELOX), is used for patients in high-risk stage II and III CRC (de Gramont *et al.*, 2000; Labianca *et al.*, 2010) Advances in the understanding of CRC molecular pathways has led to the discovery of monoclonal antibodies, such as panitumumab and cetuximab to target *EGFR*, thus inhibiting the activation of signalling pathways such as RAS, PI3K-AKT-*PTEN* and SRC induced by this ligand (Amado *et al.*, 2008). Anti-*EGFR* therapies have been shown to be effective in phase II and III clinical trials, but some downstream components in the *EGFR* pathway are resistant to the therapy(Yiu and Yiu, 2016). Further analysis showed that patients

with a *KRAS* mutation in exon 2 had no response to anti-*EGFR* treatment (Amado *et al.*, 2008; Lièvre *et al.*, 2008)

The predictive role of MSI as marker of response to 5-FU and other chemotherapeutic agents remains contradictory (Popat, Hubner and Houlston, 2005). Meta-analysis for the predictive value of MSI status concluded MSI tumours did not benefit from 5-FU-based treatment and could be harmful for stage II patients (Ribic *et al.*, 2003; Sargent *et al.*, 2010). Conversely, other studies showed that MSI was associated with a better response to 5-FU than MSS tumours (Elsaleh and Iacopetta, 2001). Recently, the PETACC-3 study, which involved a large number of stage II and III CRC patients, explored the effect of 5-FU-based therapy in patients with a 5-year disease free survival (DFS), showing significant differences between MSI and MSS CRC in the 5-year DFS (Tejbar *et al.,* 2010). This study suggested that maintaining 5-FU based therapy in MSI tumours improved the prognosis and had a significantly stronger prognostic impact in stage II than stage III (Bogaert and Prenen, 2014). Furthermore, MSI tumours exhibiting CIMP-high have been reported to increase the sensitivity to 5-FU, which might be related to CIMP-high subtypes not associated with MSI, such as *MGMT* hypermethylation (Sinicrope *et al.*, 2011). Further investigations are required to identify new mechanisms that may impact on the resistance of CRC to targeted chemotherapeutic or combined regimens, which would help to improve the survival rate for this disease (Bogaert and Prenen, 2014).

1.5 Prognosis and survival rates for CRC

The prognosis of CRC is based on staging at diagnosis. The relative 5-year survival for CRC is 65% in many developed countries and has remained static during the past decades (Sankaranarayanan *et al.*, 2010; Brenner *et al.*, 2012; Brenner, Kloor and Pox, 2014). Patients with stage I/II tumours have relatively good prognosis after surgical removal with 5-year survival of 90%. The 5-year relative survival of CRC patients with

stage III (tumour spread to regional lymph node) tumours have poorer prognosis with a 5-year survival of 70% and usually treated with adjuvant chemotherapy. CRC patients with distant metastasis (e.g. liver or lung) have worse prognosis and the 5-year survival rates drop sharply to 10% (Brenner, Kloor and Pox, 2014).

1.6 Aims and Hypothesis:

Metastasis CRC is a sequence of tumour cells detachment, migrating through lymphatic node into blood circulation establishing new tumour at a distant site/organ. In this process, tumours exhibit many molecular changes to invade aggressively and form secondary site/metastasis. Identifying these events and determine their role in advanced CRC is essential to improve prognosis and diagnosis in practice labs. The main aim of this thesis was to investigate these molecular events and their role in metastatic CRC.

Chapter 1

Aim (i): *Genotype CRC and identify association with lymphatic and distant metastasis.*

Hypothesis: Lymph node status is one of the most important prognostic factors in colorectal carcinoma. Tumour cells invade lymphatic vessels which enables the detached tumour cells to spread through the lymphatic system forming distant metastasis. Many molecular mechanisms, including abnormal gene expression and signalling pathways dysregulation, play important roles in tumorigenesis and tumour progression in CRC. The identification of these molecular alterations involved in CRC transition to metastasis is essential to improve the prognosis and diagnosis for CRC patients.

Aim (ii): *Develop a simple assay to identify tumours with MSI and discriminate Lynch Syndrome CRC from sporadic CRC.*

Hypothesis: CRCs with MSI have a different biology and prognosis to tumours which are MSS. CRCs arising in Lynch syndrome have MSI as well as approximately 15% of sporadic CRCs. BRAF mutation and MLH1 promoter methylation occur in sporadic MSI CRC but are rarely detected in Lynch Syndrome. A simple test to discriminate these three groups would be improve diagnosis for CRC patients and could be used on cfDNA for tumour screening/surveillance.

Chapter 2

Aim: *Investigate the role of cfDNA as biomarker for monitoring tumours.*

Hypothesis: Contents from tumour cells such as DNA and miRNA enter into the bloodstream during apoptosis and necrosis in tumours. These can be used to genotype the tumour, detect the presence of tumour (e.g. for tumour surveillance / screening, and for detecting metastasis following surgery) and possibly predict the response of the tumour to therapy.

Chapter 3

Aim: *Determine the influence of GNAS1 on CRC cell proliferation and motility.*

Hypothesis: *GNAS1* has been found to be mutated in CRCs. It is a G-protein coupled receptor (GPCR), found to have crucial roles in cancer progression including proliferating cells, angiogenesis and tumour cell growth. It is possible that *GNAS1* may influence CRC cell biology and possibly promote metastasis.

2 Materials and Methods

2.1 Cell Culture

Two colorectal cancer cell lines, RKO and SW620 were obtained from the Molecular and Population Genetics Laboratory, London Research Institute, Cancer Research UK, London (Table 2-1). The identity of these cell lines was confirmed by HRM-PCR screening for known mutations (Figure 8-1) and authenticated by short tandem repeat (STR) profiling using PCR-single locus technology, performed by Eurofins (certificate attached in the appendix).

2.1.1 Cell Maintenance

Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific) antibiotic free supplemented with 10% FBS (fetal bovine serum, Sigma) and maintained at 37ºC in incubator under a humidified 5% CO_2 atmosphere. The growing cells were monthly tested for mycoplasma and frequently (every 1-2 days) checked under the microscope for cells viability and for any unusual growth such as contamination. Cell were fed twice weekly by changing the media to a fresh DMEM (containing FBS).

2.1.2 Cell Freezing and Thawing

For cells thawing, vials containing cells were heated up at 37^{o}C in waterbath, then transferred into universal tubes containing 9ml of media and centrifuged at 1000rpm for 10 minutes. Supernatant was then aspirated, and cell pellets were resuspended in 12ml of media and transferred to a clean T75 flask. Cells were then incubated and observed under microscope frequently to check confluency and contamination.

When cells reached approximately 100% of confluency, cells were washed twice with phosphate buffered saline (PBS) (Sigma) and detached by incubating them with 2 ml of trypsin/EDTA (Sigma) for 5 minutes at 37°C. The cells were then suspended in 8ml of DMEM to neutralise the trypsin and pelleted by centrifugation at 1,500 rpm for 5 mins.

Cell pellet was then resuspended in 3ml of freezing mixture (3.6ml FBS, 400μl DMSO) and transferred into Cryo-Vial and stored in Liquid nitrogen storage container.

2.1.3 Cell Passage

Cells were allowed to grow until they reached approximately 70%-80% confluency and then passaged. Cells washed twice with phosphate buffered saline (PBS) (Sigma) and detached by incubating them with 2 ml of trypsin/EDTA (Sigma) for 5 minutes at 37°C. The cells were then suspended in 8ml of DMEM to neutralise the trypsin and pelleted by centrifugation at 1,500 rpm for 5 mins. The media were then discarded, and cells were re-suspended in a volume of media based on the split ratio (i.e. 10ml for a 1:10 spilt ratio). One ml of cell suspension was transferred into a new flask containing 10ml of DMEM (containing FBS). To reduce the effect of prolonged passage, experimental work was undertaken on both cell lines with a passage number of less than 12 from the first defrosting.

Table 2–1: Features of CRC cell lines.

Cell line	Gender	Age	Dukes' Stage	Differentiation	Origin	MSI Status	CIN	KRAS	BRAF	PIK3CA	PTEN	TP53
RKO	Male	64	C	Poor	Colon	MSI	NO	Wt.	V600E	H1047R	Wt.	Wt.
SW620	Male	51	C	Moderate	Lymph node	MSS	Yes	G12V	Wt.	Wt.	Wt.	R273H;P309S

2.2 Transfection and Knockdown

Small interfering RNA (siRNA) duplexes (Thermo Fisher) were used for gene knockdown and transfection was performed according to the standard manufacturer's protocol. For optimisation, Lipofectamine 2000 (Thermo Fisher Scientific) volumes of 10 µl were incubated together with 15-60 nM of GNAS siRNA duplexes (table 2-2). The transfection efficiency was assessed by western blot and the condition that gave the greatest inhibition in protein expression was considered optimal. Luciferase targeting siRNA was used as a control and transfected at the same concentration as the target siRNA.

Transfection was carried out in a 6-well plate in a total volume of 2 ml. Briefly, cells were seeded in a six well plate (Costar) with a total volume of 2 ml per well and incubated for 24 hours at 37^0C. Twenty-four hours post seeding in DMEM, after cells reached 40-50% confluence, the media was replaced with 1.5 ml of Opti-MEM reduced serum media and cells were incubated for 1 hour at 37^0C. Following this, 10 µl of Lipofectamine 2000 was added to 250 µl of Opti-MEM for each condition and incubated for 5 minutes. The optimal concentration of siRNA (60 nM) was added to 250 µl of Opti-MEM. After 5 minutes, the diluted Lipofectamine 2000 was mixed with the siRNA to give a total volume of 500 µl and incubated at room temperature for 20 minutes. The mixture was then added dropwise to the cells and incubated with the transfection reagents for 6 hours and experimentation performed 48 hours post transfection

Table 2–2: siRNA Sequences.

Target	siRNA Sequence
GNAS1	GCGCCGCCAAAGACGTGAAATTACT
LUCIFERASE	CGU ACG CGG AAU ACU UCG A

2.3 Western Blotting

2.3.1 Protein preparation and quantification

Western blotting was performed to identify and analyse protein expression. To prepare cell lysates, the media was removed from the cells and then, the cells were washed with PBS. Cell lysates were prepared using chilled radio immunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, UK) and Phosphatase and Protease Inhibitor (Thermo Fisher Scientific, UK), added directly to the cells and incubated on ice for 15 minutes. The lysate was then collected into 1.5ml eppendorf using scraper (Costar, UK) and centrifuged at 13,000rpm at 4ºC for 30 minutes. The protein lysis was collected, aliquoted into 0.5ml eppendorf tubes and store at -20ºC.

BCA Protein Assay Kit (Thermo scientific, UK) was used for protein quantification reaction according to the manufacturer's protocol. Bovine Serum Albumin (BSA) solution was used in series as a standard reference in the concentration range of 25–2000 µg/ml. The buffer solution was used as a blank sample. The 96 microplate wells (Costar, UK) contained 12.5µl of each sample and 100µl of the working reagent (1:50 reagent A to reagent B) in each well in duplicate. In order to mix the samples with working reagent, the plate was shaken for 30 second and then incubated for 30 minutes. Then, the absorbance at 550nm was measured on SpectraMax microplate reader (Molecular Devices, USA).

2.3.2 SDS-PAGE Gel and Western blot

NUPAGE LDS Sample Buffer (Thermo Fisher Scientific) containing 5% β-mercaptoethanol was added to 50 µg of protein. The protein samples were boiled for 5 minutes and then run on a NuPAGE 4-12% bis-tris gel (Invitrogen, UK) at 125 V for 90 minutes in NuPAGE running buffer (Invitrogen, UK). Protein samples were then transferred to a methanol activated PVDF membrane (GE Healthcare, UK) using the Trans-Blot Semi-Dry Transfer Cell (Bio-Rad) at 20 V for 30 minutes. After the transfer, the membranes were blocked

with 5% milk TPBS or 5% BSA TBST (depending on antibody) for 40 minutes at room temperature. Following the membrane blocking, the primary antibodies (*GNAS*, Thermo scientific, UK CAT #PA5-19315) were diluted (1/500) in 5% BSA+ PBS-T or β-actin (Sigma, UK) 1:50,000 (0.5ul in 25 ml) in 5% milk TPBS incubated with membrane overnight at 4°C. The membranes were then washed 3 times in TPBS or TBS-T at room temperature and incubated with a 1:30,000 (β-actin) anti-mouse (Sigma, UK, CAT #A4416) or 1:5000 (*GNAS*) anti-goat secondary antibody (Alpha diagnostic Intl Inc, UK CAT #30365-200) for 60 minutes at room temperature. Subsequent to this, the membranes were washed twice in TPBS or TBS-T and once in PBS or TBS each for 5 minutes at room temperature. The membranes were then developed using ECL Prime detection solution (Abersham, UK) for 5 minute, sealed in saran wrap and exposed to film (Amersham GE healthcare, UK) for 1 sec to 5 minutes. The films were then scanned after drying. Bands were quantified using image J software.

2.4 Functional Assays

2.4.1 Transwell Migration Assay

Cell migration was measured using the transwell migration system (Figure 2-2). The transwell migration assay was performed using 24 transwell chambers containing a polycarbonate filter with an 8 μm pore size (Costar, UK). The Transwell inserts (8 μm pore size) were incubated in DMEM at 37°C for 1 hour prior to use. Subsequent to this, 200μl of DMEM supplemented with 20% FBS was added to the outer wells of the Transwell plate and the Transwell inserts placed inside. Following this, a total of 1 $x10^5$ cells in DMEM supplemented with 10% FBS were seeded onto the Transwell insert. The plate was incubated for 24 hours at 37°C. Twenty-four hours post incubation, the cells migrated through the membrane towards the higher FBS concentration chemoattractant and cells on the bottom of the well were manually counted. Triplicate wells were seeded for each experimental condition.

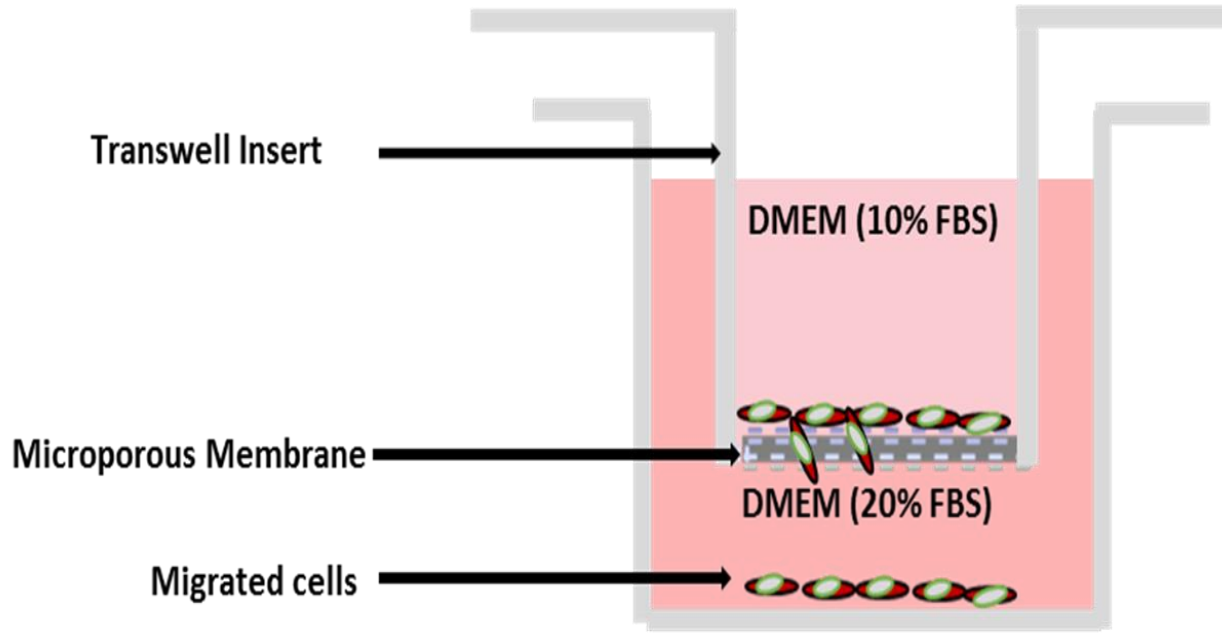

Figure 2-1: The Transwell migration assay.
Cell migration assay was performed using Transwell system with FBS as chemoattract for migrating cells

2.4.2 Proliferation assay

PrestoBlue Cell Viability Reagent (Thermo Fisher Scientific) was used as an indirect method to measure the total number of live cells. The experiment was performed according to the manufacturer's protocol. Briefly, each well of a 24 well plate (Costar, UK) was seeded with $2X10^4$ cells in triplicate and allowed to adhere for 24 hours. Following this, 200 µl of PrestoBlue Cell Viability Reagent was applied to the cells and without cells as control and incubated at 37^0C for 1 hour. Following incubation, one hundred µl of the PrestoBlue Cell Viability Reagent was removed from the cells and transferred to a 96 well plate. The fluorescent unit (OD) of each well was measured using the BMG FLUOstar Optima Plate reader (569 nm/586 nm) at different time points (Day 1, Day 2 and Day 3 respectively).

2.5 DNA Preparation

2.5.1 Samples Collection:

2.5.1.1 Nottingham Cohort

Tumour blocks from 82 primary CRCs cases were retrieved between January and July 2012 and patients' information were available at diagnostic histopathology database at the Queen's Medical Centre (QMC). Approval for the study and access to the tissues were granted by Nottingham Health science Biobank (Reference ID: ACP00000147). It was initially agreed to use 100 CRC cases to screen for specific mutations and investigate the associations with clinical features of the patients, only 82 of those were available for this analysis. The cases were randomly screened and those with proved diagnosis of Primary CRC were retrieved by Nottingham Health Science Biobank from the NUH Histopathology archive. Those samples with sufficient tumour content (more than 30%) and (less than 10% necrosis) were selected following reviewing of the H&E slides by a pathologist (Professor MI). Clinical data were not available for 1 patient for stage of primary tumours, Dukes staging, vascular invasion, and lymph node involvement and 3 patients for gender, local recurrence and distant metastasis, and these cases were not included in the final analysis. Patients Characteristics are summarized in Table 2-3.

2.5.1.2 Edinburgh Cohort:

The archives of Edinburgh Pathology Department were searched for cases which had previously been tested by immunohistochemistry for expression of MMR proteins or PCR and CE for MSI. Eighty eight of CRC were retrieved consisting of 45 cases which were dMMR/MSI and 43 cases which were pMMR/MSS. Access to tissues and ethics approval were granted by Nottingham Health Sciences Biobank, which has approval as an IRB from North West—Greater Manchester Central Research Ethics Committee (REC reference: 15/ NW/0685). Access to anonymised use of tissues was granted by Tissue Governance NHS Lothian under ethics approval number SR783.

Table 2–3: Patients characteristics.

Feature		*Frequency*	*Percentage (%)*
Gender			
	Male	*56*	*70%*
	Female	*23*	*29%*
	Not Available	*3*	*3%*
Stage of Primary tumours			
	T1	*2*	*2%*
	T2	*12*	*14%*
	T3	*45*	*55%*
	T4	*22*	*27%*
	Not Available	*1*	*1%*
Dukes' stage			
	A	*12*	*14%*
	B	*36*	*44%*
	C	*31*	*38%*
	D	*2*	*2%*
	Not Available	*1*	*1%*
Vascular Invasion			
	V0	*43*	*53%*
	V1	*38*	*46%*
	Not available	*1*	*1%*
Lymph Node Involvement			
	N0	*51*	*62%*
	N1	*24*	*29%*
	N2	*6*	*7%*
	Not Available	*1*	*1%*
Local Recurrence			
	Yes	*5*	*6%*
	No	*74*	*90%*
	Not Available	*3*	*3%*
Distant Metastasis			
	Yes	*21*	*25%*
	No	*58*	*70%*
	Not Available	*3*	*3%*
Total		*82*	

2.5.2 DNA extraction and quantification

DNA was extracted from tumour section using QIAamp DNA FFPE Tissue Kit following the kit instructions. Tumour-containing sections were microdissected from regions corresponding to slides stained with hematoxlin and eosin (H&E). Slides containing tumour sections were deparaffinized with 3 rounds of 10minutes with xylene followed by incubation with 100%, 90%, and 70% of ethanol, 10minutes each with final incubation of 30second with distilled water. Tumour area was then removed by scalpel into a 1.5ml tube. Samples were then re-suspended in 180 µl Buffer ATL and 20µl proteinase K were added to the clear lower clear phase, then the samples were mixed by vortexing. The samples were then incubated at 56°C (350 rpm) overnight, to ensure the samples are completely lysed. Following that, incubation at 90°C for 1 hour was performed. 200µl AL buffer with 200µl ethanol (100%) were then added to the lysate and mixed by vortexing. The entire lysate was transferred to a QIAamp MinElute column with 2ml collection tube followed with a subsequent centrifugation step (10,000 rpm, 1 minute). 500µl AW1 Buffer (with ethanol) was added to the column and centrifuged (8000 rpm, 1 minute). A new collection tube was added and the same procedure was repeated for buffer AW2. Centrifugation at 14,000 rpm for 3 minutes afterward was required to dry the membrane. Elution was achieved via the addition of ATE. The samples were then quantified using nanodrop technology (NanoDrop 2000 Spectrophotometer, The absorbance obtained by the instrument was kept around 2.0 (260/280 ratio) for optimal purity of the extracted DNA. Thermo scientific) maintaining average of 397 ng/µl (range: 36-1544 ng/µl) and concentrations were then diluted with H^2O to obtain 20 ng/µl for each sample before performing PCR. (Figure 2-2)

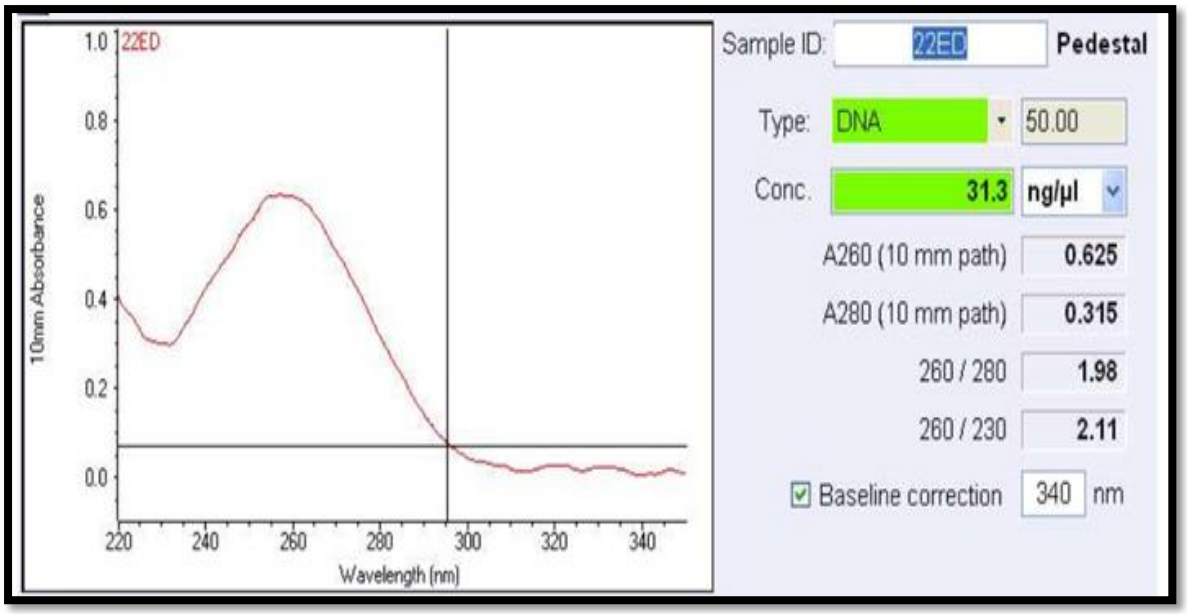

Figure 2-2: diagram represents quantified DNA generated by NanoDrop 2000 Spectrophotometer, Thermo scientific

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2.5.3 Quality of DNA by HRM

DNA quality was analysed using PCR followed by High-resolution melting (HRM) analysis. HRM is post-PCR-based test involving the quantitative analysis of the melt curve of target DNA fragment; it is considered as the next generation method for amplicon melting screening (Erali and Wittwer, 2010). Fluorescent DNA analysis is performed in the presence of saturation dyes that fluoresce strongly with double stranded DNA (Reed, Kent and Wittwer, 2007; Erali and Wittwer, 2010). The melting curve is generated when the sample is heated up in a range of temperatures in the presence of fluorescence. The temperature has a negative feedback on the fluorescence, meaning as the temperature increases, the fluorescence decreases reaching the melting point where the fluorescence drops rapidly, indicating the melting of double stranded DNA into single strands (Figure 2-3) (Reed, Kent and Wittwer, 2007). DNA melt curve profiles generated by HRM are sensitive and specific, and can be used in different analytical tests, including mutation screening, methylation analysis and genotyping, to detect single based sequence change or identify unknown genetic mutations based on small sequence differences (Garritano *et al.*, 2009).

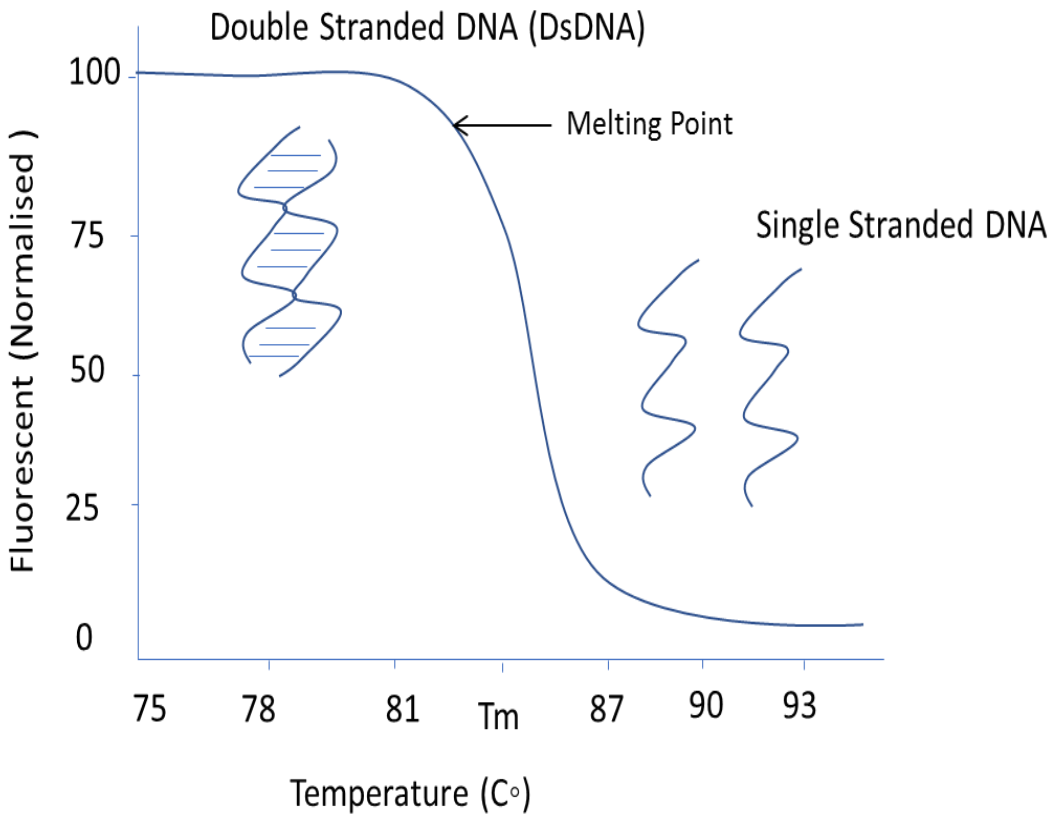

Figure 2-3: Principle of HRM analysis.
Double stranded DNA (DsDNA) is heated up until reaching the melting point where the fluorescence dye is burst and decreased with single stranded DNA at low temperature.

DNA quality was analysed using multiplexed PCR containing four sets of primers (100, 200, 300 and 400bp in length) for the housekeeping gene GAPDH. Each PCR reaction contained 1x HotShot Master-Mix (Cadama Medical Ltd), 0.4 uM of each primer, 1µl of 20ng DNA template, all made up to total volume of 10 µl with PCR grade water. PCR thermal cycling was applied as follows: (95°C 3 minutes) x 1 cycle; [(95°C/10 seconds) / (62°C/30 seconds) / (72°C/20 seconds)] x 45 cycle. PCR products were then melted by HRM (Lightscanner). (Figure 2-4)

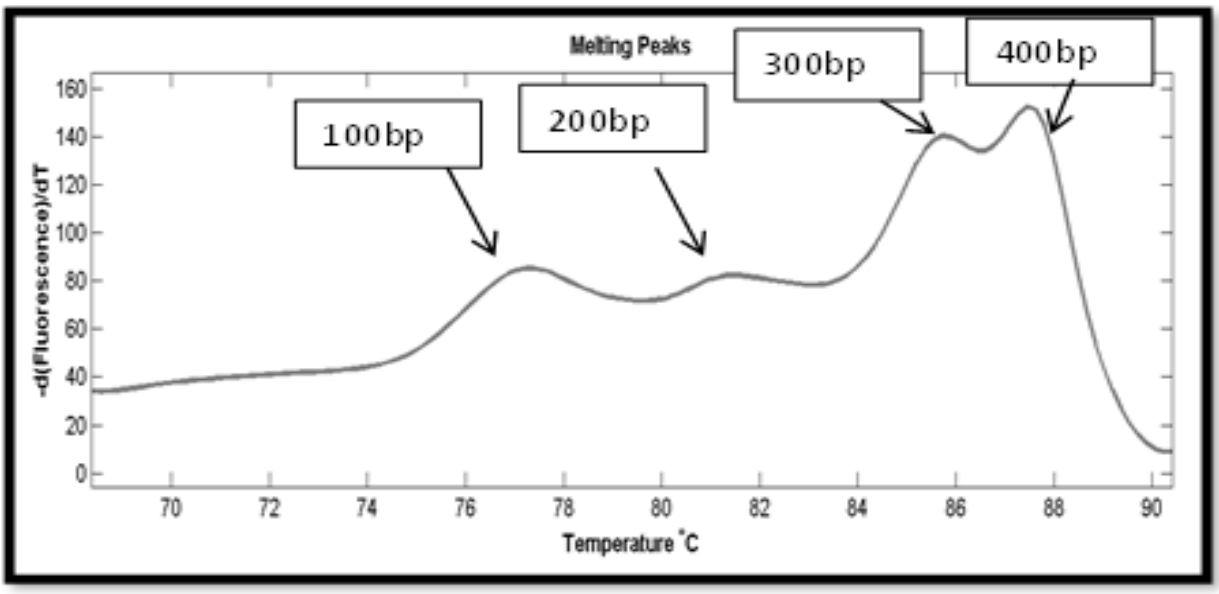

Figure 2-4: DNA quality analysis by HRM using housekeeping gene GAPDH
Each peak represents specific primer binding depending on the amplicon size.

2.6 Polymerase Chain Reaction (PCR)

2.6.1 Target genes selection

The selection of targets genes was based on their mutations recurrence in CRC. Selected genes and hotspots have been routinely investigated; *KRAS* (exon 2, 3, and 4), *PIK3CA* (exon 9 and 20), *BRAF* (exon 11 and 15), *TP53* (exon 6 and 8), *PTEN* (exon 3 and 8), *SMAD4* (exon 9). Primers were previously designed in our lab and published (Fadhil *et al.*, 2010) Primers sequences are shown in appendix.

2.6.2 Annealing Temperature (Ta) Optimization

Gradient thermal cycler has been used for PCR optimization by detecting the annealing temperature gradient. The predicted annealing temperature of each primer pair was set with temperature range of ±4. Following this step, the PCR products were evaluated by high resolution melting (HRM) analysis.

2.6.3 Mutation screening by QMC-PCR followed by High-resolution melting (HRM)

Mutation screening were conducted using quick-multiplex consensus (QMC) followed by HRM which has been developed in our lab for mutation detection in FFPE tissue (Fadhil *et al.*, 2010). QMC-PCR is a nested procedure in which PCR is initially performed in pre-diagnostic multiplex (PMD) reaction followed by a single specific diagnostic (SSD) reaction. PDM PCR is performed in a single reaction containing outer primer pairs up to 10 different target sequences, whereas SSD reaction contains a single and specific inner primer pair to each target sequence. PCR products are then melted in HRM (Lightscanner) for mutation screening (Fadhil *et al.*, 2010).

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For the purpose of detection of mutations in all CRC cases previously amplified by PCR, HRM was carried out using lightscanner instrument (BioFire Diagnostics, Inc). All samples were examined in triplicates and. For mutation analysis, the PCR reaction volume of 10µl contained 1x HotShot Master-Mix (Cadama Medical Ltd), 0.4 uM of each primer, and 1µl of 20ng DNA template. PDM-PCR was carried out at starting temperature of 95^{O}C for 5 minutes for 1 cycle followed by 25 cycles of 95^{O}C for 10 second, 55^{O}C for 10 second. The PCR products from the PDM reactions were diluted 1:100 in PCR-grade water, and 1 µl of diluted PCR produced was used as a template for a single specific diagnostic (SSD) PCR in which each of the hotspots was tested individually. The SSD PCR reaction was performed in a final volume of 10µl containing 1x HotShot Master-Mix (Cadama Medical Ltd), 0.25 µM of each primer, 0.4 x of Eva Green (Biotium, Inc), and 1µl of 20ng DNA template. PCR amplifications were conducted using the same cycling condition used for PDM. The products were then melted using lightscanner instrument with a rate of temperature increase of 0.3^{O}C per second. The difference plot was generated after normalising the temperature shift to a reference sample. Known wild-type samples were used as reference samples and all mutant samples were validated 3 times. A threshold point of 4% difference in fluorescence was used to separate out the mutants from the wild-type samples, which defines the difference of melting within both sequences. The data were analysed using lightscanner melt analysis tool software Version 2.5.0.3057 (BioFire Diagnostics, Inc).

2.6.4 Testing for Microsatellite Instability (MSI)

To study the MSI status, a panel of six markers (BCAT-25, BAT-25, BAT-26, NR21, NR22, and NR24) were screened for all cases using PCR followed by high-resolution melting (HRM) analysis as previously described (Fadhil et al. 2012), primers sequences are shown in appendix. PCR was carried out in 10µl containing 1x HotShot Master-Mix (Cadama Medical Ltd), 0.25 µM of each primer, 0.4 x of Eva Green (Biotium, Inc), and 1µl of 20ng DNA template. The products were then melted using the lightscanner instrument with a rate of temperature increase of 0.3^{O}C per second. The difference plot was generated after normalising and temperature shifting. Microsatellite stable samples were used as reference samples and all mutant samples were validated 3 times. A threshold point of 4% difference in fluorescence was used to separate out the aberrant samples from the wild-type samples. The data were analysed using Lightscanner melt analysis tool software Version 2.5.0.3057 (BioFire Diagnostics, Inc). Instability at 2 or more MSI markers refers to MSI-high tumours, whereas instability at 1 locus was regarded as microsatellite stable (MSS) tumours.

2.6.5 HRM-methylation specific analysis

Genomic DNA (400ng) was modified sodium bisulfite using EZ Methylation Lightening Kit (Zymo Research), according to manufacture protocol. HRM post-PCR was used to assess methylation analysis for promoter genes (*MLH1* and *P16*), primers used in this analysis were previously published in (Li et al., 2014) and primers sequences are listed in appendix Table 8-3 . PCR reaction was performed in a final volume of 10µl containing 1x HotShot Master-Mix (Cadama Medical Ltd), 0.25 µM of each primer, 4x of Eva Green (Biotium, Inc), and 2µl of bisulfite converted DNA. PCR amplification was performed at 95^{O}C for 5 minutes for 1 cycle followed by 40 cycles of 95^{O}C for 10 second, annealing for 30 second (*MLH1*: 58^{O}C& *P16*: 62^{O}C) and extension at 72^{O}C for 20 second. EpiTec Methylated and Unmethylated sets (Commercially available controls, Qiagen) were used as 100% methylated and 0% unmethylated controls. Spike-in experiment for

methylation analysis included different percentages of methylation of 10%, 50% and 100% and 50% was used as cut-off threshold for hypermethylated samples (Figure 2-5 and 2-6).

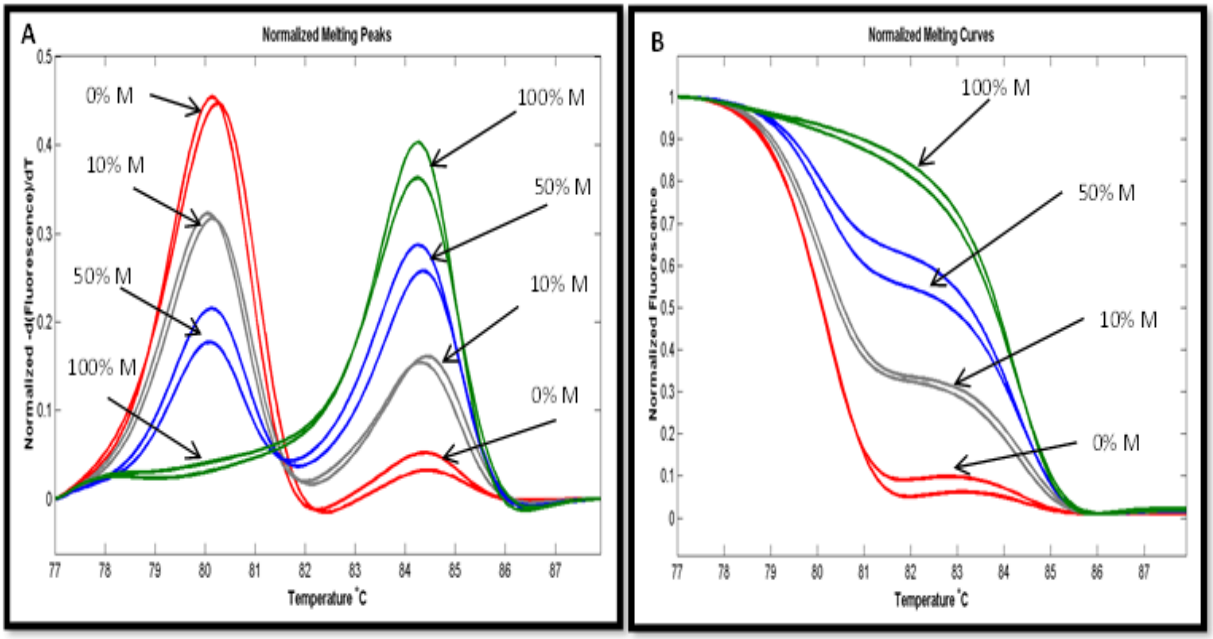

Figure 2-5: Normalized melt curve generated by HRM Lightscanner for MLH1 optimization.
Methylated and Unmethylated controls (Qiagene) used in spike-in experiment for methylation analysis including different percentages of methylation of 10%, 50% and 100%. 50% was used as cut-off threshold for hypermethylated samples. A) Normalized melting peaks, B) normalized temp shift curve; for methylated and unmethylated controls.

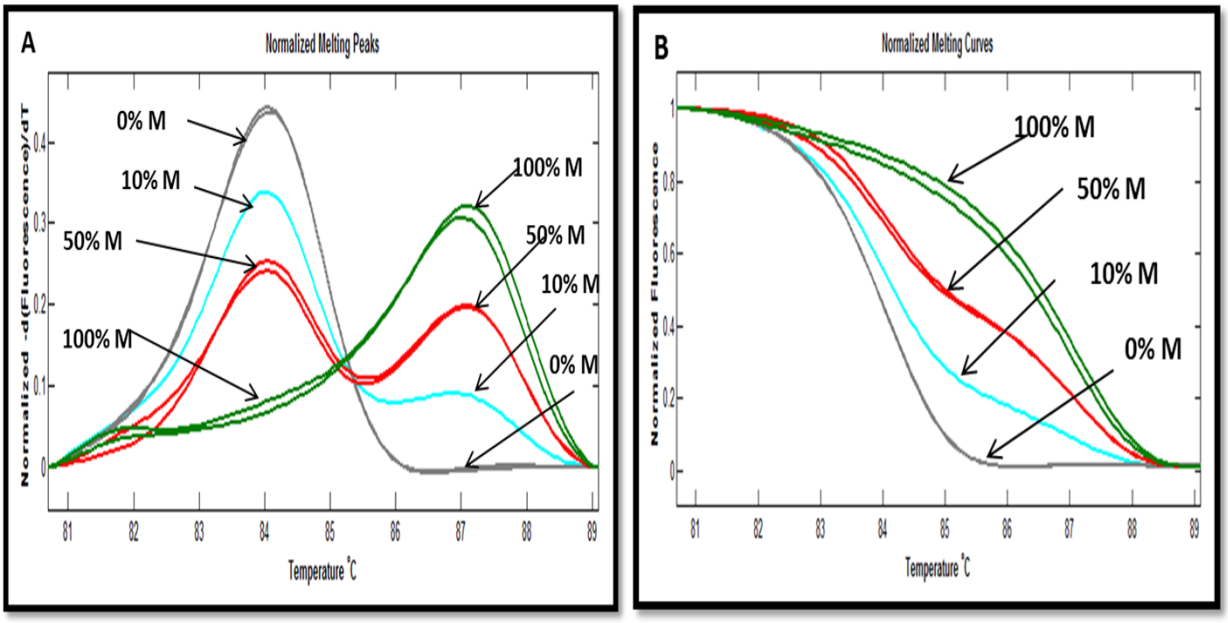

Figure 2-6: Normalized melt curve generated by HRM Lightscanner for P16 optimization.

Methylated and Unmethylated controls (Qiagene) used in spike-in experiment for methylation analysis including different percentages of methylation of 10%, 50% and 100%. 50% was used as cut-off threshold for hypermethylated samples. A) Normalized melting peaks, B) normalized temp shift curve; for methylated and unmethylated controls.

2.7 Investigating Circulating tumour DNA/miRNA

2.7.1 Primer design and optimisation

Primers were designed to specifically amplify short, fragmented DNA template (<150bp) found in *cfDNA* and FFPE tissue. Primers were designed according to standard rules except for amplicon length (Dieffenbach et al, 1993). The catalogue of somatic mutations in cancer (COSMIC) was used to find the most common mutation hotspots for each chosen gene in the large intestine. Genes were chosen based on previous data (Fadhil et al, 2010). Ensembl was used to browse the genome for hotspot specific DNA sequence. Mutation status of *KRAS* and *BRAF* were chosen as the biomarkers for this study because mutations in these genes are recurrent in CRC. Furthermore, the mutation for these genes are localised to few hotspots - exon 2 of *KRAS* contains which account for >90% of CRC *KRAS* mutations, while exon 15 of *BRAF* which account for >90% of CRC *BRAF* mutations.

A 2-step system (details below) was used for primer optimisation. The online software uMelt was used to determine the approximate location of the correct melting temperature location. Gradient PCR followed by high resolution melting analysis (HRM) was performed to determine the optimal primer annealing temperature (Ta). The optimal Ta was regarded as the temperature which gave the highest single fluorescent melting peak which was also in the correct location by HRM. Gradient PCR cycling conditions (Peqlab Primus96 Advanced Gradient Thermal Cycler) are as follows: [(95°C 5min) x1; (95°C 10sec, 60+/-5°C 30sec, 72°C 20 sec) x40]. Reaction conditions: [5µl Hotshot Diamond mastermix (Clent Life Science); 1µl 20 x EvaGreen Dye in water (Biotium, inc.); 250nM of each primer (Eurofins); 40ng cell line DNA template; addition of Nuclease-Free water (Qiagen) to give a total reaction volume of 10µl]. HRM analysis was performed on LightScanner system. The PCR products were first transferred into a LightScanner 96-well hard-shell plate and spun down in a Megafuge centrifuge (2000 rpm, 5min). HRM was performed between 65°C and 95°C with sample equilibration at

62°C. Exposure was set to 'Auto' and data was captured at a ramp rate of 0.10°C/sec. The acquired melting data was analysed with the LightScanner Call-IT software version 2.0.0.1.331.

2.7.2 Blood samples and plasma separation

Twenty-six (26) CRC patients were recruited to this study. Fifty (50) CRC patients were initially planned for this study but only 26 of those were recruited due to time constraints of the PhD. The patients had undergone surgery in either 2015 or 2016 at the Queen's Medical Centre, Nottingham. Blood was collected in EDTA tubes pre-operatively on the morning of surgery (called day 1) and post-operatively the following days which were named day 2 onwards (weekends permitting). A summary of the number of post-operative samples can be found in table 2-4 below. Plasma from patient 1 was exhausted during optimisation and could not undergo final analysis. Approval for the study and access to the blood and FFPE tissues were granted by Nottingham Health science Biobank (Reference ID: ACP00000147).

Table 2–4: summary of samples collected after surgery (n=44 plasma sample from 26 case).

No. of samples collected post-operatively	No. of samples
1 sample	26
2 samples	11
3 samples	5
4 samples	2

Plasma was separated from blood within 2 hours after collection. For plasma separation, blood samples were centrifuged at 1900g for 10mins at room temperature, and transferred into 1.5ml Eppendorf tubes. The Eppendorf tubes were then centrifuged at 16,000g for 10mins at 4°C to remove the remaining cell debris. The plasma was stored at -80°C until DNA extraction took place.

2.7.3 FFPE DNA extraction and quantification

The corresponding tumour blocks or biopsies to match the blood samples were collected from the Queen's Medical Centre FFPE archive in 2016 for all the patients included in the final analysis (n=26). Haematoxylin and Eosin stained slides were also collected and analysed by a pathologist (WF) to determine the presence and percentage of tumour in each FFPE block before DNA extraction. FFPE blocks were not processed until the plasma had undergone final analysis to stop bias in result interpretation.

Approximately 20µm thick FFPE sections were cut from resection blocks and processed using Qiagen's deparaffinization solution to prevent substantial loss of tissue. FFPE DNA was extracted via the use of Qiagen's QIAamp DNA FFPE tissue kit with no changes to the protocol. Tumour tissues were processed for digestion with proteinase K until all tissue was completely lysed to ensure the adequate presence of potential mutant target DNA. FFPE DNA was quantified via the use of the NanoDrop 2000c spectrophotometer.

2.7.4 Circulating cell free DNA extraction from plasma and quantification

DNA from plasma was extracted using circulating nucleic acid kit (Qiagen) to obtain efficient purification of these circulating nucleic acids from human plasma. The kit includes extension tubes and vacuum processing on the QIAvac 24 plus which enables starting sample volumes of up to 5ml and flexible elution volumes between 20 µl to 150 µl. Volumes of 0.4-1ml of plasma were processed for DNA isolation. 100µl of proteinase K and 0.8ml of ACL buffer (containing 1µg RNA carrier) were added to plasma in 50 ml tube and mixed thoroughly by vortexing for 30 second. Samples were then incubated at 60ºC for 30mins before adding 1.8ml of ACB buffer to the lysate in the tube and mixed thoroughly by vortexing for 15- 30 second. The mixture was then incubated on ice for 5 minutes before processing on QIAvac 24 plus. QIAamp mini column was inserted into VacConnector on QIAvac 24 plus and 20ml tube extender was inserted into the open QIAamp mini column. The lysate –buffer ACB mixture was then applied into the tube extender of QIAamp mini column and vacuum pump was switched on until all lysates have been drown through.600 µl of ACW1 buffer was then applied to QIAamp mini column and vacuum pump was switched on until all Buffer ACW1 have been drown through. 750µl of ACW2 was applied to QIAamp mini column and vacuum pump was switched on until all Buffer ACW2 have been drawn through QIAamp mini column. After that 750µl of 100% ethanol was applied to QIAamp mini column and vacuum pump was switched on until all Buffer ACW2 have been drawn through QIAamp mini column. QIAamp mini column was then removed from vacuum manifold and placed into a clean 2ml collection tube, then centrifuged at full speed (20, 000 x *g*; 14,000 rpm) for 3 minutes. QIAamp mini column was then placed into a new 2ml collection tube and incubated at 56ºC for 10 minutes with lid open to dry the membrane completely. After the incubation, QIAamp mini column was then placed into a clean 1.5 ml elution tube and a volume of 20µl-40µl of Buffer AVE was applied into the centre of QIAamp mini

membrane, and then incubated at room temperature for 3 minutes. QIAamp mini column was then centrifuged at full speed (20, 000 x *g*; 14,000 rpm) for 1 minute to elute the nucleic acids.

In general, less elution buffer was used for post-surgery samples to increase DNA concentration for PCR compared with pre-surgery samples. *cfDNA* was quantified using the DeNovixfluorometer combined with the DeNovix dsDNA high sensitivity assay kit (5pg/µl to 250ng/µl). Volume of plasma used, elution volume and DNA concentration of each sample can be found in Appendix table 8-1.

2.7.5 Outer primer PCR conditions and spiking preparation for *BRAF* exon 15 and *KRAS* exon 2

The CRC cell lines HCT116 (*KRAS* exon 2 codon 13 heterozygous mutant/*BRAF* exon 15 wild type) and RKO (*KRAS* exon 2 wild type/*BRAF* exon 15 V600E mutant) were extracted using the GenElute mammalian genomic DNA kit (Sigma Aldrich). The cell lines were quantified via a NanoDrop 2000c spectrophotometer and diluted to 20ng/µl. RKO and HCT116 were then mixed and serially diluted to give variant allele percentages of 12%, 6%, 3%, 1.5% and 0.75% for both mutations. Cell line DNA, FFPE DNA and *cfDNA* samples underwent PCR with the *KRAS* exon 2 and *BRAF* exon 15 outer primers listed in appendix. Reaction conditions (*cfDNA*): [5µl Hotshot Diamond mastermix (Clent Life Science); 1µl 20 x EvaGreen Dye (Biotium, inc.); 250nM of each outer primer (Eurofins); 5ng *cfDNA*; addition of Nuclease-Free water (Qiagen) to give a total reaction volume of 15µl]. Reaction conditions (cell line and FFPE): [5µl Hotshot Diamond mastermix (Clent Life Science); 1µl 20 x EvaGreen Dye in water (Biotium, inc.); 250nM of each outer primer (Eurofins); 40ng FFPE DNA or cell line DNA; addition of Nuclease-Free water (Qiagen) to give a total reaction volume of 10µl]. All reactions were carried out in duplicate, including negative controls. Cycling conditions (Applied Biosystem fast 7500):

[(95°C 5min) x1; (95°C 10sec, 58°C 30sec, 72°C 20 sec) x40]. PCR products were then diluted 1:100 prior processing for second stage PCR using inner primers.

2.7.6 COLD PCR-HRM optimization and determination of critical denaturation temperature for *KRAS* exon 2

Full-COLD PCR was used in this study for the enrichment of low percentage variant alleles. Co-amplification at lower temperature PCR (COLD-PCR) is a novel modification of the conventional (Standard) PCR method that preferentially enriches that low variant alleles from a mixture of wild-type and mutant sequences (Milbury *et al.*, 2011). This method is dependent on critical temperature (Tc) for each DNA sequence which is lower than the actual melting temperature (Tm). Two platforms of COLD-PCR protocol have been introduced, fast COLD-PCR and full COLD-PCR. Full COLD PCR has the potential to enrich all types of mutations, whereas Fast COLD-PCR only amplifies only mutation with lower Tm than wild-type (C/G to A/T) (Milbury *et al.*, 2011). Full COLD-PCR principles are shown in (Figure 2-7).

In order to determine Tc for full COLD-PCR, a set of COLD-PCR reactions at graded temperatures below the actual Tm were performed using gradient PCR (Peqlab Primus96 Advanced Gradient Thermal Cycler) to detect the optimal Tc. Optimisation gradients with full-COLD PCR generated data which was difficult to interpret and therefore optimisation with series of denaturation temperatures were employed in RealTime PCR (Applied Biosystem) starting at actual product Tm minus 0.2°C intervals downward. Samples included a full gradient of pre-amplified RKO (WT), HCT116 (mutant), 0.75% HCT116. Outer primer PCR conditions for cell line DNA is mentioned in section 2.8.5 of materials and methods.

The initial estimated critical temperature for the full COLD-PCR was decided using the following concept. Melting data of *KRAS* exon 2 inner primer PCR product was analysed via HRM and the centre of the melting peak -3.5°C /+0.5°C was regarded as Tm. Cycling conditions (Applied Biosystem 7500 fast) for full COLD-PCR optimisation: [(95°C 5min) x1 (95°C 10sec, 70°C 20sec, 82°C (-0.1°C) 40sec, 62°C 30sec, 72°C 20 sec) x30]. Reaction conditions: [5µl Hotshot Diamond mastermix (Clent Life Science); 1µl 20 x EvaGreen Dye in water (Biotium, inc.); 250nM of each inner primer (Eurofins); 1µl 1:100 outer primer PCR product as template; addition of Nuclease-Free water (Qiagen) to give a total reaction volume of 10µl]. All reactions were carried out in duplicate, including negative controls. To determine the critical temperature, the products generated from full COLD-PCR were analysed by HRM. The critical temperature was classed as the temperature which gave the greatest amount of enrichment without causing aberrant melting or artefacts. The greatest amount of enrichment is described as the largest fluorescence difference determined by HRM between base line wild type DNA and the 0.75% variant allele. The optimal Tc for *KRAS* exon 2 was 81.8°C and 79.9°C for *BRAF* exon 15.

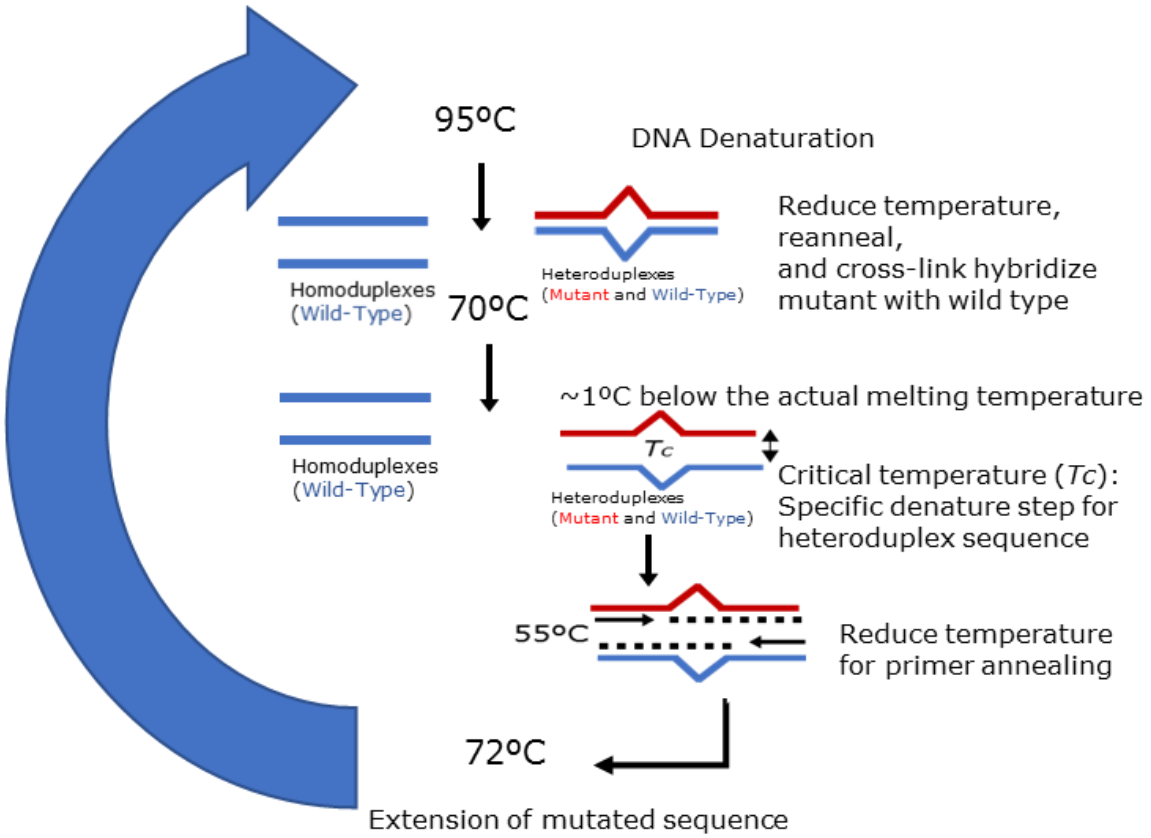

Figure 2-7: *Full* COLD PCR protocol.

2.7.7 Full-COLD PCR-HRM for detection of mutations in *cfDNA* and FFPE tissue

Full-COLD PCR was used in this study for the enrichment of low variant alleles from both *cfDNA* and FFPE tissue. A 2-step nested protocol was used. For the first step, PCR reactions were amplified using the standard PCR protocol and products were then diluted 1:100 and used as templates for full COLD-PCR as second stage. Full-COLD cycling conditions: (ABI 7500 FAST): [(95°C 5min) x1 (95°C 10sec, 70°C 20sec, Tc 40sec, 62°C 30sec, 72°C 20sec) x25]. Tc for *KRAS* exon 2 = 81.8°C; Tc for *BRAF* exon 15 = 79.9°C. Reaction conditions full-COLD PCR: [5µl Hotshot Diamond mastermix (Clent Life Science); 1µl 20 x EvaGreen Dye in water (Biotium, inc.); 250nM of each inner primer (Eurofins); 1µl 1:100 outer primer PCR product as template; addition of Nuclease-Free water (Qiagen) to give a total reaction volume of 10µl]. All reactions were carried out in duplicate, including negative controls. HRM was performed as described in section of the materials and methods.

2.7.8 Squirrel primer assay and sequencing protocol for determining absolute proof FFPE mutations

Inner primers used in this study were <100bp amplicons long which was difficult to analyse by Sanger sequencing. We therefore used "Squirrel primers" (5' 40 nucleotides long tail added to the specific sequence of the primers) to Sanger sequence directly <100bp of genomic amplicons. Primers were designed in our lab (Ebili *et al.*, 2017) and primer sequences are listed in appendix. Squirrel primer assay concepts have been successfully applied to this study to sequence <100bp amplicons by Sanger method. Inner primers were tagged with a long, non-specific, "squirrel" tail which has a 5' end that is complementary to the sequencing primers, Useq. Cycling conditions (Peqlab Primus96 Advanced Gradient Thermal Cycler): [(95°C 5min) x1 (95°C 10sec, 60°C 30sec, 72°C 20 sec) x30]. Reaction conditions: [5µl Hotshot Diamond mastermix (Clent Life Science); 1µl 20 x EvaGreen Dye in water (Biotium, inc.); 250nM of each squirrel primer (Eurofins); 1µl (undiluted PCR product from full COLD-PCR) as template; addition of Nuclease-Free water (Qiagen) to give a total reaction volume of 10µl]. All reactions were carried out in duplicate, including negative controls.

For sequencing, PCR products were purified using the GenElute PCR Clean-up kit. The purified products were diluted to 3ng/µl following initial quantification in a NanoDrop 2000 UV Spectrophotometer. Sequencing was performed with the dye terminator chemistry (BigDye version 3.1) on the 3130xl ABI PRISM Genetic Analyzer, using the USeq primer pairs as universal sequencing primers.

2.7.9 ctmiRNA Extraction

miRNA was isolated from 200µl of plasma sample following the modified protocol for miRNeasy serum/plasma kit (Qiagene) for the purification of total RNA. Synthetic miR-39 spike-in control was used prior the extraction to provide stable reference for accurate and normalised measurement. 1000µl QIAzol lysis reagent was added to the sample and briefly vortexed. The samples were then incubated for 5min at room temperature before adding 3.5µl of miRNeasy serum/plasma spike-in control and thoroughly vortexed. A volume of 200µl of chloroform were added to the sample, vortexed for 15 seconds and incubated at room temperature for 3 mins. The samples were then centrifuged for 15 mins at 12,000 xg at 4ºC. The lower phase was then transferred into a new 2ml microcentrifuge tube and mixed thoroughly with 900 µl of 100% ethanol. The entire lysate was then transferred into RNeasy MinElute spin column and centrifuged for 15 seconds at 8,000 xg at room temperature and the flow-through was discarded. 700µl of RWT buffer was then added and centrifuged for 15 seconds at 8000 xg to wash the column and the flow-through was discarded. Following that, 500µl of buffer RPE was added to the sample and centrifuge for 2mins at 8,000 xg using the same collection tube with discarding the flow-through. 500µl of 80% ethanol was then added and centrifuged for 2 mins at 8,000 xg to wash the column membrane. Samples were then transferred to new RNeasy MinElute spin column and centrifuged at full speed for 5 mins with the lid open to dry the membrane and discarding the flow-through. The RNeasy MinElute spin column was place in a new 2 ml collection tube, then centrifuged at full speed for 5 mins. Finally, using new 1.5 ml collection tube, 14 µl RNeasy-free water was added directly to the spin column membrane and centrifuged for 1 min at the full speed to elute the RNA.

2.7.10 RNA concentration and purity assessment

The concentration and purity of extracted RNA containing miRNA was quantified using Thermo Scientific NanoDrop™ 1000 Spectrophotometer. The absorbance obtained by the instrument was kept around 2.0 for optimal purity of the extracted RNA. The quantified samples were then kept at -80°C till processing.

2.7.11 Reverse Transcription

Reverse transcription was performed to create cDNA (Complementary DNA) to be quantitatively measured by qPCR. 5µl of total RNA containing miRNA (as recommended by the kit for extracted plasma) was reverse transcribed using miScript II RT kit (Qiagen) and relative miRNA expression levels were determined using miScript SYBR Green PCR kit (Qiagen). The PCR product obtained 5x miScript HiSpec buffers (4µl), 5µl of Total RNA, 10x of Nucleics mix (2µl), 2µl of miScript Reverse transcriptase mix and RNease-free water up to 20 µl of total PCR reaction. The PCR were then incubated at 56°C for 1 hour and at 90°C for 5 mins. PCR products were then placed on ice of 5 mins. cDNA was then diluted and processed for RT-PCR quantification.

2.7.12 Generation of Standard Curve

Standard curve was generated to estimate PCR assay efficiency for controls and targeted miRNAs. Serial dilutions of 1:10, 1:100, and 1:1000 of cDNA were obtained to create a proportional amplification for each dilution. Perfect PCR efficiency will demonstrate linear standard ($R>0.98$), high amplification efficiency (95-105%) and 3.3 cycle fold change between 10 fold dilutions. Figure 2-7

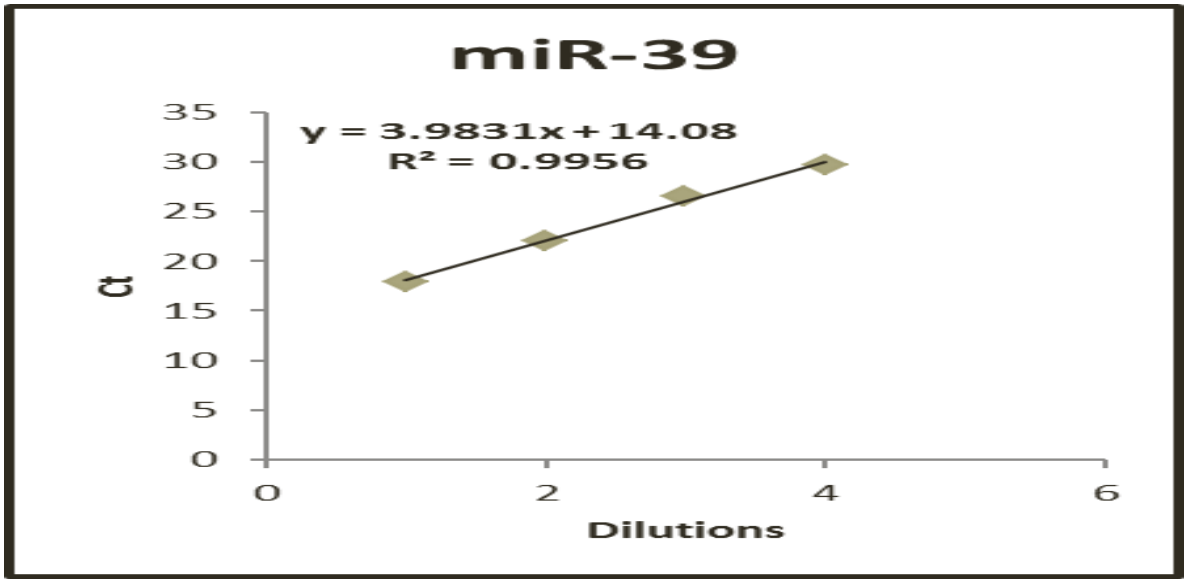

Figure 2-8: Standard curve for miR-39 assay efficiency.

2.7.13 miRNAS Expression Quantification by RT-PCR

The RT-PCR quantification was performed using real time thermal cycler PCR instrument (Applied Biosystem). The PCR reaction was carried out in 10µl. Each reaction contained 2µl of template (cDNA), 2x QuantiTect SYBR green PCR master mix (5µl), 10x miScripts universal primers (1µl), 10x miScripts primer assay (1µl), and RNease-Free water up to 10µl.The cycling conditions were conducted at initial activation step at 95ºC for 15 mins, followed by 3-step cycling for 40 cycles; 94ºC for 15 seconds, 55ºC for 30 seconds and final extension at 70ºC for 30 seconds. The Ct values were calculated automatically by Applied Biosystem software. Optimal Ct values were kept below 30 Ct, and exceeded Ct values were excluded. The relative expression level was calculated using the equation 2-ΔΔCT in relative to the synthetic control (miR-39). Figure 2-8

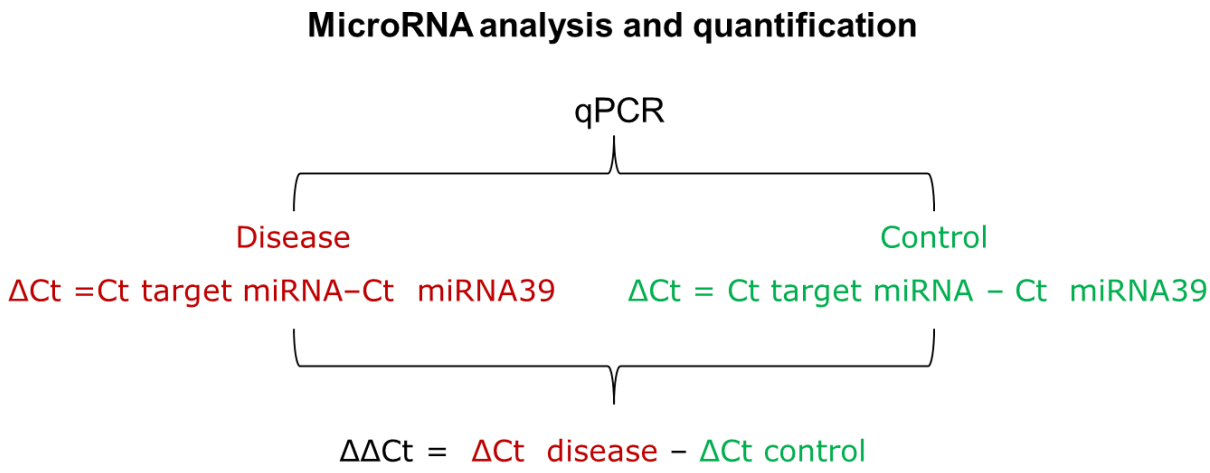

Figure 2-9: 2-ΔΔCT calculation

2.8 Statistical Analysis

2.8.1 Data Analysis for *in Vitro* Studies

GraphPad Prism software version 6 was used for statistical analysis. All evaluations were done using unpaired 2-tailed Student t test. For cell counting studies, cell numbers as represented by absorbance values from the presto blue assay were analysed. For transwell migration studies, the cell numbers were directly counted and analysed. For quantification of Western blots, x-ray films were scanned and converted into binary images using ImageJ software. Pixel counts for each antibody of interest (normalized to β-actin) were evaluated using the one-sample t test. The data are measured in triplicate and presented as means ± SD

2.8.2 Data Analysis for DNA investigation

All statistical analyses were performed with SPSS. The student chi square test (P value≤0.05 significance) was used to investigate the associations between mutations found in the sample set and clinicopathological parameters of the patients.

2.8.3 Data Analysis for miRNA expression

Manger v2,3 (Applied biosystem software) and excel Microsoft were used for $2^{-\Delta\Delta}$ calculations. Student's *t* test was used to analyse miRNA expression pre and post-surgery. ANNOVA was used for comparison of miRNA expression levels at all time points.

2.8.4 Data Analysis for Sanger sequencing

Sequencing was performed with the dye terminator chemistry (BigDye version 3.1) on the 3130xl ABI PRISM Genetic Analyzer. All reactions were carried out in duplicate, including negative controls. Sequence was analysed manually via Chromas software (version2.6).

3 Genotyping of CRC for Association with Lymphatic and Distant Metastases

3.1 Introduction

Colorectal cancer (CRC) is a common cause of malignancies and second leading cause of cancer death in worldwide (Herszényi and Tulassay, 2010; Kamiyama *et al.*, 2014; Bhandari, Woodhouse and Gupta, 2017). Most deaths of cancer, including CRC, are result of metastatic disease which is found in approximately 25% of cancer patients, and half of CRC patients are at risk of developing metastasis over time (Bilchik *et al.*, 2007; Koyanagi *et al.*, 2008; Klein, 2009; Kamiyama *et al.*, 2014) . Lymph node status is considered as one of the most prognostic factors for colorectal carcinoma (Fujii *et al.*, 2011). At advance stage of CRC, tumour cells invade locally to involve consecutive layers of colon or rectum, and then spread to the lymphatic vessels which enable tumour cells to penetrate through the lymphatic system and metastasize to involve distant organs such as liver or lung (Bathe and Farshidfar, 2014). However, there is still ambiguity regarding the mechanism leading to this spread in CRC tumours involving lymph nodes. The tumour's features that define this behaviour are reflected in the TNM (Tumour, Lymph Node, and Metastasis) staging system, where higher degrees of disease signify poor prognosis. Other clinicopathological features including degree of differentiation and presence of lymph node or vascular invasion are also well known to reflect biological behaviour in tumours with these characteristic (Compton and Greene, 2004; Bathe and Farshidfar, 2014) Furthermore, developing lymph node metastasis in CRC patients may worsen the prognosis, curative effect, and enhance drug resistance, thus reduce the survival rate of the disease (Bishehsari *et al.*, 2014; Xie *et al.*, 2017). Identifying specific markers for metastasis is required to help understanding the spread of disease to surrounding tissues, lymph nodes and distant organs.

The development in molecular biology has helped to understand CRC beyond the cellular levels and further clarified the role of genetic biomarkers. The main three genetic pathways are currently accepted in the tumorigenesis of CRC; chromosomal instability (CIN), microsatellite instability (MSI), and CpG methylator phenotype (CIMP). CIN

partially overlaps with methylator phenotype pathway in CRC, whereas MSI is found frequently associated with CIMP(Al-Sohaily *et al.*, 2012; Colussi *et al.*, 2013)

CRC progression is a result of multistep process as a consequence of accumulation of several genetic alterations in various pathways including RAS-RAF-MAPK, PI3K-PTEN-AKT, TGFβ, TP53 and DNA MMR pathways (Samuels *et al.*, 2005; Engelman, 2009; Zhang *et al.*, 2015). The activation of several signalling pathways by epidermal growth factor receptor (EGFR), including RAS-RAF-MAPK and PI3K-PTEN-AKT downstream pathways, plays a key role in regulating cell proliferation and motility, angiogenesis and apoptosis (Krasinskas, 2011; Wee and Wang, 2017). Accumulation of mutations in proto-oncogenes and tumour suppressor genes involved in these pathways such as *KRAS, BRAF, PIK3CA,* and *PTEN*, are well described in adenoma-carcinoma sequence in CRC. In RAS-RAF-MAPK pathway, mutations in KRAS and BRAF are found in 35%-45% and 4%-15% of metastatic colorectal cancer (mCRC), respectively. In PI3K-PTEN-AKT pathway, PIK3CA mutations and loss of PTEN expression are present in 10%-18% and 19%-42% of mCRC, respectively (Lièvre, Blons and Laurent-Puig, 2010; Mao *et al.*, 2015). KRAS and BRAF mutations are found mutually exclusive, whereas PIK3CA mutation may co-exist with either KRAS or BRAF mutations (Rajagopalan *et al.*, 2002).

In the treatment of mCRC, monoclonal antibodies, such as cetuximab and panitumumab, against EGFR are used in clinical practice (Wilson, Labonte and Lenz, 2010). Mutations in KRAS or BRAF have been widely demonstrated as major predictive biomarkers for resistance to anti-EGFR treatments, while wild-type KRAS or BRAF is associated with poor response to the treatment in mCRC patients (Allegra *et al.*, 2009; Wilson, Labonte and Lenz, 2010). Mutations in these genes are involved in the downstream signalling pathways of EGFR which may prevent the effect anti-EGFR targeted therapies (Karapetis *et al.*, 2008; Laurent-Puig *et al.*, 2009; De Roock *et al.*, 2010). Therefore, screening for KRAS and BRAF mutations prior to anti-EGFR therapy is required in order to prevent side-effects caused by EGFR antibodies and reduce excessive treatment costs.

Despite this background, genomic changes and their significance for metastatic CRC are not fully explored. Further insight into these molecular changes may help to understand the molecular biology of CRC and may identify therapeutic targets for this disease.

Therefore, this study aimed to:

(1) Genotype CRC using HRM technology and explore the molecular associations and their correlation to lymph node and distant metastases.
(2) Investigate the association between MSI, *BRAF* mutations and *MLH1* promoter methylation in sporadic CRCs.

3.2 Results

3.2.1 Nottingham Cohort

3.2.1.1 Mutation Screening Analysis

A total of 82 CRC cases underwent screening for mutations in six different genes (*KRAS, BRAF, PIK3CA, TP53, PTEN* and *SMAD4)* using HRM technology following the QMC-PCR protocol, as mentioned in the materials and methods section, which has been established in our lab as quick, cheap and reliable method for mutation screening. Positive and negative controls for mutation screening included in this study have been previously confirmed by Next generation sequencing (NGS) for either wild-type (negative control) or mutant allele (positive control) for specific targets and used as reference controls for data normalisation. The differences in DNA melting between wild-type and mutant allele can be observed simply in the melting plots generated by the software (Figure 3-1).

The overall mutation frequencies mostly had perfect concordance with previous published data, expect for *TP53* mutation which was relatively low (2 fold difference) in comparison to those in the literature (as shown in Table 3-1). Nonetheless, *KRAS* mutations were detected in 32 (39%) of the 82 cases, with 27 (84%) of these occurring in exon 2, 2 (5%) found in exon 3 and 4 (11%) being detected in exon 4, 1 (3%) of those coexisted in exon 2 and 4 (figure 3-1). *BRAF* mutations were detected in nine cases (10%), 7 (77%) in exon 15 and 2 (22%) mutation in exon 11 (figure 3-2). *PIK3CA* mutations were detected in six cases (7%), four cases (66%) in exon 9 and two cases (33%) in exon 20 (figure 3-3). *TP53* mutations were detected in 23 (28%) cases, 13 (59%) of those were detected in exon 8 and 9 (39%) in exon 6, one (1%) mutation coexisted in both exons (figure 3-4). *PTEN* mutations were found in twelve cases (14%), four cases (33%) in exon 3 and eight cases (66%) in exon 8, and significantly associated with lymph node involvement (Chi squared test, $p < 0.009$) tumours (figure 3-5). *SMAD4* mutations in exon 9 were detected in two cases (2.46%) (Figure 3-6). No

significant association was found between *KRAS*, *PIK3CA*, *BRAF*, *TP53* and *SMAD4* mutations and any of the studied pathological categories (for summary, see Tables 3-2 & 3-3).

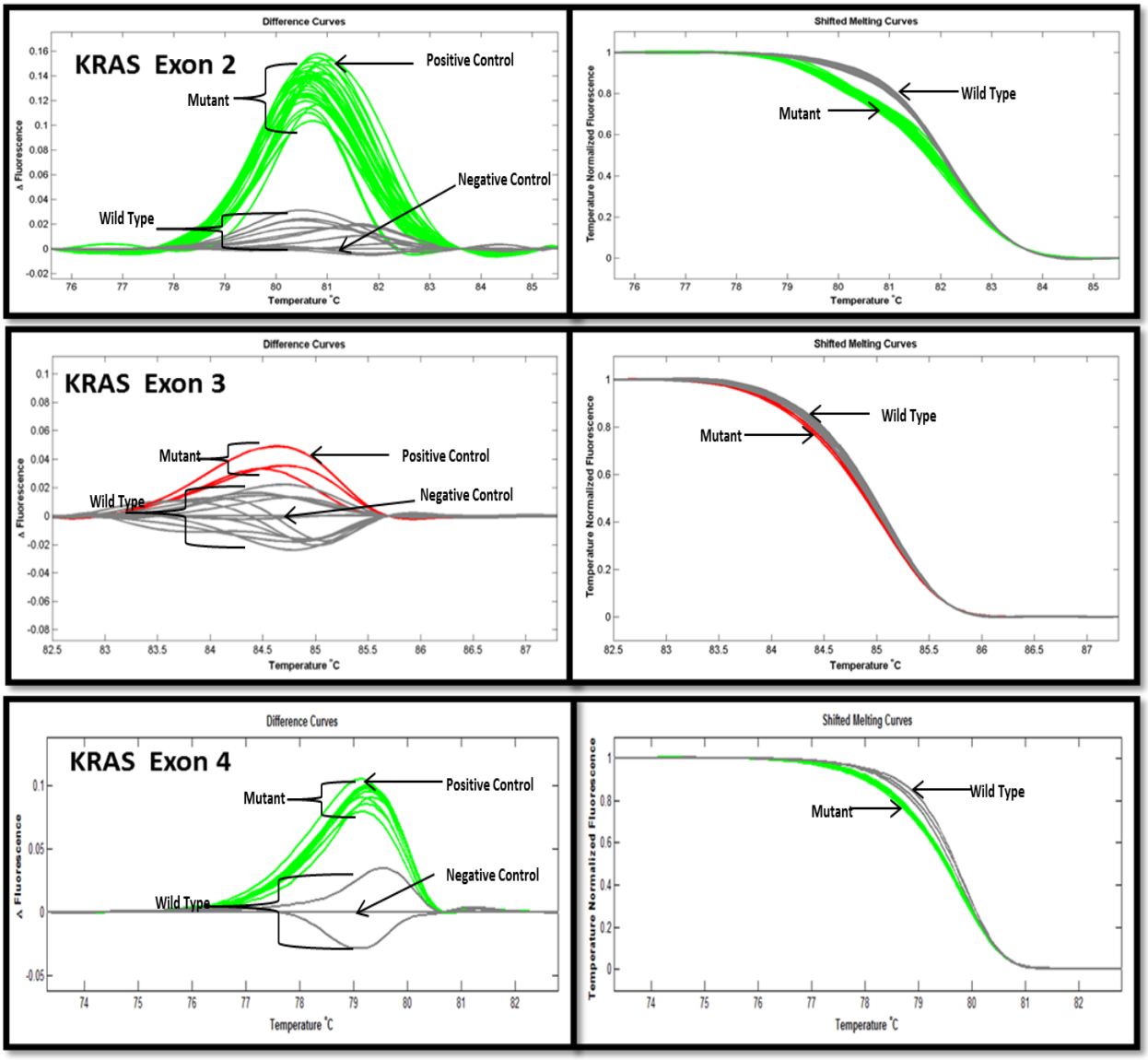

Figure 3-1: Normalized difference plot and temperature shift generated by HRM Lightscanner for KRAS (Exon 2, 3, and 4) mutations.
The generated plot shows variations between the products, containing wild-type (grey) and mutated samples (green/red). Aberrant mutations excessing the threshold 4%. Positive and negative controls were used for data normalisation and presence or absence of mutations was confirmed by screening the samples multiple times (at least three times).

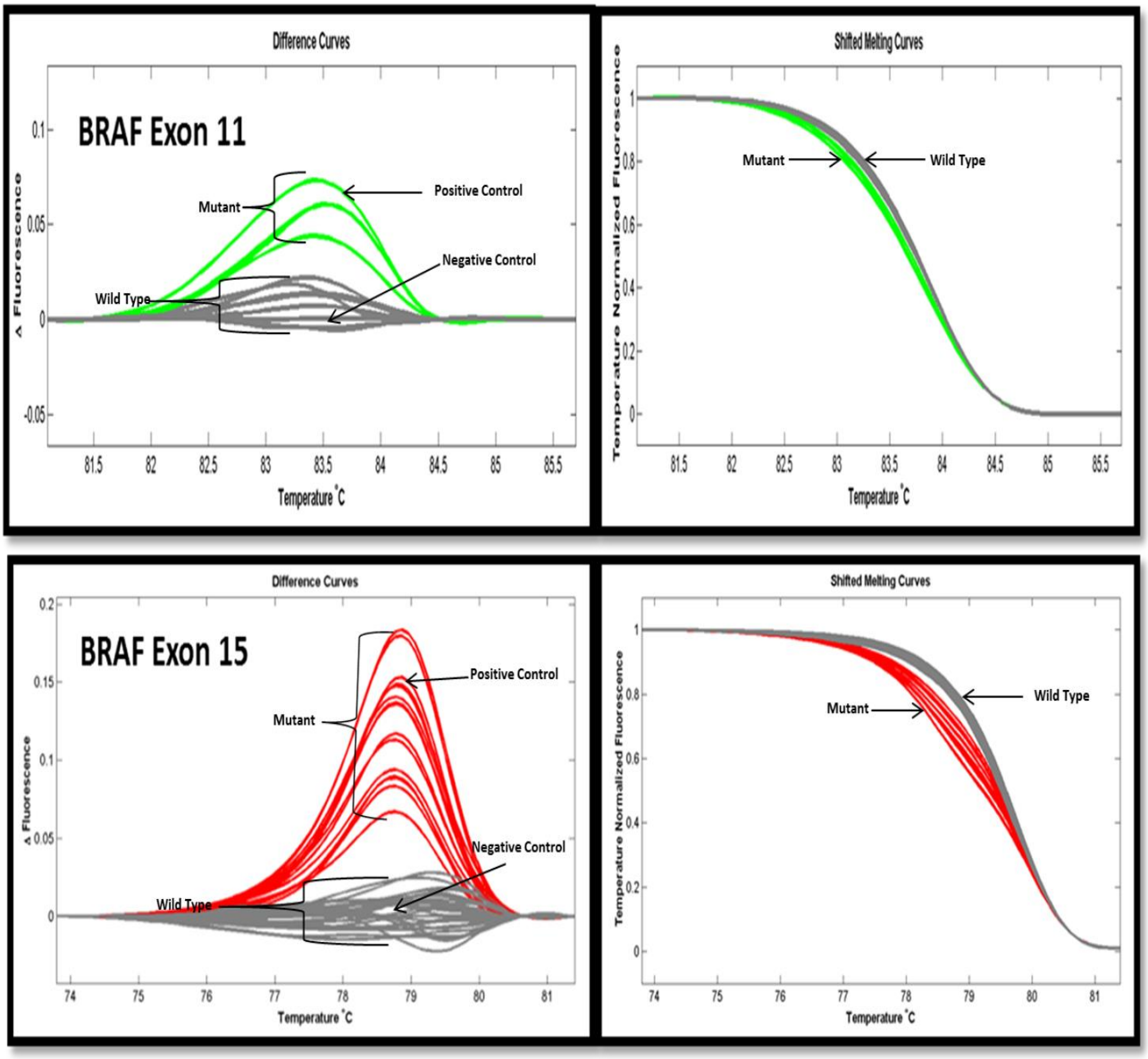

Figure 3-2: Normalized difference plot and temperature shift generated by HRM Lightscanner for BRAF (Exon 11 and 15) mutations.
The generated plot shows variations between the products, containing wild-type (grey) and mutated samples (green/red). Aberrant mutations excessing the threshold 4%. Positive and negative controls were used for data normalisation and presence or absence of mutations was confirmed by screening the samples multiple times (at least three times).

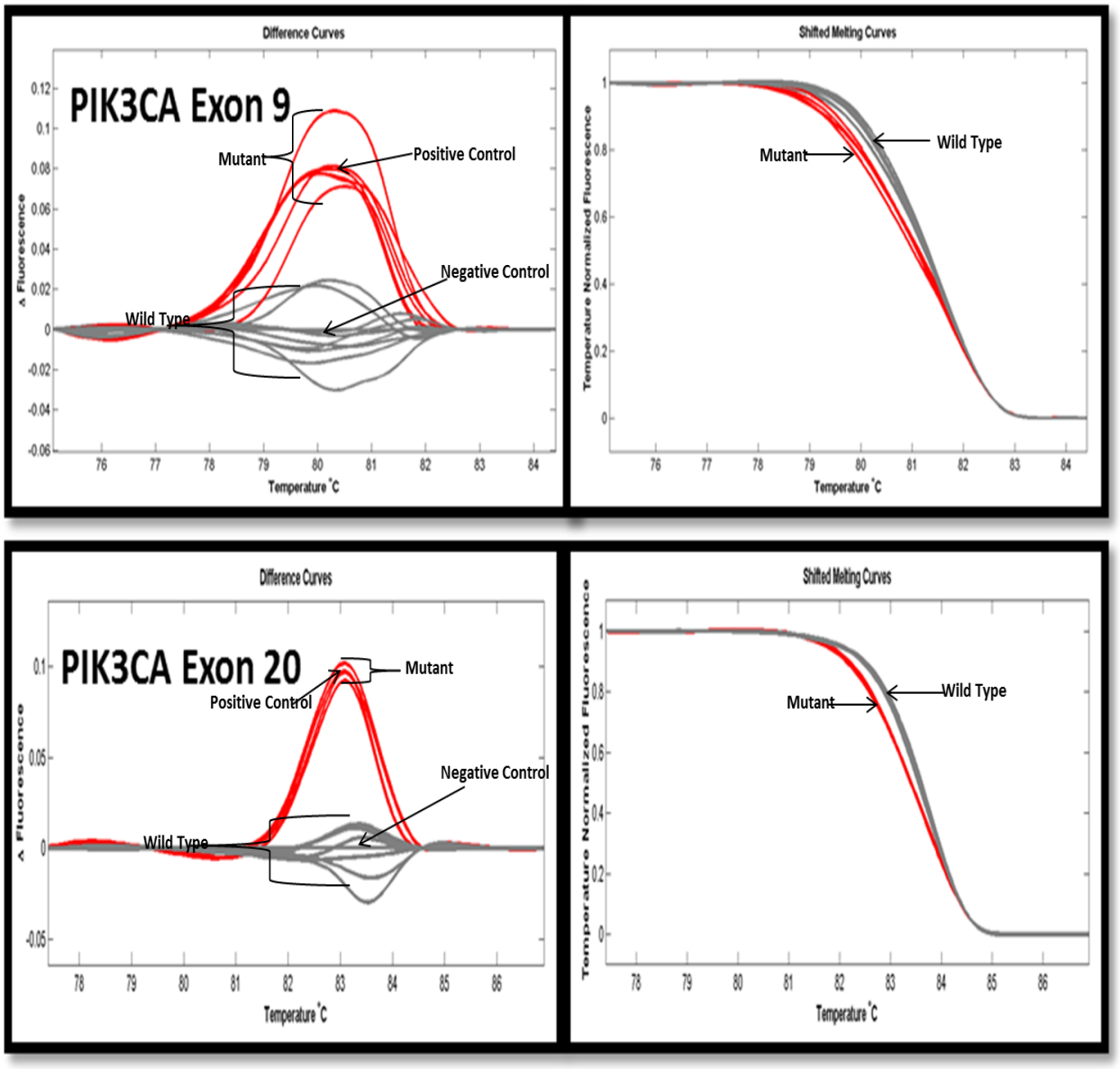

Figure 3-3: Normalized difference plot and temperature shift generated by HRM Lightscanner for PIK3CA (Exon 9 and 20) mutations.
The generated plot shows variations between the products, containing wild-type (grey) and mutated samples (red). Aberrant mutations excessing the threshold 4%. Positive and negative controls were used for data normalisation and presence or absence of mutations was confirmed by screening the samples multiple times (at least three times).

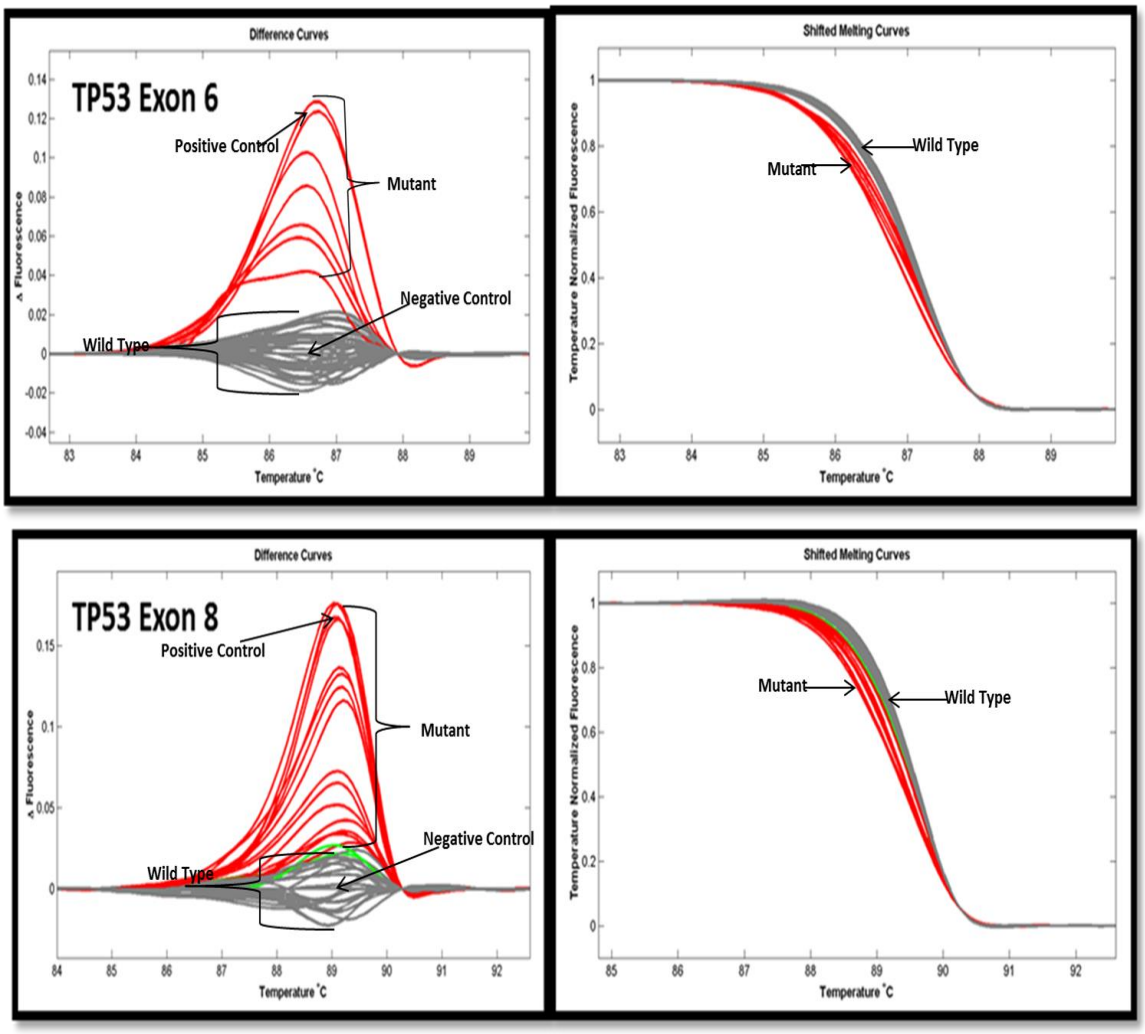

Figure 3-4: Normalized difference plot and temperature shift generated by HRM Lightscanner for TP53 (Exon 6 and 8) mutations.
The generated plot shows variations between the products, containing wild-type (grey) and mutated samples (red). Aberrant mutations excessing the threshold 4%.Samples on green colour represent borderline mutations. Positive and negative controls were used for data normalisation and presence or absence of mutations was confirmed by screening the samples multiple times (at least three times).

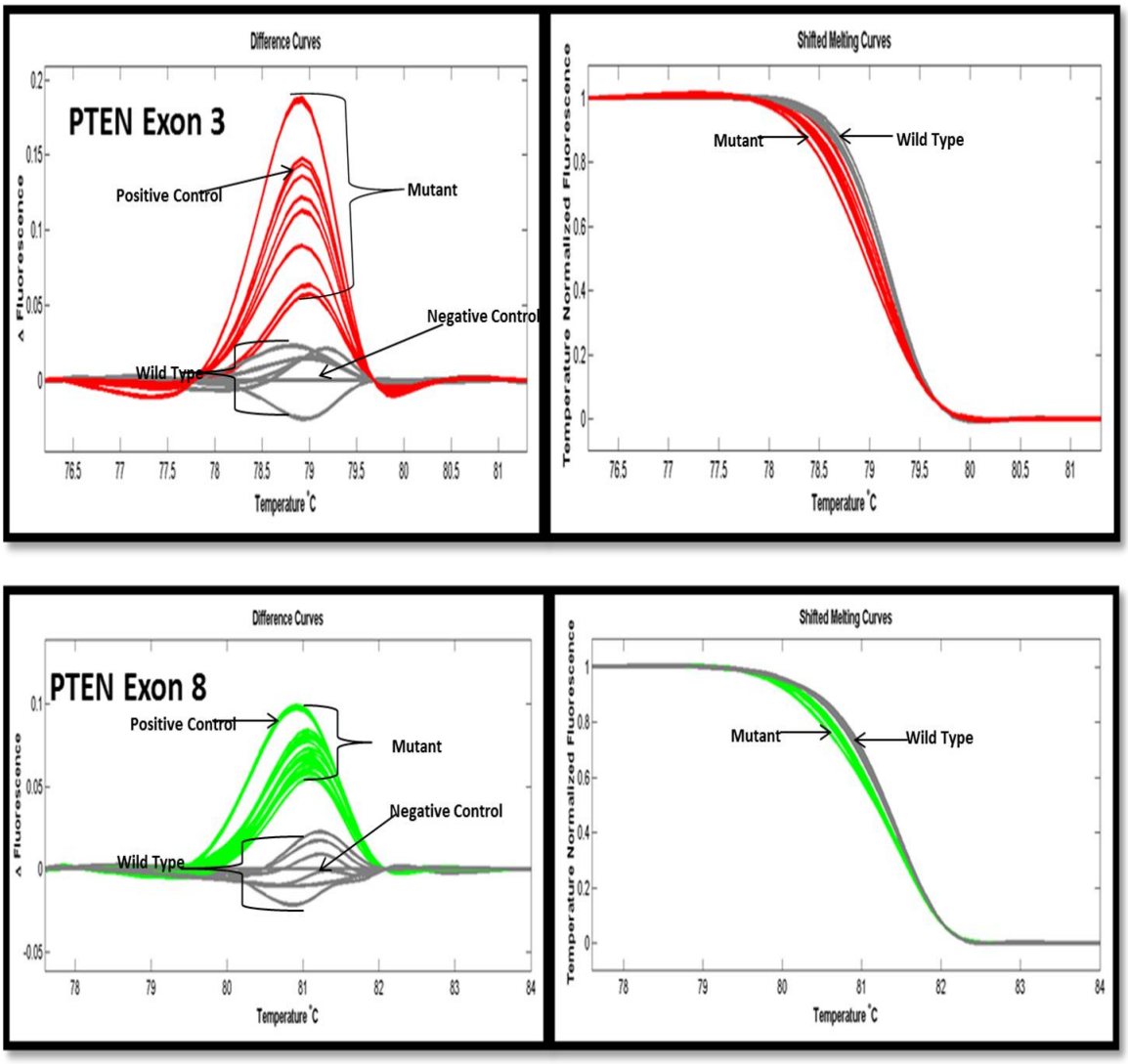

Figure 3-5: Normalized difference plot and temperature shift generated by HRM Lightscanner for PTEN (Exon 3 and 8) mutations.
The generated plot shows variations between the products, containing wild-type (grey) and mutated samples (green/red). Aberrant mutations excessing the threshold 4%. Positive and negative controls were used for data normalisation and presence or absence of mutations was confirmed by screening the samples multiple times (at least three times).

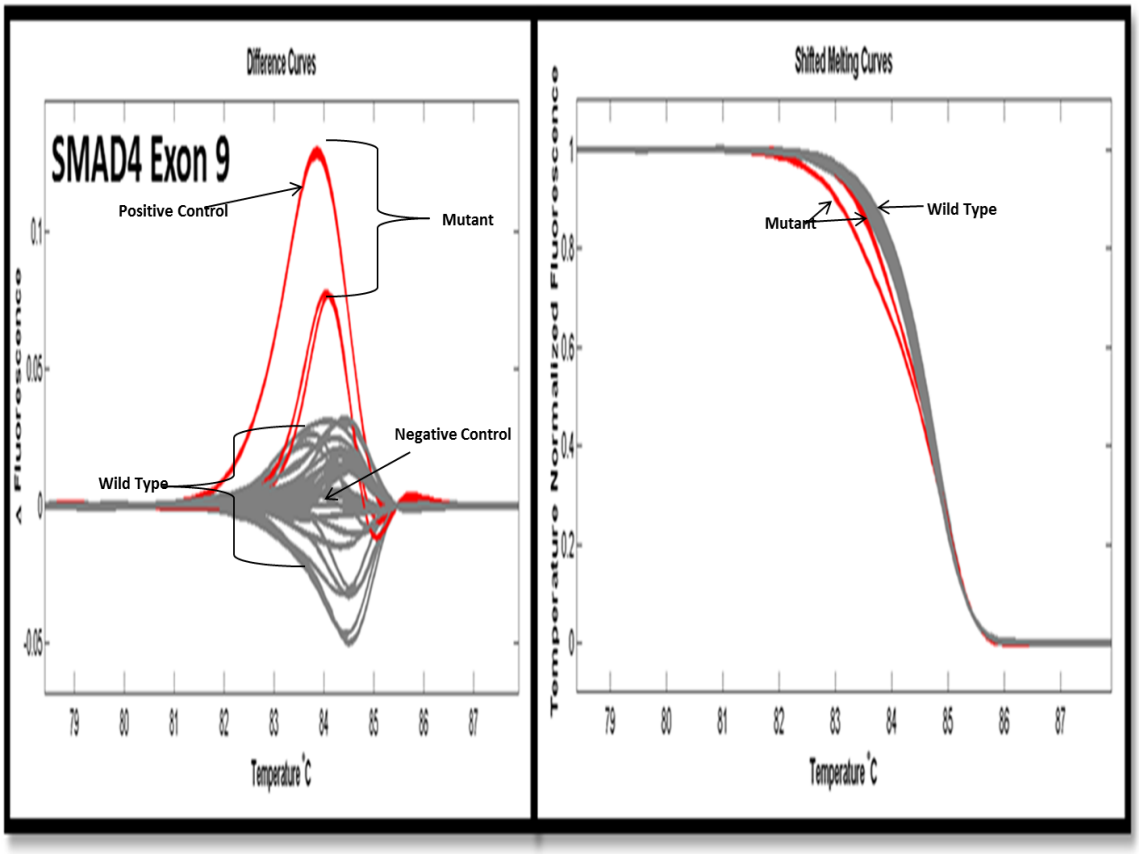

Figure 3-6: Normalized difference plot and temperature shift generated by HRM Lightscanner for SMAD4 (Exon 9) mutations.
The generated plot shows variations between the products, containing wild-type (grey) and mutated samples (green/red). Aberrant mutations excessing the threshold 4%. Positive and negative controls were used for data normalisation and presence or absence of mutations was confirmed by screening the samples multiple times (at least three times).

3.2.1.2 HRM Analysis for MSI Screening

A total of 82 CRC cases underwent screening for MSI using HRM following standard PCR was used as a quick and reliable method for MSI analysis. Positive and negative controls for MSI screening included in this study have been previously confirmed by Immunohistochemistry (IHC) for either MSS (negative control) or MSI (positive control) and used as reference controls for data normalisation MSI status was assessed by analysing the instability of a panel of six markers (BCAT-25, BAT-25, BAT-26, NR21, NR22, and NR24) depending on two categories of MSI status: two or more (≥30%) loci out of six loci with instability were defined as MSI-positive and one locus or none with instability was considered as MSS. HRM analysis revealed 13% (11/82) of all patients were MSI-H, 90% (10/11) of these were detected by the BCAT marker. Most cases showed instability at three markers (81%) of total MSI-H CRC (Figure 3-7). MSI-H CRCs were found to be significantly associated with Dukes' staging (Chi squared test, $p < 0.025$) (for summary, see Tables 3-2).

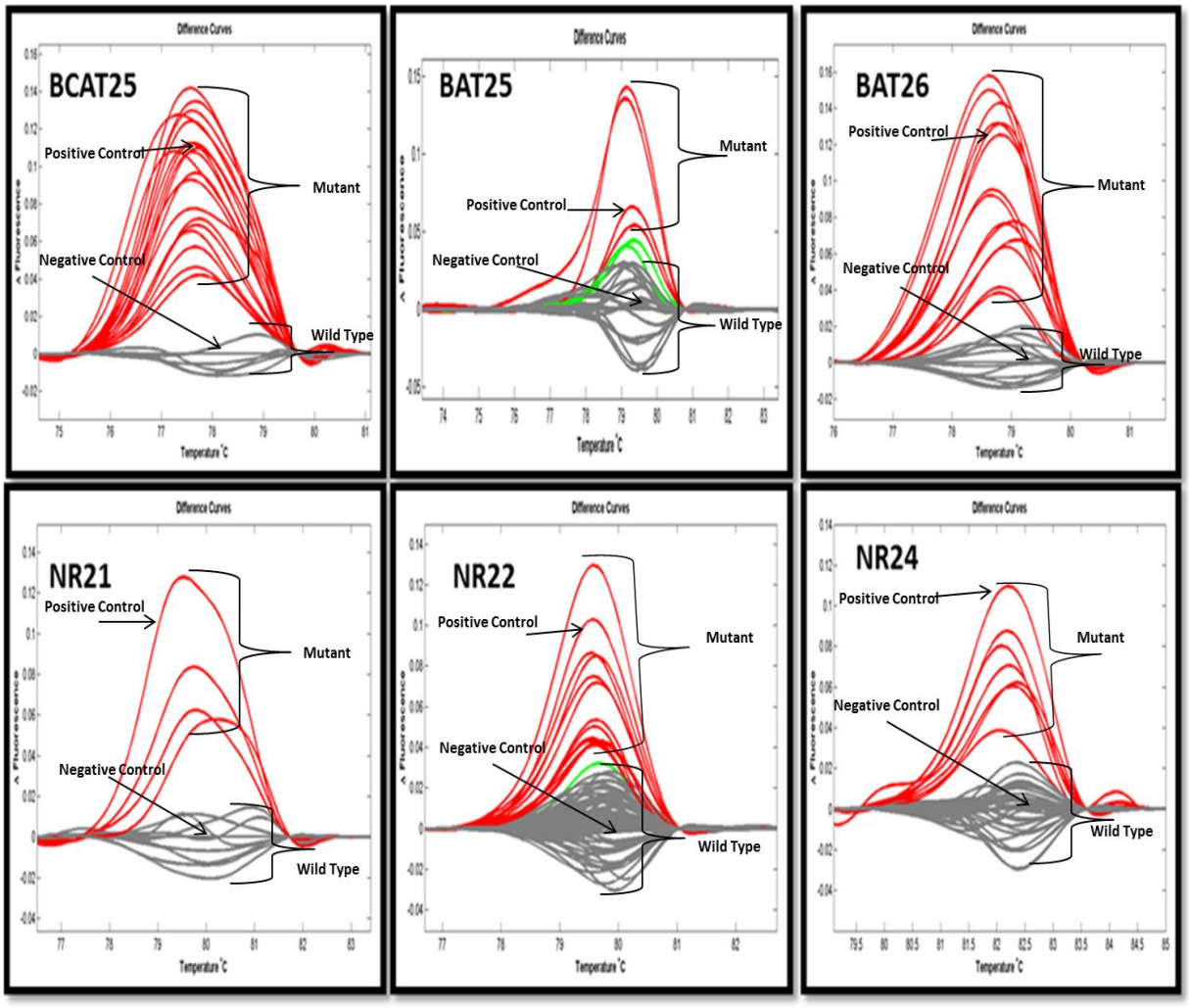

Figure 3-7: Normalized difference plot generated by HRM Lightscanner for MSI markers (BCAT25, BAT25, BAT26, NR21, NR22, and NR24).
Difference shows variations between the products, containing wild-type (grey) and mutated samples (Red). Aberrant mutations excessing the threshold 4%. Samples on green colour represent borderline mutations. Positive and negative controls were used for data normalisation and presence or absence of mutations was confirmed by screening the samples multiple times (at least three times).

3.2.1.3 Analysis of *MLH1* and *P16* Promoter Methylation by HRM

Methylation analysis by HRM following standard PCR was performed to detect the methylation status of *MLH1* and *P16* genes in 82 CRC cases. Methylated and unmethylated DNA set (Qiagen) were used as 0% unmethylated and 100% methylated controls. Standard curves generated from known methylated and unmethylated standards were used for the detection of methylated samples, with reference to 50% or above methylation levels regarded as methylated. Methylation in promoter regions was detected in 22% (18/82) of all analysed samples. 19% (16/82) of total CRC cases were exhibiting *MLH1* methylation (Figure 3-8; Table 3-1). Only 4% (4/82) with *P16* methylation, which was relatively low (3-12 fold difference) in comparison to those in the literature (Figure 3-9; Table 3-1). *MLH1* promoter methylation was significantly associated with gender female (Chi squared test, p=0.011) but no significant association with other clinicopathological characteristics was observed. In contrast, *P16* promoter methylation was significantly associated with the stage of primary tumour and Dukes' staging (Chi squared test, p= 0.025 and p=0.012, respectively). (for summary, see Tables 3-2 and 3-3).

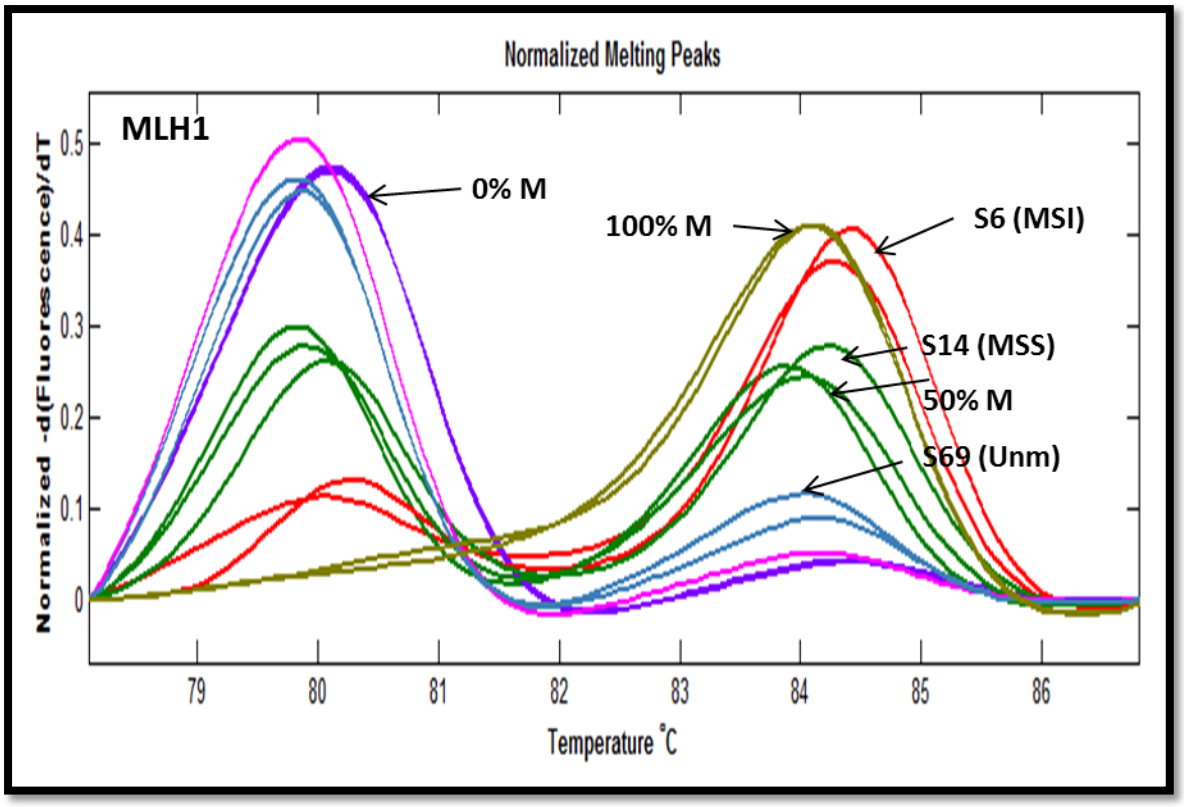

Figure 3-8: Normalized melt curve generated by HRM Lightscanner for MLH1 promoter methylation analysis .
Methylated and Unmethylated controls used in spike-in experiment for methylation analysis including different percentages of methylation of 50% and 100%. 50% was used as cut-off threshold for methylated samples.

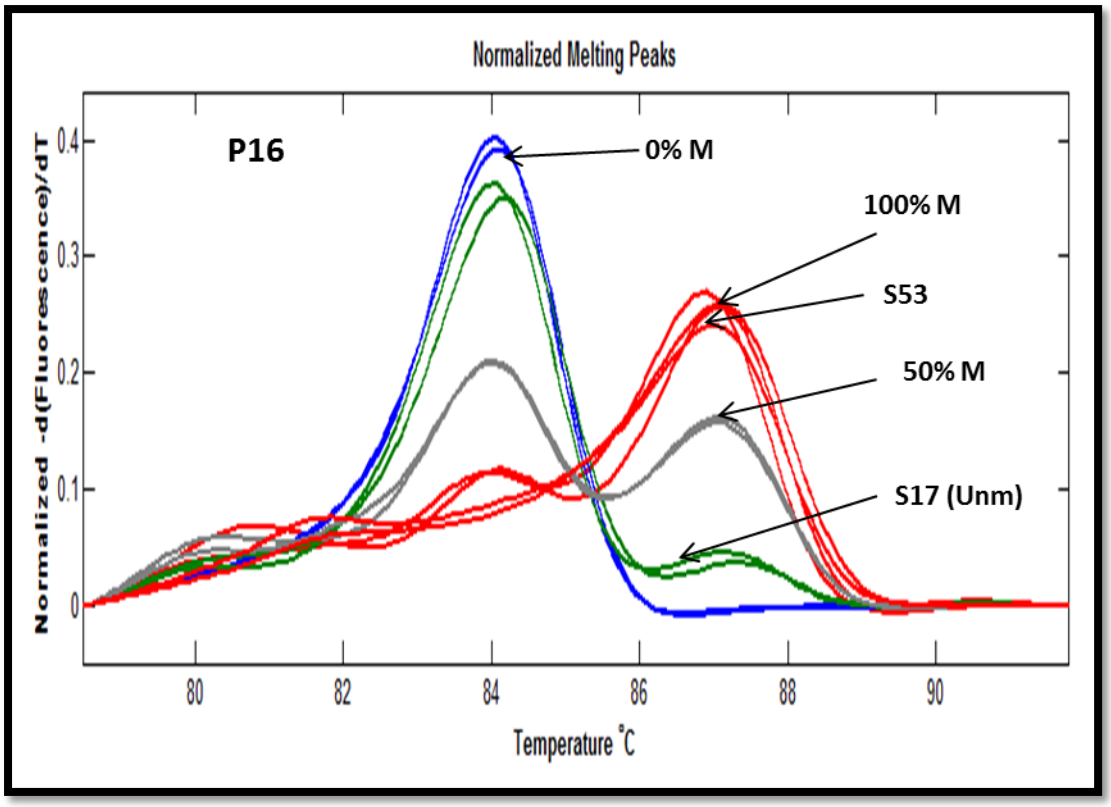

Figure 3-9: Normalized melt curve generated by HRM Lightscanner for P16 promoter methylation analysis.
Methylated and Unmethylated controls used in spike-in experiment for methylation analysis including different percentages of methylation of 50% and 100%. 50% was used as cut-off threshold for methylated samples.

Table 3–1: Summary for all genes mutations frequency and previous published data

Gene	Exon	Frequency (%)	Total Frequency (%)	Expected %	Reference
KRAS	*2*	*27 (32%)*	*32 (39%)*	*30%-50%*	(Brink *et al.*, 2003; Pajkos *et al.*, 2000)
	3	*2 (2%)*			
	4	*4 (4%)*			
PIK3CA	*9*	*4 (4%)*	*6 (7.4%)*	*7%-32%*	(Rosty *et al.*, 2013)
	20	*2 (2%)*			
BRAF	*11*	*2 (2%)*	*9 (10%)*	*10%*	(Lea, Allingham-Hawkins and Levine, 2010; Corcoran *et al.*, 2015)
	15	*7 (8%)*			
PTEN	*3*	*4 (4%)*	*12 (14.6%)*	*5%-20%*	(Barton, Starling and Swanton, 2010; Molinari and Frattini, 2014)
	8	*8 (9%)*			
SMAD4	*9*	*2 (2%)*	*2 (2.4%)*	*2.1%- 20%*	(Fleming *et al.*, 2013; Yu *et al.*, 2015; Malapelle *et al.*, 2016; Sarshekeh *et al.*, 2017)
TP53	*6*	*10 (12%)*	*23 (28%)*	*40%-50%*	(Olivier *et al.*, 2002; A Russo *et al.*, 2005; Takayama *et al.*, 2006; X. L. Li *et al.*, 2015; Yu *et al.*, 2015)
	8	*14 (17%)*			
MSI-H	----	----	*11 (13%)*	*10%-15%*	(Morrison *et al.*, 2011; Al-Sohaily *et al.*, 2012)
MLH1 Promoter methylation	----	----	*16 (19%)*	*20%-80%*	(Poynter *et al.*, 2008; Li *et al.*, 2013)
P16 Promoter methylation	----	----	*4 (4%)*	*12%-50%*	(Shima *et al.*, 2011; Bihl *et al.*, 2012; X. Li *et al.*, 2014)

3.2.1.4 Correlation with local and metastatic recurrences

In the 82 patients with CRC, the median follow up time was 8.6 months (0.5-49 months). Five out of eighty-two patients experienced a local recurrence (6%) and 21/82 developed distant metastasis (25%). Among the five patients with local recurrence, all patients were MSS (100%), 3/5 were harboring *KRAS* mutation (60%), 3/5 were harboring *TP53* mutation (60%), 2/5 with *PIK3CA* mutation (40%), and no patient exhibited mutations in *BRAF*, *PTEN* or *SMAD4* genes, had MSI, *MLH1* or *P16* promoter methylation. For the twenty-one patients with distant metastasis, 13/21 had *KRAS* mutation (61%), 6/21 with *PTEN* mutation (28%), 2/21 with *SMAD4* mutation (9%), 2/21 with *PIK3CA* mutation (9%), 3/21 with *BRAF* mutation (14%), 6/21 with *TP53* mutation (28%), 20/21 were MSS (95%), one had MSI (4%), 4/21 had *MLH1* promoter methylation (19%), one had *P16* promoter methylation (4%). No significant correlation observed between local or metastatic recurrences and mutations in *BRAF* or *TP53* genes, MSS or MSI status, promoter methylation in *MLH1* or *P16* genes. However, *PIK3CA* mutations were significantly associated with local recurrence CRC (P=0.005). In addition, mutations in *KRAS*, *PTEN*, and *SMAD4* were significantly associated with distant metastasis (P=0.02, P=0.046, and P=0.017, respectively) (Table 3-3).

3.2.1.5 Overall CRC Molecular Subgroups

The findings showed that *KRAS* and *BRAF* mutations are mutually exclusive, but *KRAS* and *PIK3CA* mutations associated with each other. *KRAS* exon 2 and *PIK3CA* exon 9 mutations were significantly associated (Chi squared test, $p < 0.004$).

The pooled prevalence of MSI and *BRAF* mutation status showed that 45% (5/11, Chi squared test, p= 0.00) of MSI-H CRCs were found to have mutant *BRAF*. MSI-H CRCs with Mutant *BRAF* were significantly associated with the stage of primary tumours (Chi squared test, p= 0.013) and Dukes' staging (Chi squared test, p =0.029).

For total CRC, *MLH1* promoter methylation was detected in 45% (5/11) of overall MSI-H CRC (Chi squared test, p =0.02), one of which had a *P16* methylation. MSI-H tumours with *MLH1* promoter methylation were significantly associated with the stage of primary tumour and Dukes' staging (A and B) (Chi squared test, p =0.041 and p =0.012, respectively). *MLH1* promoter methylation was frequently associated with MSI CRCs exhibiting *BRAF* mutations than MSI CRCs with *BRAF* wild-type and were also significantly associated with the stage of primary tumour and Dukes' staging (A and B) (Chi squared test, p= 0.001 and p=0.003, respectively). The frequency of *MLH1* promoter methylation in *BRAF* mutant CRCs was 45% (4/9, Chi squared test, p =0.045). *P16* promoter methylations were detected in two tumours with *BRAF* mutation and significantly associated with the stage of primary tumour and Dukes' staging (C and D) (Chi squared test, $p < 0.031$, and $p = 0.046$, respectively). *P16* promoter methylation frequency in *PTEN* mutations detected in 16% (2/12), and were associated with Dukes' staging (C and D) (Chi squared test, $p = 0.04$). (Table 3-2)

Table 3–2: Summary of Molecular Associations

		BRAF			***PIK3CA***			***TP53***			***PTEN***			***SMAD4***			***MSI***			***MLH1***			***P16***		
		M	*WT*		*M*	*WT*		*M*	*WT*		*M*	*WT*		*M*	*WT*		*MSI*	*MSS*		*M*	*Um*		*M*	*Um*	
KRAS	*M*	*1*	*31*	*p=0.69*	*4*	*28*	*p=0.14*	*10*	*22*	*p=0.64*	*7*	*25*	*P=0.14*	*2*	*30*	*P=0.73*	*0*	*32*	*p=0.004*	*4*	*28*	*P=0.2*	*1*	*31*	*P=0.55*
	WT	*8*	*42*		*2*	*48*		*13*	*36*		*5*	*45*		*0*	*50*		*11*	*39*		*12*	*38*		*3*	*47*	
BRAF	*M*				*0*	*9*	*p=0.37*	*1*	*7*	*p=0.29*	*2*	*7*	*p=0.49*	*0*	*9*	*P=0.61*	*5*	*4*	*p=0.00*	*4*	*5*	*P=0.045*	*2*	*7*	*P=0.01*
	WT				*6*	*67*		*22*	*51*		*10*	*73*		*2*	*71*		*6*	*67*		*12*	*61*		*2*	*71*	
PIK3CA	*M*							*3*	*3*	*p=0.22*	*0*	*6*	*P=0.292*	*0*	*6*	*P=0.68*	*0*	*6*	*p=0.31*	*1*	*5*	*p=0.85*	*0*	*6*	*P=0.56*
	WT							*20*	*55*		*12*	*64*		*2*	*74*		*11*	*65*		*15*	*61*		*4*	*62*	
TP53	*M*										*3*	*20*	*P=0.79*	*0*	*23*	*P=0.37*	*1*	*22*	*P=0.13*	*4*	*19*	*P=0.76*	*0*	*23*	*P=0.2*
	WT										*9*	*50*		*2*	*57*		*10*	*49*		*12*	*47*		*4*	*55*	
PTEN	*M*													*0*	*12*	*P=0.55*	*1*	*11*	*P=0.57*	*3*	*9*	*P=0.6*	*2*	*10*	*P=0.04*
	WT													*2*	*68*		*10*	*60*		*13*	*57*		*2*	*68*	
SMAD4	*M*																*0*	*2*	*P=0.57*	*0*	*2*	*P=0.48*	*0*	*2*	*P=0.74*
	WT																*11*	*69*		*16*	*64*		*4*	*76*	
MSI	*MSI-H*																			*5*	*6*	*P=0.02*	*1*	*10*	*P=0.486*
	MSS																			*11*	*60*		*3*	*68*	
MLH1	*M*																						*2*	*14*	*P=0.11*
	Um																						*2*	*64*	

Table 3–3: Summary for Molecular alterations and their association with clinicopathological Features.

		KRAS mutant	KRAS WT	BRAF mutant	BRAF WT	PIK3CA mutant	PIK3CA WT	PTEN mutant	PTEN WT	TP53 mutant	TP53 WT	SMAD4 mutant	SMAD4 WT	MSI	MSS	MLH1 M	MLH1 Unm	P16 M	P16 Unm
Gender	Male	24 (75%)	32 (68%)	2 (22%)	54 (77%)	3 (50%)	53 (72%)	4 (33%)	52 (77%)	16 (69%)	40 (71%)	2 (100%)	54 (70%)	4 (44%)	52 (74%)	6 (42%)	50 (76%)	2 (50%)	54 (72%)
	Female	8 (25%)	15 (31%)	7 (77%)	16 (22%)	3 (50%)	20 (27%)	8 (66%)	15 (77%)	7 (30%)	16 (288%)	0 (0%)	23 (29%)	5 (55%)	18 (25%)	8 (57%)	15 (23%)	2 (50%)	21 (28%)
			p=0.5		p=0.001		p=0.24		p=0.002		p=0.86		p=0.35		p=0.064		p=0.01		p=0.34
Dukes' stage	A/B	17 (54%)	31 (63%)	6 (66%)	42 (58%)	5 (83%)	43 (57%)	4 (33%)	44 (63%)	16 (69%)	32 (55%)	1 (50%)	47 (59%)	9 (90%)	39 (54%)	9 (56%)	39 (60%)	1 (25%)	47 (61%)
	C/D	14 (45%)	18 (36%)	3 (33%)	30 (41%)	1 (16%)	32 (42%)	8 (66%)	25 (36%)	7 (30%)	26 (44%)	1 (50%)	32 (40%)	1 (10%)	32 (45%)	7 (43%)	26 (40%)	3 (75%)	30 (38%)
			p=0.33		p=0.24		p=0.47		p=0.16		p=0.48		p=0.93		p=0.02		p=0.74		p=0.01
Primary Tumour	T1/T2	4 (12%)	10 (20%)	1 (11%)	13 (18%)	2 (40%)	12 (15%)	1 (8%)	13 (18%)	7 (30%)	7 (12%)	0	14 (17%)	3 (27%)	11 (15%)	3 (18%)	11 (16%)	1 (25%)	13 (16%)
	T3/T4	27 (87%)	40 (80%	8 (88%)	59 (81%)	3 (60%)	64 (84%)	11 (91%)	56 (81%)	16 (69%)	51 (87%)	2 (100%)	65 (82%)	8 (72%)	59 (84%)	13 (81%)	54 (83%)	3 (75%)	64 (83%)
			p=0.6		P=0.11		P=0.43		P=0.81		P=0.07		P=0.85		P=0.3		P=0.73		P=0.02
Lymph Node Involvement	N0	17 (54%)	34 (68%)	6 (66%)	45 (62%)	5 (100%)	46 (60%)	4 (33%)	47 (68%)	17 (73%)	34 (58%)	1 (50%)	50 (63%)	10 (90%)	41 (58%)	11 (68%)	40 (61%)	1 (25%)	50 (64%)
	N1	11 (35%)	13 (26%)	3 (33%)	21 (29%)	0	24 (31%)	8 (66%)	16 (23%)	6 (26%)	18 (31%)	1(50%)	23 (29%)	1 (9%)	23 (32%)	5 (31%)	19 (29%)	3 (75%)	21 (27%)
	N2	3 (9%)	3 (6%)	0	6 (8%)	0	6 (7%)	0	6 (8%)	0	6 (10%)	0	6 (7%)	0	6 (8%)	0	6	0	6 (7%)
			P=0.48		P=0.66		P=0.2		P=0.009		P=0.2		P=0.78		P=0.11		P=0.44		P=0.12
Vascular Invasion	V0	16 (51%)	27 (54%)	6 (66%)	37 (51%)	3 (60%)	40 (52%)	5 (41%)	38 (55%)	15 (65%)	28 (48%)	1 (50%)	42 (53%)	6 (54%)	37 (52%)	11 (68%)	32 (49%)	2 (50%)	41 (53%)
	V1	15 (48%)	23 (46%)	3 (33%)	35 (48%)	2 (40%)	36 (47%)	7 (58%)	31 (44%)	8 (34%)	30 (51%)	1 (50%)	37 (46%)	5 (45%)	33 (47%)	5 (31%)	33 (50%)	2 (50%)	36 (46%)
			P=0.83		P=0.3		P=0.55		P=0.29		P=0.12		P=0.72		P=0.58		P=0.13		
Local Recurrence	Yes	3 (9%)	2 (4%)	0 (0%)	5 (7%)	2 (33%)	3 (4%)	0 (0%)	5 (7%)	3 (13%)	2 (3%)	0 (0%)	5 (6%)	0 (0%)	5 (7%)	0 (0%)	5 (7%)	0 (0%)	5 (6%)
	No	29 (90%)	45 (95%)	9 (100%)	65 (92%)	4 (66%)	70 (95%)	12 (100%)	62 (92%)	20 (86%)	54 (96%)	2 (100%)	72 (93%)	9 (100 %)	65 (92%)	14 (100%)	60 (92%)	4 (100 %)	70 (93%)
			P=0.35		P=0.4		P=0.005		P=0.32		P=0.11		P=0.71		P=0.4		P=0.28		P=0.5
Distant Metastasis	Yes	13 (30%)	8 (17%)	3 (33%)	18 (25%)	2 (33%)	19 (26%)	6 (50%)	15 (22%)	6 (26%)	15 (26%)	2 (100%)	19 (24%)	1 (11%)	20 (28%)	4 (28%)	17 (26%)	1 (25%)	20 (26%)
	No	19 (59%)	39 (82%)	6 (66%)	52 (74%)	4 (66%)	54 (73%)	6 (50%)	52 (77%)	17 (73%)	30 (73%)	0 (0%)	58 (75%)	8 (88%)	50 (71%)	10 (71%)	48 (73%)	3 (75%)	55 (73%)
			P=0.02		P=0.62		P=0.69		P=0.046		P=0.94		P=0.01		P=0.26		P=0.85		P=0.9

3.2.2 Edinburgh cohort

88 CRC samples were screened for MSI and *BRAF* mutations using a simple assay to identify tumours with MSI and further validate the association between MSI and *BRAF* mutations. Screening CRCs for Lynch syndrome (LS) is recommended by Guidance from the National Institute of Clinical and Healthcare Excellence (NICE). For the first step, the process involves identifying cases with MSI by testing MMR deficiency by immunohistochemistry (IHC) and MSI by MSI involves PCR followed by capillary electrophoresis. The second step, excludes sporadic cases by testing for *BRAF* mutation by PCR followed by mutation screening or sequencing and *MLH1* promoter methylation by PCR on modified DNA followed by sequencing or gel electrophoresis. This strategy involves multiple tests and requires downstream analysis of the PCR products on different platforms. We believed that testing for MSI can be simplified by HRM analysis. This test can be performed in-closed tube, where HRM is performed at the end of a PCR without needing to transfer PCR products to another tube. This test was introduced to provide high sensitive test for MSI analysis and avoid any sample manipulation with PCR products which may cause contamination. 88 CRC samples (45= MSI & 43=MSS) were tested previously for MSI and MSS by IHC. MSI test was performed by screening 5 mononucleotides markers (BCAT25, BAT25, BAT26, MYB, and EWSR1) recently designed in our lab and published (Susanti et al, 2018). Primers sequences are shown in appendix. The analysis was initially performed in a close-tube test by HRM (Applied Biosyestem) and then melted in HRM (Lightscanner) system for further validation (Figure 3-10). HRM-PCR screening was able to differentiate between MSI and MSS with 100% and 95% sensitivity and specificity, respectively.

BRAF (exon15) mutation was screened by HRM-PCR to analyse the association between these mutations and MSI. *BRAF* mutations were detected in 34% (30/88) of CRCs patients. Mutant *BRAF* was significantly associated with MSI (P square test, 0.000), 62%

(28/45) of MSI tumours and 4% (2/43) of MSS tumours. The clinicopathological data for the patients were not available to study the association with these mutations.

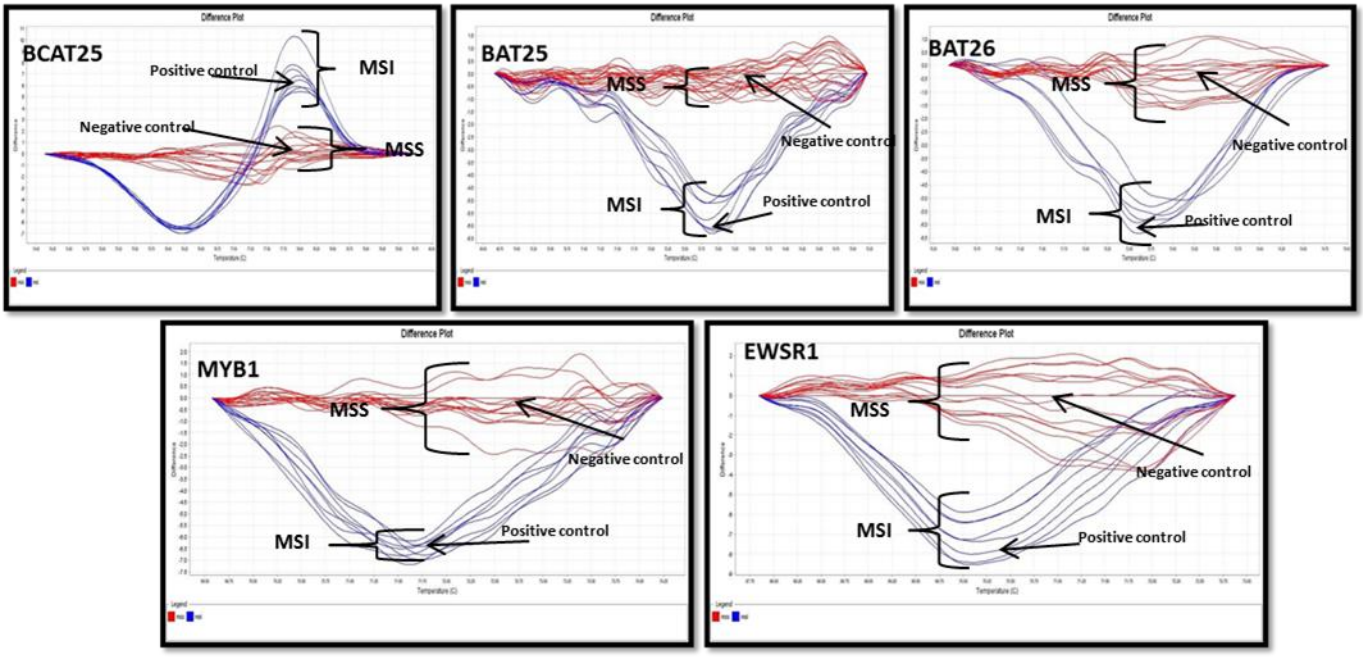

Figure 3-10: Normalized difference plot generated by HRM (Applied Biosystem) for MSI markers (BCAT25, BAT25, BAT26, MYB1, and EWSR1).
Difference shows variations between the products, containing MSS (red) and MSI samples (blue). Aberrant mutations excessing the threshold 4%.

3.3 Discussion

This study was carried out using QMC-PCR protocol followed by HRM analysis which has been established in our lab for mutation detection and found this to be robust assay producing accurate and reproducible data for FFPE derived DNA template (Fadhil *et al.*, 2010). QMC-PCR protocol is very rapid (cycling between 95^0C and 55^0C), inexpensive and simple method which involves two-step nested procedure in which initially a pre diagnostic multiplex (PDM) reaction containing up to 10 primer pairs followed by single specific multiplex (SSD) reaction for single hotspot analysis. The protocol showed great sensitivity in comparison to direct sequencing (gold standard method) and therefore it was applied in this study to screen for specific point of mutations (Fadhil *et al.*, 2010, 2012). In this study, 82 cases of CRC were collected and screened for *KRAS*, *BRAF*, *PIK3CA*, *TP53*, *PTEN*, and *SMAD4* mutations. All aberrations investigated in this study are part of PI3K-AKT-PTEN, RAS-RAF-MAPK, TP53, and TGFβ pathways which are involved in stimulating cell proliferation, survival and invasion in CRC tumours. The main objective of this analysis was to screen for specific mutations associated with metastatic CRC (mCRC).

The frequencies of mutations within the samples were concordant to other published studies (Table 3-1). However, *TP53* mutations were 2 fold difference and *P16* promoter methylation were 3-12 fold difference in comparison to published data (Table 3-1). Failure to detect mutation or methylation in tumour samples may thus be a technical artefact resulting from the limit of the detection of the methods. We have used HRM technology, which have been previously shown to be able to detect 5% of mutant alleles (Ibrahem *et al.*, 2010; Fadhil *et al.*, 2012). It is possible that these cases contained small numbers of mutant alleles or low level of methylation that cannot be detected by either QMC-PCR or standard PCR followed by HRM and these are theoretically may be regarded as false-negative. It was demonstrated previously that QMC-PCR is more sensitive than gold standard method (direct sequencing) which may indicate the results

of the screen cannot be validated by the current gold standard method for mutation detections (Fadhil *et al.*, 2010, 2012). Therefore, more sensitive methods for mutation detection and promoter methylation analysis such as pyrosequencing or NGS may resolve this problem.

Previous studies have shown the association between *PIK3CA* and *KRAS* mutations or that between *PIK3CA* mutation and the presence of either *KRAS* or *BRAF* mutations in CRC patients. The data herein confirmed that *KRAS* and *PIK3CA* coexist and *KRAS/PIK3CA* and *BRAF* mutations are mutually exclusive. The significant association between *KRAS* and *PIK3CA* mutations may suggest a synergistic effect of mutations in both genes in activating PI3K-AKT pathway during CRC development.

The objective had been to find mutations or combinations of mutations which may be associated with clinical metastasis. There was no significant association observed between *TP53* mutations and the clinicopathological parameters of the patients. This possibly due to the low frequency of *TP53* mutations detected in this study as discussed above. On the other hand, *BRAF* mutations were only associated with female gender, which agrees with previous studies (English *et al.*, 2008; Rozek *et al.*, 2010; Kalady *et al.*, 2012). Furthermore, mutations in *KRAS*, *PIK3CA*, *PTEN*, and *SMAD4* genes were significantly correlated with local recurrence, lymphatic or distant metastasis. 40% (2/5) of those developed local recurrence were detected with *PIK3CA* mutations showing a significant association between mutants *PIK3CA* and local recurrent CRC. Previous studies have shown contradictory findings regarding the predictive and prognostic value of *PIK3CA* mutation in CRC patients. The findings were limited by using independent validation group to support their final conclusions. Some reported that *PIK3CA* mutations were not associated with overall survival in CRC patients, while others found strong association between *PIK3CA* mutations and worse overall survival (Kato *et al.*, 2007; Abubaker *et al.*, 2008; Ogino, Nosho, Gregory J Kirkner, *et al.*, 2009; Fariña Sarasqueta

et al., 2011; Liao *et al.*, 2012; Day *et al.*, 2013; Manceau *et al.*, 2015). It has also been shown that *PIK3CA* mutations were strongly associated with increased local recurrence rate, revealing that tumours with *PIK3CA* mutations may develop local recurrence more rapidly after surgery (He *et al.*, 2009). However, this may support the finding of this study but our data are limited by small number of cases with local recurrence to conclude the association with *PIK3CA* mutations in CRC and further validation is required. *KRAS* mutation occurred in 61% (13/21) of those developed distant metastasis. This finding agrees with previous published data which showed that the presence of *KRAS* mutation correlates with advanced stage of tumour and metastatic CRC (Li *et al.*, 2011; Modest *et al.*, 2011; Mannan and Hahn-Strömberg, 2012; Boutin *et al.*, 2017). It has also been shown previously great concordance of mutant *KRAS* status in primary and metastatic CRC, which suggests that *KRAS* mutations are maintained throughout carcinogenesis (Zauber *et al.*, 2003; Artale *et al.*, 2008; Pritchard and Grady, 2011). This evidence may suggest the utility of *KRAS* mutational analysis on archived primary tumour tissues in patients with metastatic CRC and eliminate additional biopsy tissues from the patients (Pritchard and Grady, 2011). S*MAD4* mutations were detected in only two cases (2.4%) and both found to have distant metastasis. The presence of same *SMAD4* mutations has been previously identified in both primary and distant metastases in the same patients (Miyaki *et al.*, 1999). This may indicate that *SMAD4* mutations occurred in the primary carcinoma and some tumour cells acquiring mutant *SMAD4* metastasize to distant tissue. Therefore, the presence of *SMAD4* mutation in primary tumour tissues may be a prognostic biomarker. Although *SMAD4* mutations frequency were in range with those in the literature but it was relatively low, however, further validation for the presence of *SMAD4* mutations in distant metastasis is required. Furthermore, *PTEN* mutation was significantly correlated with female gender, involvement of the lymph node and distant metastasis. 50% (6/12) of those exhibiting *PTEN* mutation have developed distant metastasis and 66% (8/12) of total *PTEN* mutant CRCs showed involvement of at least one to three regional nodes according to TNM

staging. The association of *PTEN* mutations with lymph node metastasis suggests that *PTEN* may be involved in invasion and metastasis. Other studies have reported similar findings showing significant correlation between *PTEN* gene mutations and locally advanced or metastatic CRCs (Sawai *et al.*, 2008; X. Li *et al.*, 2009; Molinari and Frattini, 2014). It has also been reported that all tumours with a *PTEN* mutation showed low or absence of *PTEN* expression, which was correlated with the advanced stage of tumours (Nassif *et al.*, 2004; Molinari and Frattini, 2014) This association was confirmed in further studies indicating a link between *PTEN* dysregulation and an aggressive phenotype (X. Li *et al.*, 2009; Lin *et al.*, 2011; Molinari and Frattini, 2014).

P16 promoter methylation is one of the main elements of a classic panel for detection of CIMP which has been found to lead to silencing of the tumour suppressor *P16* gene, which possibly further result in the development, progression and invasion of CRC (Lee *et al.*, 2008; Mitomi *et al.*, 2010; Mojarad *et al.*, 2013). Aberrant activation of *P16* by methylation has been found correlated to more advanced tumours and overall poor survival rates (Bai *et al.*, 2004; Karamitopoulou *et al.*, 2010; Mitomi *et al.*, 2010; Nakayama *et al.*, 2011). It is noteworthy that *P16* promoter methylation is correlated with protein expression of *P16*, supporting the argument that HRM-PCR for methylation analysis could be used as a surrogate marker for *P16* protein expression (Payá *et al.*, 2009). In this study, we examined the methylation status of *P16* and association with metastatic CRC. HRM-PCR analysis showed aberrant methylation of the *P16* promoter region in only four (4%) tumours, MSI-H was only detected in one tumour (25%). No significant association was found between *P16* promoter methylation and the clinicopathological features including vascular invasion, lymph node involvement, tumour grade, and local/distant recurrences in this study. However, *P16* methylation was associated with the stage of primary tumours and Dukes' staging, with 3/4 (75%) of those detected in Dukes' stage C and D tumours. Similar findings have been previously reported (Yi *et al.*, 2001). Associated *P16* methylations and other mutant genes, such as

BRAF and *PTEN* genes, were detected in only two patient samples and were also associated with Dukes' staging C and D. Different studies have shown that CIMP-positive driven by *BRAF* mutations demonstrated the worse outcome in CRC patients which may indicate that these molecular interactions may be implicated in advanced disease (Samowitz *et al.*, 2005; Kim *et al.*, 2009; Ogino, Nosho, Gregory J. Kirkner, *et al.*, 2009). Furthermore, since both *P16* promoter methylation and *PTEN* mutations have been found to be associated with a late stage of tumour, it may suggest that *P16* promoter methylation and *PTEN* mutation may have related effect in metastatic disease. These findings are required to be further validated to confirm the association between *P16* promoter methylation and mutant *BRAF/PTEN* in CRC and identify their role in metastatic tumours. Moreover, it would be suggested to utilise further analysis approaches, such as Network-Based Stratification (Hofree-NBS), ccpwModel, and xGeneModel, which have been established to identify genetic sub-types within cancer type and relate the sub-types to clinical outcomes and pathologic features of patients (Hofree *et al.*, 2013; Kim, Sael and Yu, 2015; Zhang, Flemington and Zhang, 2018). Furthermore, performing basic statistical principle such as power calculations for sample size is crucial to avoid bias in interpreting the results (Kadam and Bhalerao, 2010). The calculation of sample size is performed to identify the optimum number of participants in order to obtain ethically and scientifically valid results (Kadam and Bhalerao, 2010). However, including such analysis is suggested to detect the true effect of identified molecular alterations in association with patients' features.

In this study, we have also analysed MSI status using HRM-PCR methods. The aim of this analysis was to screen for MSI-H within CRC patients and observe the association with *BRAF* mutation. It has been shown previously that *BRAF* mutations are less frequent in MSS CRCs than in MSI-H in sporadic CRCs, particularly in tumours with *MLH1* promoter methylation (Koinuma *et al.*, 2004; Nagasaka *et al.*, 2004; Goel *et al.*, 2007). The link between *BRAF* mutations and *MLH1* promoter methylation suggests the

possibility of using this marker in the discrimination between MSI-H in sporadic CRC and MSI-H due to Lynch syndrome. HRM-PCR analysis revealed 13% (11/82) of CRCs showed instability at two MSI markers, most of these, 81% (9/11) had instability at three MSI markers. The frequency of *BRAF* mutations in MSI-H CRC was 45% and 5% in MSS tumours and the significant association of MSI and *BRAF* mutation (Chi squared test, $p < 0.000$), in line with the published data (Jensen *et al.*, 2008; Capper *et al.*, 2013; Thiel *et al.*, 2013). These findings are closely concordant with those of our collaborators, Edinburgh Molecular Pathology Node, showing a *BRAF* mutation frequency of 61% in MSI tumours and 11% in MSS tumours, as well as the significant association of MSI with the *BRAF* mutation (Chi squared test, $p < 0.0001$) (Susanti *et al*., 2018). The *BRAF* mutant and MSI CRCs were significantly associated with moderate differentiated tumours and early Dukes' stage (A and B). These findings confirm that *BRAF* mutations are frequently associated with MSI-H CRCs and may be useful biomarkers for primary sporadic CRC tumours.

MLH1 promoter hypermethylation is a crucial event which leads to silencing of the *MLH1* gene and inhibits the formation of *MLH1* protein and normal activation of the DNA repair gene. This process induces genomic instability and cell proliferation in CRC formation. *MLH1* promoter methylation is commonly found in sporadic CRC with MSI , rather than HPNNC MSI, in association with *BRAF* mutation (Raedle *et al.*, 2001; Parsons *et al.*, 2012; Chen *et al.*, 2014). In this analysis, we aimed to investigate the association between *MLH1* promoter methylation, MSI-H, and *BRAF* mutation in CRC tumours. The frequency of *MLH1* promoter methylation in 82 CRC cases was 16 (19%) and was significantly associated with female gender in concordance with published data (Li *et al.*, 2013; Coppedè, Migheli, *et al.*, 2014). *MLH1* promoter methylation was observed in 45% of MSI-H CRCs and 15% of MSS tumours. The possible explanation for MSI in the group with no detected *MLH1* methylation is that might be a result of germline mutation in one of the MMR genes. As it has been stated previously that *MLH1* methylation is frequently

associated with loss of expression which leads to MSI and rarely observed in MMR germline mutations (Deng *et al.*, 1999; Miyakura *et al.*, 2001; Vilkin *et al.*, 2009). Partial methylation (Methylation around 50%) of *MLH1* promoter was observed of in 55% (6/11) within the group with MSS, whereas all MSI with *MLH1* methylation (5/5) showed full methylation (Methylation around 100%). This needs to be further validated by different methods such as pyrosequencing to identify the level of methylation within these samples. Different studies have demonstrated that partial methylation of the *MLH1* promoter is not sufficient for *MLH1* silencing. Extensive methylation of the *MLH1* promoter is necessary of the inactivation of *MLH1* which leads to the development of MSI in CRC (Deng *et al.*, 1999; Miyakura *et al.*, 2001; Vilkin *et al.*, 2009). These findings are similar to those from other studies, which observed that only full methylation of the *MLHI* promoter is essential for *MLH1* inactivation. Conversely, patients who had tumours with full *MLH1* methylation were correlated with MSI-H CRCs with *BRAF* mutations than MSI-H CRCs with *BRAF* wild-type. This study suggests that the frequency of *MLH1* promoter methylation was significantly associated with MSI-H CRC with *BRAF* mutations which may indicate that tumours with this subtype are sporadic CRC. The correlation between MSI-H CRC and *BRAF* mutations were further validated in different cohort to gain deeper insight into their association in CRC tumours.

In this study we have also developed a simple test as a single panel closed-tube test for MSI screening. Our panel of 5 microsatellite markers which includes two well-known markers (BAT25, BAT26) and 3 novel markers (BCAT25, MYB and EWSR1) (Susanti *et al.*, 2018). When tested in 88 CRCs (Edinburgh cohort), there was perfect concordance with IHC analysis. Our assay showed that all MSI cases with loss of expression in one or more of *MLH1*, MSH2, PMS2 or MSH6 by IHC were perfectly categorised as MSI or MSS CRC. Furthermore, the mutational status of *BRAF* was screened in the same cohort to evaluate the association between these mutations and MSI status. *BRAF* mutations were significantly associated with MSI, which are concordant with published data. This

association is consistent with observations in the Nottingham cohort, which confirms that MSI and *BRAF* mutation are strongly associated. Given the absence of mutant *BRAF* with MSI in Lynch Syndrome, this may indicate that all cases in both cohorts with MSI and *BRAF* mutations are sporadic CRC. MSI analysis involves a panel of 5 PCRs which are performed in a closed-tube run using single cycling program. HRM analysis is performed at the end of the run, thus eliminate the risk of laboratory contamination with PCR products which may be caused by sample transfer to another platform for further analysis (such as capillary electrophoresis). Therefore, implying this test for Lynch syndrome and sporadic CRC in diagnostic pathology lab would provide information can be used to stratify patients into groups eligible for treatment with 5-Fluorouracil based therapy or immunotherapy (Bertagnolli *et al.*, 2009; Des Guetz, Schischmanoff, *et al.*, 2009; Roth *et al.*, 2010; Llosa *et al.*, 2015).

In summary, this study revealed good concordance of mutations frequency of *KRAS*, *BRAF*, *PIK3CA*, *SMAD4*, *PTEN*, *MSI*, and *MLH1* promoter methylation in comparison to those reported in the literature, except for *TP53* mutations and *P16* promoter methylation. Finding herein indicates that colorectal cancers with *KRAS*, *PIK3CA*, *PTEN*, or *SMAD4* mutations are more likely to develop into metastasis recurrence. It is known that patients with metastatic CRC have a poor prognosis, which may indicate those with mutation in *KRAS*, *PIK3CA*, *PTEN*, or *SMAD4* gene may have a poor prognosis and these points of mutations may become potential predictive and prognostic biomarkers. *P16* promoter methylation and *PTEN* mutations were associated and both were correlated with locally advanced CRC tumours depending on Dukes' staging and lymph node involvement. Whether these genes share a similar mechanism or act individually through other molecular pathways in CRC progression remains unclear. Therefore, further investigations and analysis of this genetic sub-type (*P16* promoter methylation and *PTEN* mutations) are required to fully understand their role in metastatic CRC cells. The findings herein confirm that *MLH1* promoter methylation is significantly associated with

MSI-H CRCs with *BRAF* mutations and this molecular subgroup can be used as marker for primary sporadic CRC tumours.

4 Screening Liquid Biopsies for Tumour-Derived Biomarkers

4.1 Introduction

Surgery is the main treatment modality for colorectal cancer although, increasingly, other therapeutic modalities are being used such as neoadjuvant therapy (Labianca *et al.*, 2013). Having a sensitive and accurate method of monitoring tumour clearance after surgery or tumour response to treatment would allow patients to be managed in a more sophisticated way. Monitoring of tumours is possible via the analysis of circulating nucleic acids (CNA) (Chaudhuri *et al.*, 2015). Nucleic acids, including cell-free DNA (*cfDNA*), circulating microRNA (c-miRNA) and circulating RNA (cRNA), are released into the bloodstream when cells undergo any form of cell death (Wan *et al.*, 2017). These can therefore be analysed to provide information on the tumour and this is known as "liquid biopsy" (Schwarzenbach, 2013).

Liquid biopsies can be tested for the presence of tumour related features such as gene mutation, expression profile etc. and therefore are regarded as a surrogate for the main tumour. They can be tested for mutation in order to provide predictive information i.e. which therapy the tumour will respond to (for example, the presence of the KRAS mutation in the cfDNA of a patient with colorectal cancer will mean that that patient is not eligible for treatment with anti-EGFR therapy). They can also be tested simply for the presence of tumour-derived mutant DNA as a marker of presence of tumour. For example, cfDNA has a half-life of 15 minutes and, it would be expected that if surgical clearance is successful, tumour-derived mutations would not be detectable in the cfDNA. Similarly, if there has been successful surgery, liquid biopsy can be used for surveillance since tumour-derived mutations may be detected in the cfDNA in recurrent disease prior to a clinically detectable recurrence (Matikas *et al.*, 2016; Reinert *et al.*, 2016; Nadal *et al.*, 2017; Khakoo *et al.*, 2018). Finally, liquid biopsies can be used to monitor response of tumour therapy by quantification of tumour-derived mutant DNA. Thus, the ratio of the chosen tumour biomarker (for example a mutant sequence) to normal biomarker (for example, wild type sequence), can be plotted over time to create a decay curve which

may reflect the response to therapy. For example, if the mutation load decreases (mutant: wild type ratio), this would indicate response to therapy and vice versa if there was unchanging or increased mutation load. Complete loss of mutant DNA in the liquid biopsy over a long-time course may indicate a complete pathological response.

Currently, many studies are investigating the utility of liquid biopsy for cancer screening, treatment response monitoring, surveillance of metastases and cancer recurrence. Testing liquid biopsies has many advantages such as (non-invasive methods with easy access to blood which can be withdrawn at any time and fresh source of molecular testing carries potential genetic information for tumour burden). There are however also a number of limitations which limit the techniques which can be used. Firstly, there is the issue of exceptionally low template concentration with cfDNA concentrations reported to be 1–100 ng/ml (Volik et al., 2016). Secondly, there is the issue of fragmentation with *cfDNA* reported to be highly fragmented, with a mean fragment size of 166 bp (Underhill et al., 2016). Finally, there is an issue with low levels of tumour biomarker present in the blood especially cfDNA with tumour-derived mutations often being reported to be present in frequencies <1% (Chaudhuri et al., 2015; Mouliere et al., 2013b). At such levels, the detection of mutant alleles becomes impossible with standard techniques such as Sanger Sequencing and this has prompted many investigators to use techniques such as Next Generation Sequencing (NGS) and Digital Droplet (DD) PCR (Aravanis, Lee and Klausner, 2017; Barata *et al.*, 2017; Gutteridge *et al.*, 2017; van Ginkel *et al.*, 2017).

We believed that information from liquid biopsies could be used for the management of patients. However techniques such as NGS and DD-PCR are complex and require sophisticated template preparation; the turnaround time from these would be too long for many situations. This study aimed to develop a robust and rapid assay to screen liquid biopsies for tumour-derived biomarkers in CRC. We therefore:

(1) Optimized extraction and quantification of *cfDNA* from plasma.

(2) Devised assays to screen for mutations in *KRAS*, *BRAF*, *PIK3CA, SMAD4, PTEN,* and *TP53* in *cfDNA* using HRM. Given that HRM has a limit of detection of around 5%, we optimised protocols for mutation enrichment using COLD-PCR (see material and methods).

(3) Devised assays to screen liquid biopsies for the following cfmiRNA: mir-20a, mir-21, mir-29a, mir-31, and mir-92a.

(4) Tested our biomarkers in liquid biopsies obtained from patients prior to surgery and several days following surgery. This evaluated the utility of this method as means of testing for surgical clearance

4.2 Results

4.2.1 Quantification of *cfDNA*

Blood samples were collected in EDTA (anti-coagulants) tubes and plasma was separated within 2 hours of collection to obtain valid results. Initially, plasma samples were extracted using DNA Blood mini kit (Qiagen) and DNA concentrations were quantified using Nanodrop (Thermo Fisher scientific). Concentrations measurement by Nanodrop was not reproducible which affected the mutation analysis. Therefore, DNA concentration analysis was then performed by DeNovix QFX flourometer (DeNovix) for fluorescence quantification which enables quick, sensitive and reproducible measurement of DNA concentration from 0.5pg/µl to 4000 ng/µl. Conversely, Nanodrop spectrophotometer provides a limit of detection from of 2ng/µl for double stranded DNA. A comparison between the quantifications (n=16) using both methods is shown in table 4-1. It was observed that Nanodrop was generating false measurements for DNA concentrations and most were undetectable by DeNovix. However, it was confirmed that these discrepancies within the results are artefacts due to low quantities of DNA. This indicates that DNA extraction from plasma using DNA Blood mini kit may generate insufficient yields of circulating nucleic acid which cannot be quantified by DeNovix. Efficient purification with greater yields may provide reproducible analysis of circulating DNA. Therefore, plasma samples were extracted using other method, as described in materials and methods chapter 2, (Circulating Nucleic Acid kit, Qiagen) which enables for efficient purification for circulating nucleic acid up to 5ml of human plasma, whereas DNA Blood mini kit processes only sample sizes of up to 200µl, therefore increases the concentration of extracted DNA. Most plasma samples (n = 45) produced extracted *cfDNA* yields within a 1 ng/ml to 100 ng/ml range, except day 1 samples 2, 3, 4 and 6. All patients had sufficient extracted DNA for final analysis, with a minimum yield of 3.78 ng/ml and maximum of 294 ng/ml (Table 4-2).

Table 4–1: A comparsion of Nanodrop and DeNovix quantificantions for cfDNA extracted from DNA Blood mini kit (Qiagen).

Sample	***Nanodrop Measurement (ng/µl)***	***DeNovix Measurement (ng/µl)***
1	*19.9*	*Undetectable*
2	*4.6*	*Undetectable*
3	*4.1*	*Undetectable*
4	*3*	*Undetectable*
5	*2.5*	*Undetectable*
6	*2.9*	*Undetectable*
7	*2.6*	*Undetectable*
8	*3.2*	*Undetectable*
9	*3.8*	*Undetectable*
10	*4.4*	*0.094*
11	*2.3*	*Undetectable*
12	*2.2*	*Undetectable*
13	*3.8*	*Undetectable*
14	*8.3*	*0.013*
15	*3*	*Undetectable*
16	*2.2*	*0.012*

Table 4–2: Concentration of cfDNA from patient blood samples extracted with the Qiagen circulating nucleic acid kit. Total DNA (ng) per ml of plasma = (elution volume (µl) x DNA concentration (ng/ml)) ÷ volume of plasma (ml).

Sample (elution 1)	*Vol of plasma (ml)*	*Vol of elution (µl)*	*Concentration (ng/µl)*	*Total DNA per ml of plasma (ng/ml)*
Case 2 Day 1	*0.75*	*40*	*3.02*	*161.06*
Case 2 Day 2	*1*	*20*	*1.43*	*28.6*
Case 3 Day 1	*0.55*	*40*	*2.11*	*153.45*
Case 3 Day 4	*1*	*20*	*2.67*	*53.4*
Case 4 Day 1	*0.4*	*40*	*2.44*	*244*
Case 4 Day 2	*1*	*20*	*3.61*	*72.2*
Case 5 Day 1	*1*	*40*	*1.29*	*51.6*
Case 5 Day 2	*1*	*20*	*4.15*	*83*
Case 6 Day 1	*1*	*40*	*7.35*	*294*
Case 6 Day 2	*1*	*20*	*4.9*	*98*
Case 7 Day 1	*1*	*40*	*1.99*	*79.6*
Case 7 Day 2	*1*	*20*	*2.02*	*40.4*
Case 8 Day 1	*1*	*40*	*1.84*	*73.6*
Case 8 Day 2	*1*	*20*	*1.85*	*37*
Case 9 Day 1	*1*	*40*	*1.77*	*70.8*
Case 9 Day 2	*1*	*20*	*2.63*	*52.6*
Case 10 Day 1	*1*	*40*	*1.65*	*66*
Case 10 Day 2	*1*	*20*	*2.61*	*52.2*
Case 11 Day 1	*1*	*40*	*1.26*	*50.40*
Case 11 Day 2	*1*	*20*	*1.87*	*37.40*
Case 12 Day 1	*1*	*40*	*1.43*	*57.20*
Case 12 Day 2	*1*	*20*	*1.82*	*36.40*
Case 13 Day 1	*1*	*40*	*1.06*	*42.40*
Case 13 Day 2	*1*	*20*	*0.92*	*18.48*
Case 14 Day 1	*1*	*40*	*1.13*	*45.20*
Case 14 Day 2	*1*	*20*	*0.96*	*19.34*
Case 15 Day 1	*1*	*40*	*1.49*	*59.60*
Case 15 Day 2	*1*	*20*	*2.31*	*46.20*
Case 16 Day 1	*0.6*	*20*	*1.13*	*37.60*
Case 16 Day 2	*0.6*	*20*	*1.83*	*61.00*
Case 17 Day 1	*0.6*	*20*	*2.86*	*95.33*
Case 17 Day 2	*0.6*	*20*	*1.89*	*63.00*
Case 18 Day 1	*1*	*40*	*1.40*	*56.00*
Case 18 Day 2	*1*	*20*	*1.98*	*39.60*
Case 19 Day 1	*1*	*40*	*1.07*	*42.80*
Case 19 Day 2	*1*	*20*	*2.01*	*40.20*
Case 20 Day 1	*1*	*40*	*1.36*	*54.40*
Case 20 Day 2	*1*	*20*	*3.61*	*72.20*
Case 21 Day 1	*1*	*40*	*1.48*	*59.20*
Case 21 Day 2	*1*	*20*	*1.89*	*37.80*
Case 22 Day 1	*1*	*40*	*1.86*	*74.4*
Case 22 Day 2	*1*	*20*	*2.91*	*58.2*
Case 23 Day 1	*1*	*40*	*0.233*	*9.32*
Case 23 Day 2	*1*	*20*	*0.298*	*5.96*
Case 24 Day 1	*1*	*40*	*0.118*	*4.72*
Case 24 Day 2	*1*	*20*	*0.189*	*3.78*
Case 25 Day 1	*1*	*40*	*0.302*	*12.08*
Case 25 Day 2	*1*	*20*	*2.59*	*51.8*
Case 26 Day 1	*1*	*40*	*0.399*	*15.96*
Case 26 Day 2	*1*	*20*	*0.463*	*9.26*

4.2.2 Full-COLD-PCR-HRM for the Detection of Low Percentage Variant Alleles

Detection of tumour-associated mutations in plasma has been challenging due to low amount of circulating DNA and low proportion of mutant to wild-type. The fragmented DNA present in blood circulating is difficult to amplify, therefore we designed short amplicon primers (<125 bp fragments) to amplify short fragments. In this study, mutation analysis was firstly performed using standard PCR protocol followed by HRM but failed to detect any mutation which was possibly due to the low sensitivity of the standard protocol, therefore standard PCR protocol may not be suitable tool for such analysis (Figure 4-1). Optimised protocol Full-COLD PCR follow by HRM was then used for the enrichment of low variant alleles with high sensitivity that cannot be detected by standard PCR protocol. Full-COLD-PCR can be used to enrich all mutation types, whereas Fast-COLD-PCR amplifies only mutations with lower Tm (G/C to A/T). Full COLD-PCR uses an extra hybridisation step at intermediate temperature which allows cross-hybridisation of mutant and wild-type alleles throughout PCR cycling, figure 2-7 in chapter 2 materials and methods. Heteroduplexes, which have lower melting temperature than homoduplexes, are subsequently denatured at specific critical temperature (Tc) and further amplified during PCR cycling (Milbury, Li and Makrigiorgos, 2009). In nested full COLD-PCR, PCR products are initially amplified using standard PCR protocol and then diluted for full COLD-PCR analysis as a second stage. Full COLD-PCR in nested procedure allows more amplification for the low frequency variant alleles.

Figure 4-2 shows analyses of a series of limit of detection (LOD) experiments including one stage full-COLD-PCR (Figure 4-1 A), with a LOD of 1.5% and nested second stage full-COLD-PCR (Figure 4-1 B) producing a LOD of 0.75%. This series of experiments proved that full-COLD-PCR can be used to detect low percentage variant alleles and applied in numerous formats. The most sensitive protocol was nested second stage full-COLD-PCR. This was also advantageous as the first stage PCR product could be diluted to

prevent excess precious *cfDNA* stock being wasted. However, if saving time is an issue, a one stage format can be used with a slightly higher LOD. This may be particularly effective for patients with larger tumours or metastases and FFPE samples which produce abundant DNA yields. The same experiments were performed for *BRAF* (Figure 4-3).

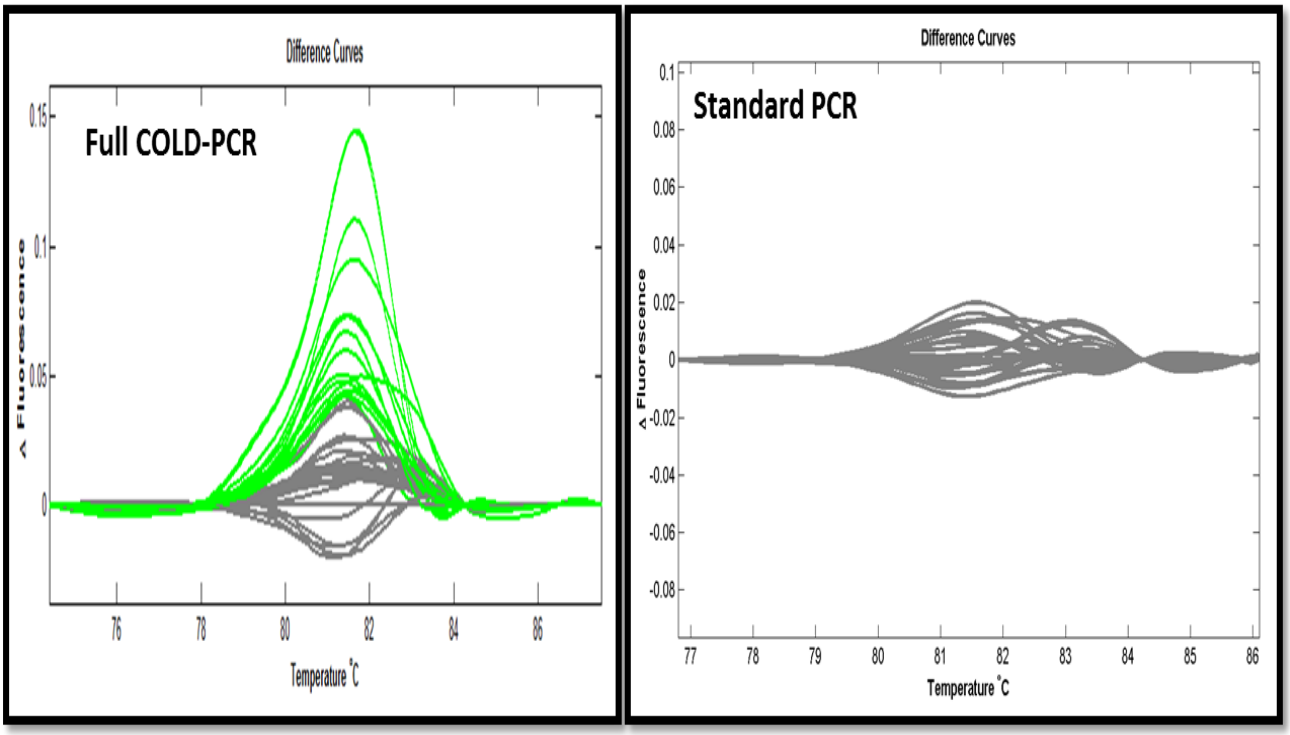

Figure 4-1: Difference Plots generated by HRM (Lighscanner).
A comparison between Full COLD-PCR and Standard PCR protocols used for mutations detection in KRAS (exon 2) in plasma samples. Screening with standard PCR protocol (right) showed all samples were wild-type (grey) for KRAS mutations. Full COLD-PCR (left) succefully detected number of plasma samples (green) harboring mutations in KRAS exon 2 (n=9).

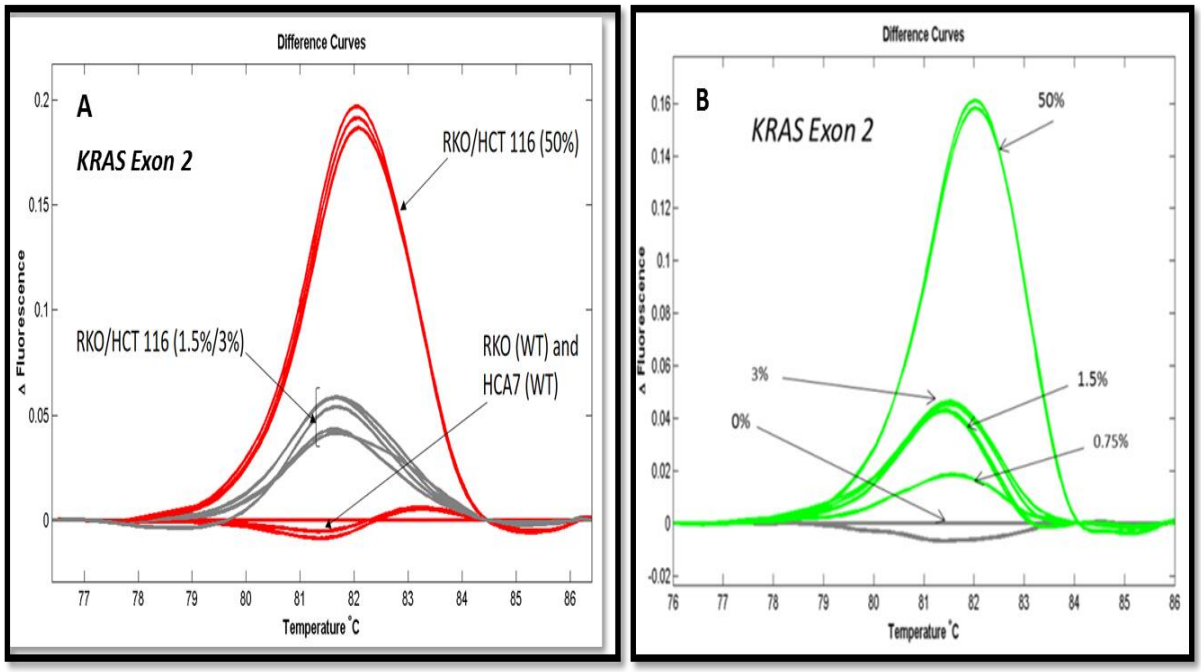

Figure 4-2: Full-COLD PCR-HRM spiking experiments:
image A - one-stage full-COLD PCR difference curve giving a LOD of 1.5% for KRAS exon 2; image B- nested full-COLD PCR difference curve giving a LOD of o.75% for KRAS exon 2.

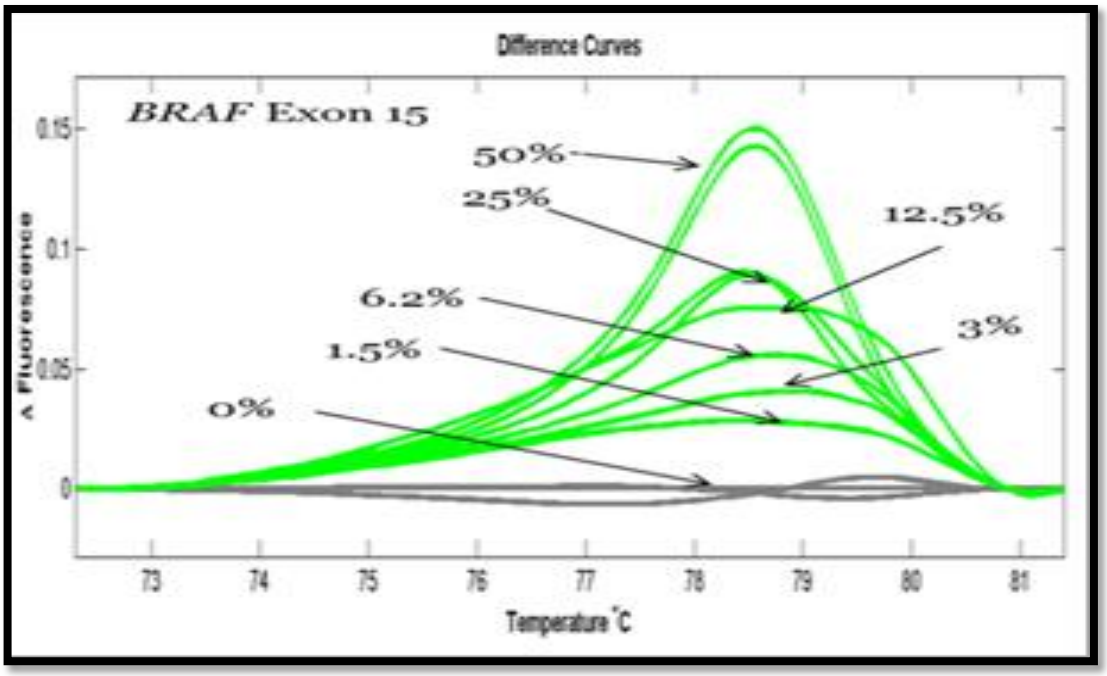

Figure 4-3: nested second stage full-COLD PCR difference curve producing a LOD of 1.5% for BRAF exon 15.

4.2.3 FFPE Sequencing

All FFPE samples were screened by HRM-PCR, using standard PCR protocol, and sequenced to confirm the presence or absence of a mutation. Nine samples were confirmed mutant for *KRAS* exon 2 by HRM, seven out of nine samples were confirmed by Sanger sequencing. The remaining two sample showed wild-type, same results obtained by full COLD-PCR followed by Sanger sequencing.

One hundred percent of wild type samples were confirmed as wild type. Six samples presented a codon 12 mutation (3 x c35:G>A mutation; 2 x c34:G>T mutation; 1 x c35.G>T mutation). Examples of sequencing analysis are shown in Figure 4-4.

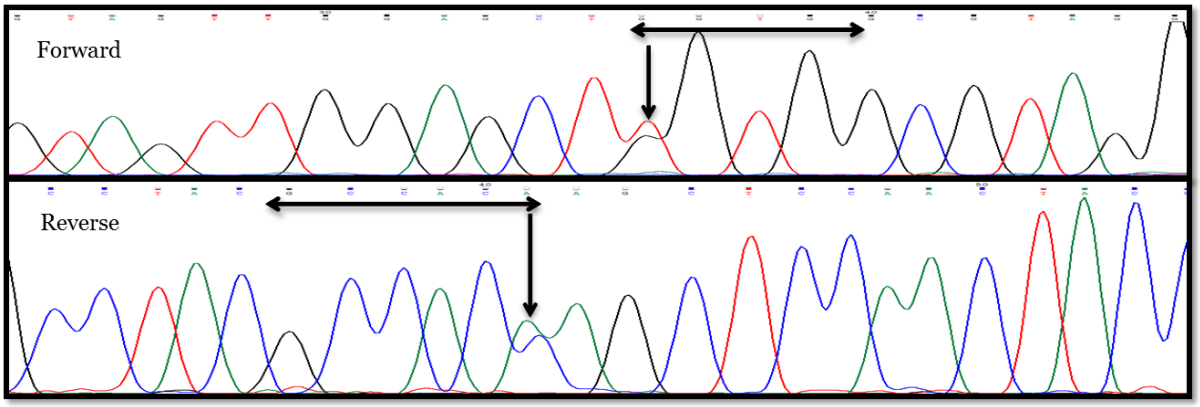

Figure 4-4: Sanger Sequencing Chromatogram.
Represents forward and reverse sequences for resected tumour sample, showing mutation detection at KRAS codon12 (GGT>TGT).

4.2.4 Comparison of mutations in Pre-surgery Plasma Samples and Corresponding Resected Tumours

Matched resection tumours (preserved in FFPE) were included in this analysis to determine the sensitivity of the method and validate *cfDNA* analysis. Extracted DNA samples from both FFPE and plasma (only pre-surgery samples) were screened for the same mutations in *KRAS* (exon 2) and *BRAF* (exon 15) genes and data were analysed separately.

As all samples were found to be *BRAF* wild type for both FFPE and *cfDNA* samples (Figure 4-6). For *KRAS* (exon 2), the final analysis of pre-surgery *cfDNA* samples with corresponding FFPE samples (Table 4-3) is sectioned into three groups: wild type (n = 14), mutant (n = 7), plasma mutant with FFPE wild type (n =2) and FFPE mutant with plasma wild type (n=2). In total, three groups were generated, wild type (56%), mutant (36%) and discrepant (16%). Relative to matched FFPE DNA, the *cfDNA* assay achieved 77.7% sensitivity (n = 7/9) and 87.5% specificity (n = 14/16) (Figure 4-5).

Table 4–3. Final analysis for plasma and matched FFPE samples.

	Plasma		***FFPE***	
Patient	*Kras*	*Braf*	*Kras*	*Braf*
2	*M*	*WT*	*WT*	*WT*
3	*M*	*WT*	*M*	*WT*
4	*M*	*WT*	*M*	*WT*
5	*M*	*WT*	*M*	*WT*
6	*WT*	*WT*	*WT*	*WT*
7	*WT*	*WT*	*WT*	*WT*
8	*WT*	*WT*	*WT*	*WT*
9	*WT*	*WT*	*WT*	*WT*
10	*WT*	*WT*	*WT*	*WT*
11	*WT*	*WT*	*M*	*WT*
12	*M*	*WT*	*M*	*WT*
13	*WT*	*WT*	*M*	*WT*
14	*M*	*WT*	*M*	*WT*
15	*M*	*WT*	*WT*	*WT*
16	*WT*	*WT*	*WT*	*WT*
17	*WT*	*WT*	*WT*	*WT*
18	*WT*	*WT*	*WT*	*WT*
19	*WT*	*WT*	*WT*	*WT*
20	*WT*	*WT*	*WT*	*WT*
21	*M*	*WT*	*M*	*WT*
22	*M*	*WT*	*M*	*WT*
23	*WT*	*WT*	*WT*	*WT*
24	*WT*	*WT*	*WT*	*WT*
25	*WT*	*WT*	*WT*	*WT*
26	*WT*	*WT*	*WT*	*WT*

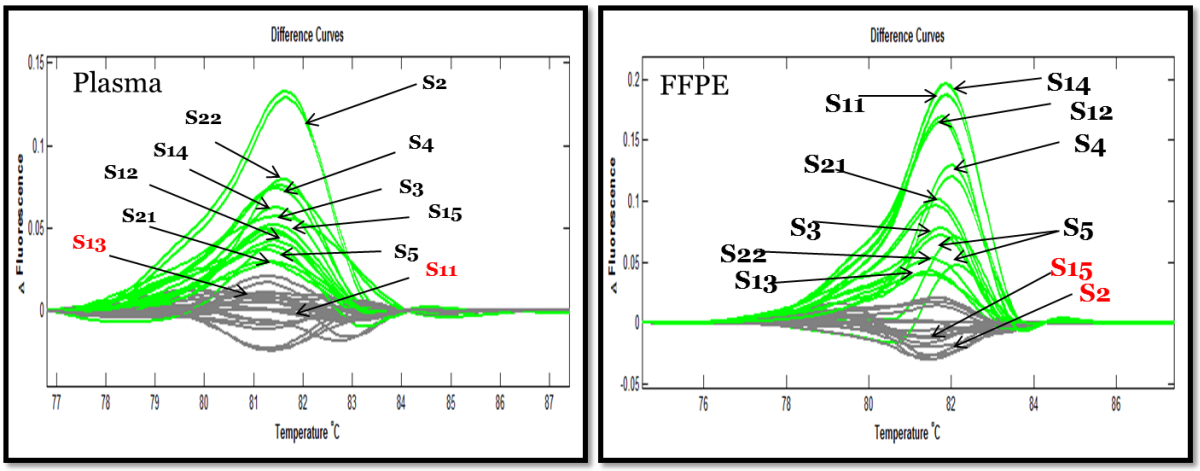

Figure 4-5: Normalised difference Plots generated by HRM (Lightscanner).
Graph on the left represents mutation in KRAS (exon 2) detected in plasma samples and on the right represents mutations detected in KRAS (exon 2) in FFPE sample for same patients. Samples on red refer to discordancy between the two sets.

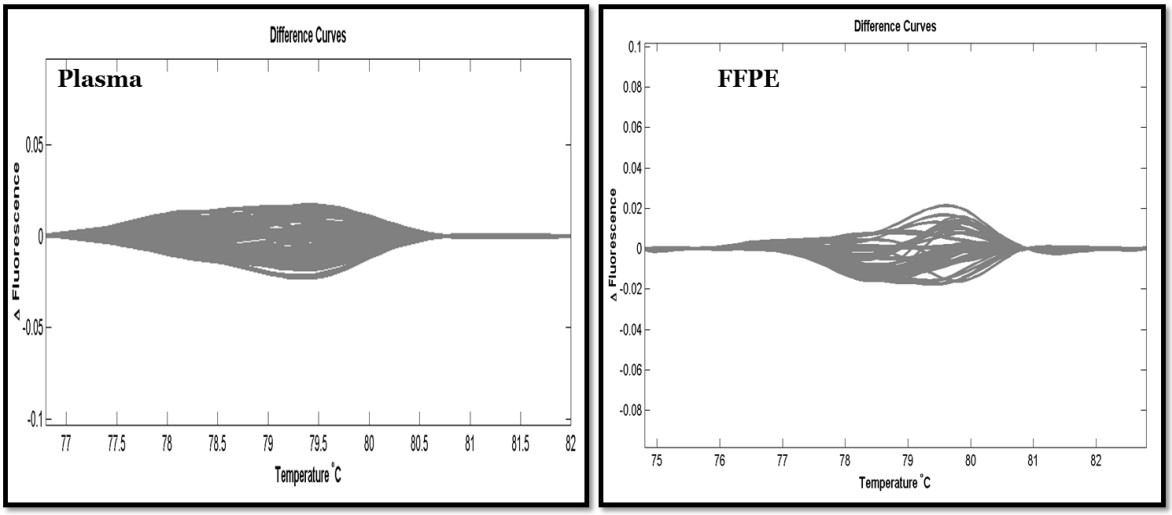

Figure 4-6: Normalised difference plots generated by HRM (Lightscanner).
Screening for mutations in BRAF gene (exon 15), graph on the left represents plasma samples and graph on the right represents FFPE samples for same patients, showing no mutation detected in both sample sets (grey).

4.2.5 Final Analysis for Post-surgery Follow Up Analysis

Part of the aim of this project was to investigate the use of liquid biopsy to exclude metastatic disease. Thus if a mutation was present in the *cfDNA* prior to surgery, then clearance of the tumour would result in loss of the mutation form the *cfDNA*. A persistent mutation would indicate that there was tumour still present. Mutations in several genes (*KRAS, BRAF, PIK3CA, TP53,* and *SMAD4*) were tested as they would all be expected to change in a similar way if they were present in the same tumour i.e. if there true clearance, the all mutations would be expected to be lost. If there wasn't clearance, all would be expected to be present.

HRM-PCR analysis of the post-surgery samples showed some discrepancies as follow: 1) mutation signal loss in day after surgery in some genes, whereas others showed mutation signal increase in comparison to the day before surgery at the same time point (Figure 4-7A); 2) fluctuated mutation signal in same gene for post-surgery samples (i.e. mutation signal loss in first day after surgery and then increases again in samples collected days after) (Figure 4-7B); 3) discrepancies within the replicates for same samples (i.e. when HRM-PCR analysis repeated for confirmation each time represents different pattern) (Figure 4-7C); 4) The shift melting curves generated by HRM showed right-shifted curves for some samples (Figure 4-7D). Taking altogether, findings were suspicious and difficult for interpretation. Therefore, it was assumed that these discrepancies may be due to low quantities of DNA which is generating artefacts and mutation analyses for these genes were excluded. Mutations in *KRAS* (exon 2) and *BRAF* (exon 15) were analysed using the developed protocol, since all samples showed *BRAF* wild-type only *KRAS* analysis is included.

For post-surgery follow up analysis, was as shown below in Table 4-4 Five samples (55%) showed potential surgical clearance of the tumour as the mutation disappeared in post-surgery analysis and four samples (44%) showed a lack of surgical clearance as the

mutation persisted. Supplementary Figure 4-8 shows an example of the potential response to surgery.

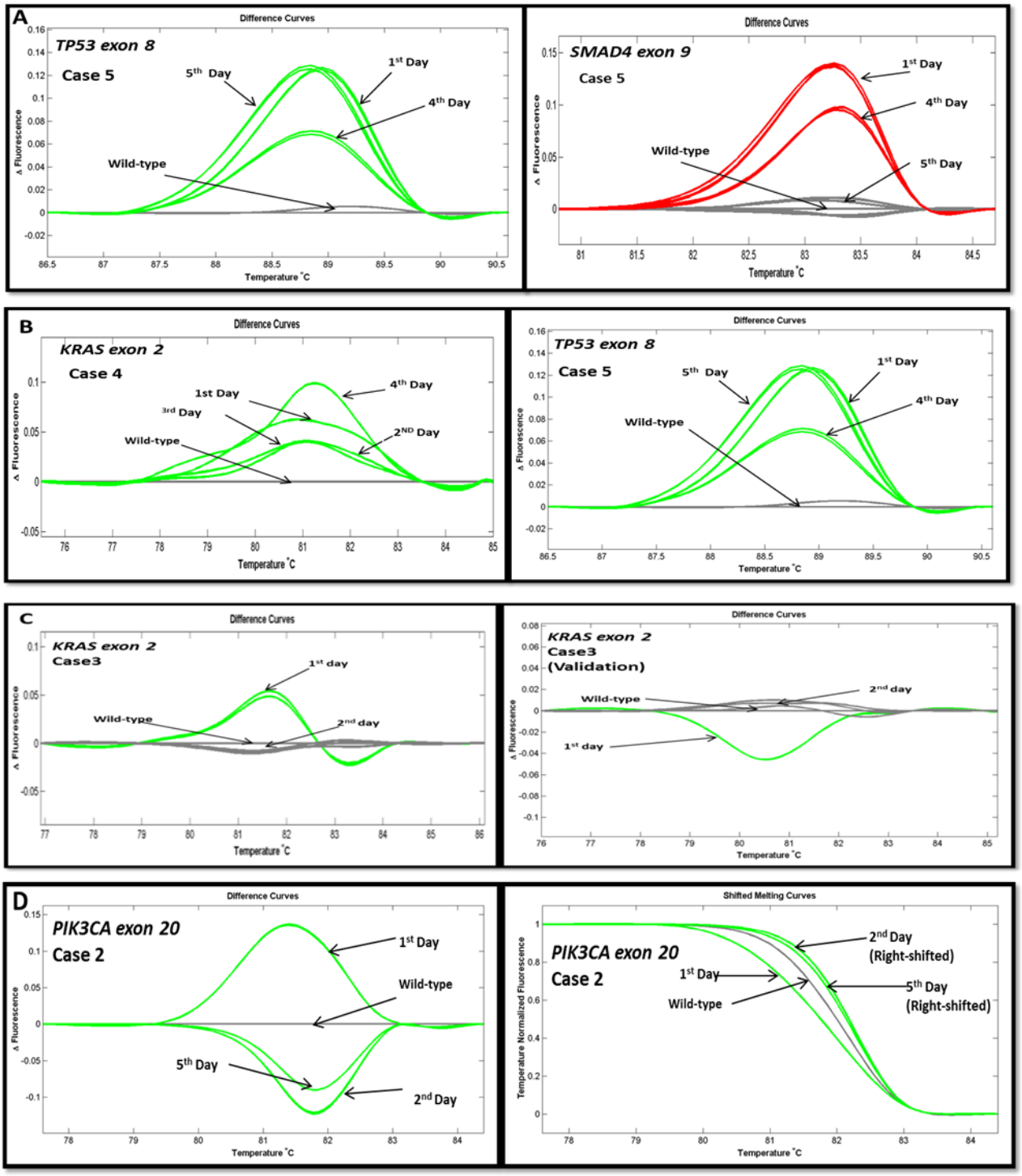

Figure 4-7: Normalised difference Plots and Temp-shift curve generated by HRM (Lightscanner).

Graphs (A-D) represent the discrepancies observed when screened different genes by old protocol (Blood mini kit and standard PCR). A) Represents mutation analysis of TP53 and SMAD4 genes for case 5 showing different patterns of both genes. B) Represents fluctuated mutational signals of KRAS (case 4) and TP53 (case 5) genes for pre and post-surgical samples. C) Represents discrepancies between original screening (left) and validation screening (right) of KRAS gene for the same case. D) Represent right-shifted curves for post-surgical samples for PIK3CA mutation analysis.

Table 4–4: *cfDNA* analysis with post-surgery follow up by full COLD-PCR followed by HRM analysis for mutant *KRAS* exon 2 samples.

Case	*Day 1*	*Day 2*	*Day 3*	*Day 4*
1	*N/A*	*N/A*	*N/A*	*N/A*
2	*Mutant*	*Wild type*	*Wild type*	*N/A*
3	*Mutant*	*Wild type*	*N/A*	*N/A*
4	*Mutant*	*Mutant*	*Wild type*	*Wild type*
5	*Mutant*	*Wild type*	*Wild type*	*N/A*
6	*Wild type*	*Wild type*	*Wild type*	*Wild type*
7	*Wild type*	*Wild type*	*Wild type*	*N/A*
8	*Wild type*	*Wild type*	*Wild type*	*N/A*
9	*Wild type*	*Wild type*	*Wild type*	*N/A*
11	*Wild type*	*Wild type*	*Wild type*	*Wild type*
12	*Mutant*	*Mutant*	*N/A*	*N/A*
13	*Wild type*	*Wild type*	*Wild type*	*N/A*
14	*Mutant*	*Mutant*	*N/A*	*N/A*
15	*Mutant*	*Wild type*	*Wild type*	*N/A*
16	*Wild type*	*Wild type*	*Wild type*	*N/A*
17	*Wild type*	*Wild type*	*Wild type*	*N/A*
18	*Wild type*	*Wild type*	*Wild type*	*N/A*
19	*Wild type*	*Wild type*	*Wild type*	*N/A*
20	*Wild type*	*Wild type*	*N/A*	*N/A*
21	*Mutant*	*Mutant*	*N/A*	*N/A*
22	*Mutant*	*Mutant*	*N/A*	*N/A*
23	*Wild type*	*Wild type*	*Wild type*	*N/A*
24	*Wild type*	*Wild type*	*Wild type*	*N/A*
25	*Wild type*	*Wild type*	*Wild type*	*N/A*
26	*Wild type*	*Wild type*	*N/A*	*N/A*

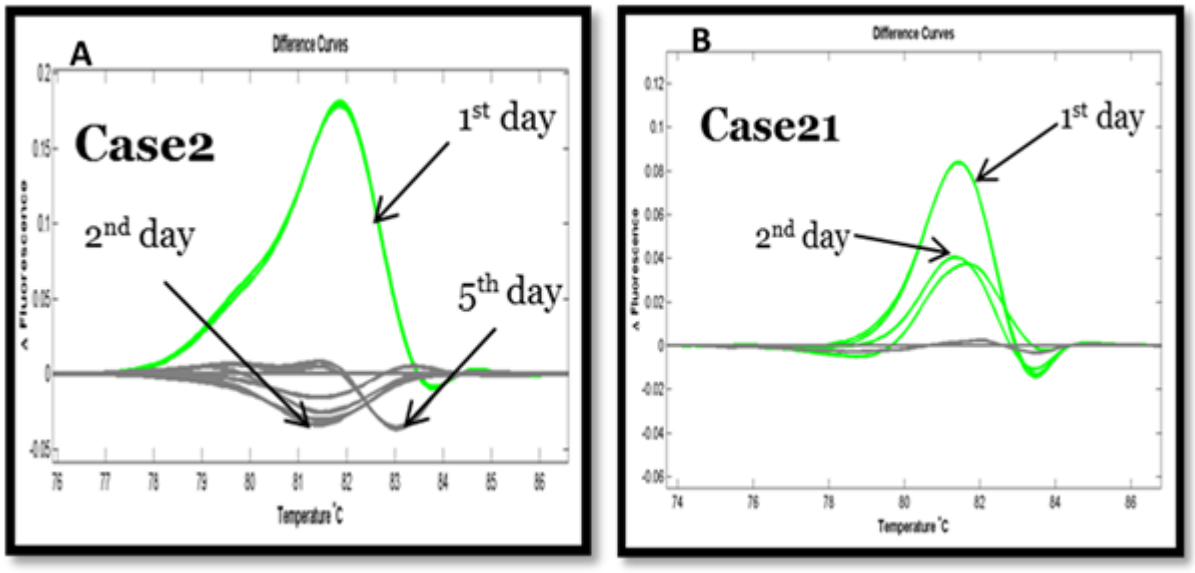

Figure 4-8: An example of the differences in fluorescence found by second stage nested full-COLD PCR-HRM for pre- (day 1) and post-surgery (day 2&5) samples.
A) Shows mutant signal loss in post-operative samples. B) Represents persistent mutant signal in samples collected after surgery.

4.2.6 *cfmiRNAs* expression in plasma

The selected five candidate miRNAs (miR-20a, miR-21, miR-29a, miR-31, and miR-92a) had been previously reported in different studies to be up-regulated in CRC, specially metastatic disease (Thiébaut *et al.*, 2013; M. Li *et al.*, 2014; Tang *et al.*, 2014; Lee *et al.*, 2016; Huang *et al.*, 2017). In Q-PCR, accurate and reproducible analysis is dependent on the Ct value. Therefore, we stratified our data based on Ct values (Ct values≤30) to obtain most accurate and reliable results. Q-PCR optimisation for all candidate miRNAs was assessed by diluting all reverse transcription reactions prior to performing Q-PCR analysis. Duplicate plates for each miRNA candidate were run to examine the reproducibility of the method. Ct values were identified for all candidate miRNAs for each sample as ≤30 Ct (Figure 4-9). The miRNA expression of a panel of five miRNAs was quantified in 32 samples of extracted plasma from 10 CRC patients, pre- and post-operatively, using a QPCR system (Applied Biosystems). Three plasma samples were obtained from healthy donors for data normalisation. For Q-PCR data analysis, $2^{-\Delta\Delta Ct}$ method was applied to analyse the relative changes in miRNAs expression after normalization to miR-39 control. Relative quantification analysis revealed that the expression levels of miR-20a, miR-21, miR-29a, miR-31, and miR-92a were upregulated at least two-fold or more in 8/32 (25%), 9/32(28%), 14/32 (43%), 11/32 (34%), and 12/32 (37%) respectively of total plasma samples. Each sample showed increased levels of at least one candidate circulating miRNA, however, only 2/32 (6%) showed the same pattern of deregulation for all targeted miRNAs. 80% of candidate miRNAs (miR-21, miR-29a, miR-31, and miR-92a) demonstrated same pattern of deregulation in 5/32 (15%) of total plasma samples. miR-21 and miR-92a deregulation was significantly associated (Chi-square test p=0.002) was observed in 8/32 (25%) of total plasma samples. Some candidate miRNAs showed same pattern of deregulation in some plasma sample but it did not significantly correlate table 4-5.

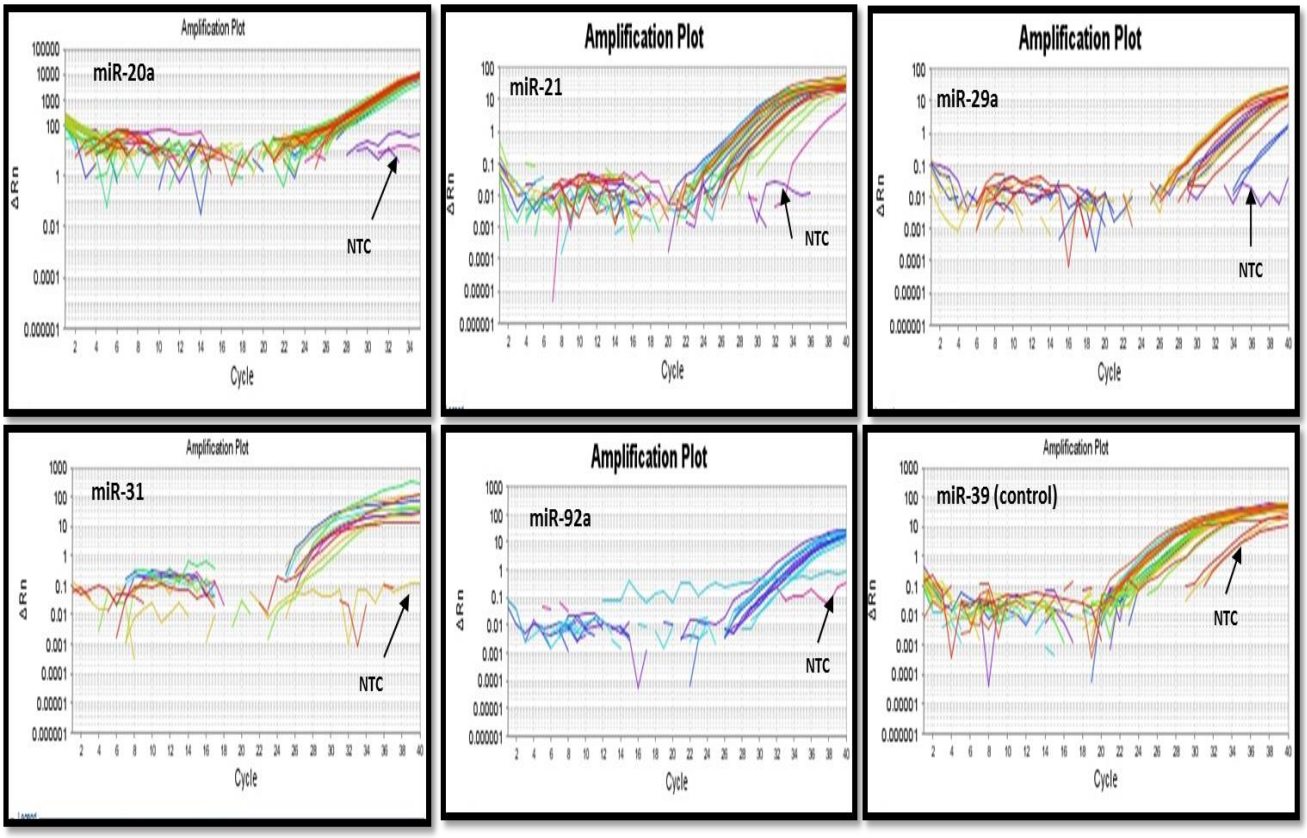

Figure 4-9: Amplification Plots for all candidate cfmiRNAs generated by Q-PCR (Applied Biosystems) based on Ct values.

Table 4–5: Correlation Between miRNAs Over-expression (Chi Square, p≤0.05).

		miR -21			**miR - 29a**			**miR -31**			**miR - 92a**		
		D	*N*		*D*	*N*		*D*	*N*		*D*	*N*	
miR - 20a	*D*	*2*	*6*	*p=0. 69*	*7*	*1*	*p=0. 06*	*3*	*3*	*p=0. 575*	*5*	*3*	*P=0. 104*
	N	*6*	*18*		*7*	*17*		*8*	*16*		*7*	*17*	
miR -21	*D*				*5*	*3*	*p=0. 205*	*5*	*3*	*p=0. 068*	*7*	*1*	*p=0. 002*
	N				*9*	*15*		*6*	*18*		*5*	*19*	
miR - 29a	*D*							*7*	*7*	*p=0. 103*	*7*	*7*	*P=0. 179*
	N							*4*	*14*		*5*	*13*	
miR -31	*D*										*6*	*5*	*P=0. 145*
	N										*6*	*15*	

4.2.7 Expression of *CfmiRNAs* in Pre- and Post-surgical CRCs

The expression of the miRNAs, miR-20a, miR-21, miR-29a, miR-31, and miR-92a, were decreased post-operatively in 4/10 (40%), 4/10 (40%), 4/10 (40%), 2/10 (20%) and 3/10 (30%), respectively, of total CRC cases. miR-21 and miR-92a were up-regulated in samples collected pre-surgery (sample day 1) for case 1 and expression was decreased in days after surgery (2^{nd} and 5^{th} day), then increased again in samples collected 6 days after operation. The pattern of deregulation of four candidate miRNAs (miR-21, miR-29a, miR-31, and miR-92a) demonstrated same patterns in case 2 and 3 (pre and post-operatively) showing over-expression (>2-fold change), only 2 of those showed miR-20a deregulation in pre-surgery sample (Day 1) in case 2 and 4^{th} day after surgery in case 3. Over-expression of miR-21 and miR-92a was observed in 3 days post-operation in case 4. Only miR-21 was up-regulated in plasma sample collected pre-surgery in case 5 and expression was decreased post-surgery. The expression levels of miR-20a, miR-31, and miR-92a were up-regulated in plasma sample collected pre-surgery in case 6 and expression levels showed decreased in first or second day after surgery, whereas the expression level of miR-29a was up-regulated in only first day after surgery. The up-regulations in the expression levels of miR-20a and miR-21 were detected in pre-surgery sample for case 7 and expression levels were decreased post-operatively. Conversely, expression levels of miR-31 showed up-regulation in only post-surgical samples. MiR-20a and miR-21 were up-regulated in only samples collected first day after surgery (Day 2) in case 8. The expression of three candidate miRNAs (miR-20a, miR-21, and miR-92a) showed up-regulation in pre-surgical samples for case 9. The expression of miR-20a and miR-21 slightly decreased in first day after surgery (sample day 2) and increased again in third day after surgery (sample day 4), whereas the expression of miR-92a decreased in samples collected after surgery. Finally, the expression levels of miR-29a and miR-31 were up-regulated in samples collected post-operatively (sample day 2) for case 10, the expression of both miRNAs slightly decreased in the following day (sample day 3) and

increased in samples collected the day after (sample day 4). Paired t test and one-way analysis of variance (ANOVA) analyses revealed no statistical significance between the circulating miRNA expression and these time points post-surgery ($P > 0.05$). A summary of miRNAs profiles are shown in Table 4-6 and Figure 4-10.

Table 4–6: A Summary of relative changes in miRNAs expression (≥2-fold change).

Case No.	Sample No	miR-20a	miR-21	miR-29a	miR-31	miR-92a
1	Day 1	1.17	2.6	0.89	0.25	2.30
	Day 2	0.37	0.96	0.29	0.02	0.45
	Day 5	0.88	1.7	0.38	0.08	1.95
	Day 6	1.24	3.82	0.93	0.32	2.12
2	Day 1	2.66	11.72	3.13	7.33	7.72
	Day 2	0.50	2.35	4.99	12.34	10.80
	Day 5	0.28	3.55	4.02	14.88	14.51
3	Day 1	0.91	3.74	5.85	33.43	13.1
	Day 4	4.10	2.08	4.00	25.65	19.90
4	Day 1	0.81	0.66	0.48	0.73	1.64
	Day 2	0.66	0.87	0.64	1.68	1.39
	Day 3	0.98	2.6	1.34	0.52	2.13
	Day 4	0.48	1.03	0.60	0.49	0.65
5	Day 1	1.53	2.05	0.78	1.07	1.56
	Day 4	0.76	0.62	0.43	0.69	1.35
6	Day 1	5.49	0.34	1.57	2.99	3.34
	Day 2	5.02	0.46	2.36	1.54	1.99
	Day 3	0.51	0.08	0.46	2.85	0.37
	Day 4	1.02	0.14	0.77	1.06	0.79
7	Day 1	2.74	0.05	2.92	0.28	1.73
	Day 2	0.58	0.06	0.23	2.23	0.33
	Day 4	0.76	0.09	0.87	3.38	0.67
8	Day 1	0.64	0.03	1.61	0.62	1.18
	Day 2	3.38	0.31	2.20	1.92	1.56
	Day 4	1.67	0.28	0.99	1.04	0.72
9	Day 1	4.85	0.12	8.02	1.36	2.46
	Day 2	0.60	0.05	4.51	1.08	0.43
	Day 4	2.38	0.15	6.48	1.71	1.46
10	Day 1	0.62	0.02	0.99	1.38	0.38
	Day 2	0.46	0.04	4.00	4.58	0.33
	Day 3	0.97	0.04	2.38	1.64	0.92
	Day 4	1.21	0.17	9.57	23.21	0.56
Total (%)	32	8/32 (25%)	9/32 (28%)	14/32 (43%)	11/32 (34%)	12/32 (37%)

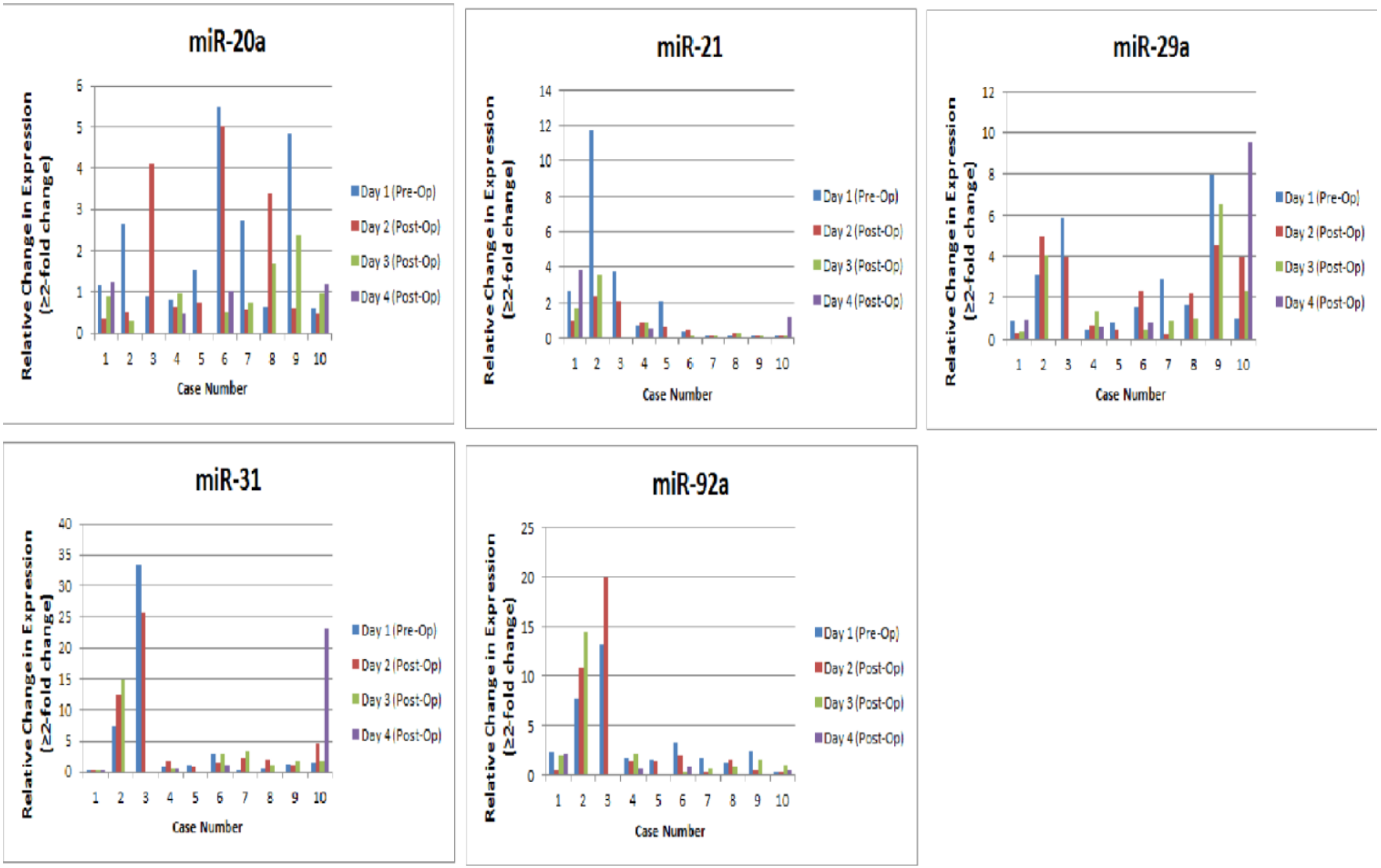

Figure 4-10: miRNAs expression. *Graphs summerise the relative change in the expression of cfmiRNAs in pre and post operation samples.*

4.3 Discussion

This study revealed important information about the characteristics of *cfDNA* and successful methods for extraction and analysis. The Qiagen circulating nucleic acid kit can generate sufficient, high quality *cfDNA*. However, the concentration of *cfDNA* is still exceptionally low and a minimum extracted quantity of 3.78 ng *cfDNA*/ml of blood is still reliably amplified, but unacceptable for clinical analysis due to the potential loss of target DNA. In this study, 1 ml of plasma was used for *cfDNA* extraction per patient, although it is possible to extract from 5 ml of plasma using the Qiagen CNAK. Qiagen's new magnetic bead extraction methodology enables *cfDNA* extraction from 10 ml of plasma. The use of a higher plasma volume would generate a higher total *cfDNA* yield for analysis, however, it is increasingly difficult to obtain large volumes of plasma since patients are bled daily for standard of care analysis leaving a small minority for research. Nonetheless, it is expected that in a clinical setting the amount of blood used for clinical *cfDNA* testing would be increased, improving the analysis of *cfDNA* for all techniques.

The fragmented nature of *cfDNA* cannot be prevented as this occurs when cell-death-mediated DNases are activated during apoptosis or necrosis (Kitazumi, Tsukahara and The, 2011). This study has provided recommendations regarding the design of PCR assays to overcome this characteristic. The reliable amplification of <125 bp fragments is possible and still allows for the detection of target variant DNA.

CfDNA has been shown to carry a wide range of variant allele percentages which are low in most cancer patients (Mouliere *et al.*, 2013). It is difficult to obtain an average expected value before conducting analysis as previous studies have used a wide range of methods and patients' samples for mutation detection. However, *cfDNA* variant allele percentages can be as low as 0.01% in a proportion of patients and this is the greatest challenge for *cfDNA* analysis (Chaudhuri *et al.*, 2015). It is generally accepted that at later stage, larger tumours or patients with metastatic disease will release more circulating tumour DNA than at early stages due to increases in the tumour cell death

rate, which will increase the variant allele percentage as well as the quantity of *cfDNA* that can be extracted (Qin *et al.*, 2016).

This study has presented one stage and nested second stage full-COLD-PCR for the detection of *KRAS* exon 2 and *BRAF* exon 15 mutations in *cfDNA*, with the latter able to detect as low as 0.75% variant alleles. As full-COLD-PCR can enrich all mutation types (Milbury *et al.*, 2011), it is recommended over fast-COLD-PCR for analysis. Although COLD-PCR is not directly quantitative, it is assumed that all the mutations detected by COLD-PCR are above 0.75% and the maximum variant allele percentage detected was <6% as no mutations were detected by the original screening by nested PCR-HRM which had a LOD of 6%. Furthermore, mutations were detected in *cfDNA* which matched with the primary tumour analysis for three Duke's stage A patients, two Dukes' stage B patients and two Dukes' stage C1 patients, indicating that early-stage tumours can be analysed via this methodology.

COLD-PCR is rapid and cheap in comparison with different technologies, such as digital PCR and next generation sequencing. Applying this technique to screen for molecular changes in *cfDNA* to monitor tumour burden can possibly provide a good diagnostic marker in cancer patients. Approximately 77.7% sensitivity (n = 9) and 87.5% specificity (n = 16) was achieved by full-COLD-PCR in comparison to FFPE primary tumour mutation analysis. Whilst these techniques are sufficient for research, it is expected that sensitivity and specificity should be nearer 100% for clinical analysis. Using a larger plasma volume of up to 10 ml would provide a larger mutant copy number pool, allowing a larger amount of template to be added to the PCR reaction due to an increase in *cfDNA* concentration, leading to increased sensitivity. Moreover, it may be necessary to target patients before screening who are likely to have high mutant allele percentages, those with high FFPE mutant allele percentages and larger tumours. Lastly, a larger selection of biomarkers other than *KRAS* exon 2 and *BRAF* exon 15 may resolve

this issue as patients will have more than one mutation and perhaps will demonstrate similar pattern if they were present in the same patient. Full-COLD-PCR generated two major forms of discrepancy: samples with a FFPE mutation but no matching *cfDNA* mutation (n = 2) and wild type FFPE samples which have mutations present in *cfDNA* (n = 2). The question as to why these discrepancies occur is major and remains largely unknown. The detection rate of *KRAS* and *BRAF* mutations in blood of CRC patients have been previously reported in different studies, using different techniques including amplification refractory mutation system (ARMS), digital PCR, and NGS. The sensitivity and specificity reported for *KRAS* and BRAF mutations were relatively high, 98% specificity and 92% sensitivity for *KRAS* mutations and 100% specificity and sensitivity for *BRAF* mutations (Thierry *et al.*, 2014). Others reported that *KRAS* mutations were higher (50%) in plasma than in primary tumour tissues (28%) (Kuo *et al.*, 2014), whereas others revealed that *KRAS* mutations were relatively low in plasma (3%) in comparison to the matched adenocarcinoma tissues (Perrone *et al.*, 2014). In addition, the reported concordance between plasma and tumour tissue variant of detection of *EGFR* variant in Non-small cell lung carcinoma (NSCLC) with leading platforms ranges between 70%-90% (Diaz and Bardelli, 2014; Thress *et al.*, 2015; Xu *et al.*, 2017). However, inconsistent results made the use of *cfDNA* in diagnostic laboratory uncertain. This discordance between *cfDNA* and tumour tissue remains unclear whether it is a result of analytical or biologic factors. There are several analytical and biological factors that may affect the concordance such as plasma volume, LOD of the method, small fraction of *cfDNA* present in plasma, tumour stage and tumour heterogeneity (Higgins *et al.*, 2012; Bettegowda *et al.*, 2014; Diaz and Bardelli, 2014; Siravegna *et al.*, 2017; Merker *et al.*, 2018). Early stage of tumour and early metastatic disease has lower number of *cfDNA* fragments present in blood circulation. Moreover, detection of *cfDNA* derived from tumours is challenging due to low levels of circulating tumour DNA which represents small fraction (<1.0%) of total *cfDNA* and this requires very sensitivity methods for *cfDNA* analysis (Diehl *et al.*, 2005; Frank Diehl *et al.*, 2008; Holdhoff *et al.*, 2009; Diaz

and Bardelli, 2014). It is possible that the two mutant FFPE samples without matching *cfDNA* mutations occurred due to *cfDNA* variant allele percentages which fall below the current LOD for COLD-PCR. As mentioned previously, reports as low as 0.01% variant alleles have been described in the literature (Chaudhuri *et al.*, 2015). Neoadjuvant therapy which has cleared the tumour may have also caused the lack of detectable variant alleles, although this clinical information is not presently known and is probably unlikely as both cases were Dukes' stage B. The cause of wild type FFPE samples that have mutations present in *cfDNA* is a slightly more complex issue. All FFPE blocks containing tumour were analysed for discrepant cases to investigate tumour heterogeneity between blocks as the probable cause, however, the same result was obtained in all blocks. There are several possibilities which may account for this result: unknown metastatic tumours which present the mutation; unknown primary tumours which harbour the same mutation; low percentage of *KRAS* mutant tumour cells in the FFPE block leading to a low percentage variant allele which was not detected by COLD-PCR HRM. To overcome these issues discussed above, new approaches such as ARMS, digital PCR, and NGS have been developed to offer high level of analytical specificity and sensitivity which can be applied in *cfDNA* analysis (Taniguchi *et al.*, 2011; Forshew *et al.*, 2012; Diaz and Bardelli, 2014; Siravegna *et al.*, 2017; Xu *et al.*, 2017). Digital PCR-based technologies enable high-throughput screening of the mutant gene of interest on the background of wild-type allele, reaching 0.0001% LOD which is mandatory for the detection of low aberrant alleles or low levels of *cfDNA* are present (i.e. early stage of disease or minimal residual disease) and cannot be detected by COLD-PCR (Ji *et al.*, 2010; Diaz and Bardelli, 2014; Siravegna *et al.*, 2017). NGS-based approaches offer broad coverage assay which have the capability to detect large number of variant in multiple genes and enables discovery of novel mutated variants (Reis-Filho, 2009; Diaz and Bardelli, 2014; Siravegna *et al.*, 2017). Therefore, utilizing Digital-PCR and NGS may resolve these limitations regarding the LOD of the method and increase the level of specificity and sensitivity for *cfDNA* analysis but, in comparison to COLD-PCR, they have

higher costs and cannot be readily applied to monitor patients longitudinally (Siravegna *et al.*, 2017). On the other hand, ARMS are sensitive methods which offer quick and easy analysis of mutant DNA and routinely being able to detect 1% of mutant DNA in background of 99% of wild-type DNA and recently has been revealed that this method was able to detect 0.1% of mutant sequences (Kimura *et al.*, 2006; Ellison *et al.*, 2010; Zhang *et al.*, 2017, 2018). This method has more advantages over Digital-PCR and NGS technology, including; it does not require new equipment for processing and analysis, it can be performed incorporation with intercalating fluorescent dyes such as SYBR green, the ability to analyse the results in closed-tube format which eliminates PCR product contamination and reduces the time to generate the results, and it can only detect targeted mutations and this could be considered as an advantage for the use of ARMS for *cfDNA* analysis in clinical setting as decision on treatment and patients-outcome results are based on known mutations (Tseng *et al.*, 1997; Boehm *et al.*, 2004; Ellison *et al.*, 2010; Zhang *et al.*, 2017). With these assumptions, ARMS assays seem more favourable than Digital PCR or NGS for *cfDNA* analysis. However, it was planned to optimize and confirm the results obtained in this study by ARMS for reasons stated above but this could not be done due time constraints.

Serial sampling of *cfDNA* pre-and post-operatively in CRC patients has been reported recently to monitor tumour changes and observe any correlation with tumour progression and clearance after surgery (Garcia-Murillas *et al.*, 2015; Guo *et al.*, 2016). In total, more than half of patients (55%) showed potential surgical clearance of the tumour as the mutation disappeared in post-surgery analysis and the remaining four samples (44%) showed a lack of surgical clearance as the mutation persisted. This indicates the potential clinical utility of *cfDNA* for measuring the clearance by surgery or the response to therapy. Ideally, a 6-month to 5-year follow up should take place to fully confirm recurrence rates and these results. The rate of survival is unknown for this study because the follow up analysis was only a maximum of six days after surgery. This

indicates that more than half of the patients with a *KRAS* mutation responded to tumour removal and the remaining patients have a persistent mutant signal, feasibly due to metastases, late stage disease, or residual *cfDNA* in the blood after surgery. Therefore, the analysis of *cfDNA* for surgical clearance is a potential diagnostic marker for prognosis and surveillance. However, a larger study which includes a panel of genes would be required to increase the sensitivity and power of this test.

In this study, we also performed *cfmiRNA* analysis in patient's plasma as a complementary form of analysis and an alternative to *cfDNA* analysis. Data from *cfDNA* and primary tumours analysis showed good reflection of the primary tumour by the *cfDNA*. An optimised protocol for Q-PCR analysis successfully identified all candidate miRNAs for each sample as <30 Ct in order to obtain more reliable and reproducible results. The expression levels of *cfmiRNAs* (miR-20a, miR-21, miR-29a, miR-31, and miR-92a) were analysed in 32 plasma samples (10 cases) and relative change of expression was evaluated by $2^{-\Delta\Delta Ct}$ method. The findings here confirm that miRNAs are present in human plasma, and the miRNA panel used in the present study are expressed in CRC patients. *cfmiRNAs* demonstrated the same patterns of deregulation in only two samples. However, the expression of four *cfmiRNAs* (miR-21, miR-29a, miR-31, and miR-92a) was upregulated in 5 plasma samples collected from 2 patients (pre and post operatively). This may indicate that the selected miRNAs may not follow same patterns of expression in CRC patients. Although some candidate *cfmiRNAs* showed deregulation in same plasma samples, there was no significant association. However, the over-expressions in miR-21 and miR-92a were significantly associated which may indicate that the two candidate miRNAs are useful markers for *cfmiRNA* analysis as they may demonstrate similar pattern of deregulation.

In the next step, the expression levels of *cfmiRNAs* were evaluated at different time points in pre- and post-operative of CRC patients. The diagnostic significance of

circulating miRNA signatures has been recently the focus in cancer research, in which the expression levels in cancer patients are compared to healthy individuals (Thomas *et al.*, 2015; Rapisuwon, Vietsch and Wellstein, 2016). Consequently, several studies investigated variation in levels of *ctmiRNA*s in plasma, evaluating differences in expression at pre- and post-operative points. Recent reports showed a significant decrease of several circulating candidate miRNAs levels in the comparison of pre- and post-surgical CRCs (Ogata-Kawata *et al.*, 2014; Ristau *et al.*, 2014; J. Li *et al.*, 2015; Jo *et al.*, 2017). Therefore, it was of interest to investigate the role of these *cfmiRNAs* for surgical clearance in CRC patients. The analysis in this study revealed that 70% (7/10) of CRC cases demonstrated up-regulation (≥2 fold change) in the expression of at least 1 or more of candidate miRNAs in pre-operative samples. The expression levels of candidate *ctmiRNA*s decreased at least 1.5-fold or more post-operatively in 42% (3/7) of these cases. Furthermore, statistical analysis showed there was no significant difference in miRNA expression at these time points. Despite that most of them were downregulated at the 2nd or 3rd day (the day after operation), the expression of some increased on the 4rd day and onwards after the operation. This may be as a result of the inflammatory impact on miRNA expression after tumour removal (Bravo-Egana *et al.*, 2012; Poon *et al.*, 2017). Additionally, the origin or source of miRNA in the blood circulation has also been one of the challenging issues with investigating miRNAs in plasma (Pritchard *et al.*, 2012). *CfmiRNA* in blood plasma can be derived from blood cells from different organs which may be mixed with cancer-specific miRNAs from other sources (Ma, Jiang and Kang, 2012; Pritchard *et al.*, 2012). Moreover, the increased levels of specific organ miRNAs in blood plasma may be an indication of toxicity in a particular organ or tissue (Penman, Kaufman and Daniels, 2014). However, this study is limited by sample size and number of biomarkers which may be the possible explanation of lack of statistical differences between the miRNA expression and these time points. Based on the findings in this study, it is not possible to state with certainty the useful role of miRNAs in blood circulation as biomarkers for surgical clearance in CRC patients.

Ambiguity still remains regarding the diagnostic and prognostic value of *ctmiRNA* in patients' plasma, so a larger study involving more patients and other miRNAs is required to clarify their value.

In summary, patients can be dichotomised into a group which either loses or retains mutant *cfDNA* or *cfmiRNA* expression following tumour removal. Detection of mutations in *cfDNA* is a good means of non-invasive screening for colorectal cancer and as COLD-HRM is a sensitive, fast and reliable method of detection, this may provide a novel method of assessing surgical clearance and testing for recurrence.

5 The potential role of *GNAS1* signalling on cell motility and metastasis in colorectal cancer

5.1 Introduction

The development of metastasis is a complex process which involves epithelial-mesenchymal transition (EMT) of the cells in the primary tumour to get into the lymphatic and blood vessels. In order to establish new colonies at the metastatic sites, the cells must undergo mesenchymal-epithelial transition (MET) of the cell when they arrive at their metastatic site. In our initial study of CRCs in chapter 1, we found lymph node and metastasis to be associated with mutation of *KRAS, PIK3CA, SMAD4,* and *PTEN* and with promoter methylation of *P16*. The processes of EMT and MET, however, require de-regulation of several different functions and neither of the genes that we have identified appear to be directly involved in the regulation of these functions.

One of the students in the lab, in a separate project, identified mutations in *GNAS1* in a small proportion of CRCs. This was thought to be a more likely candidate as a metastasis inducing genes as *P16* promoter methylation and *KRAS*, *PIK3CA*, *SMAD4*, *PTEN* mutations occur early in the process of malignancy and are therefore probably promoting local growth rather than metastasis. Although there are few GNAS1 mutations, it is located at 20q21 – are region usually amplified in CRCs; genomic instability such as amplification is a late event. *GNAS1* was of interest because it encodes numerous proteins due to a variety of alternative promoter and splice regions within it. The most well documented form of the *GNAS1* gene is the 13-exon long portion that codes for the stimulatory alpha G protein subunit (Gsα) of the heterotrimeric G protein family (Weinstein, Chen and Liu, 2002; Weinstein *et al.*, 2004) Gsα is commonly found as a vital component in cellular signalling (Bastepe, 2007). Moreover, adenylyl cyclase is activated by Gsα through which cAMP (cyclic adenosine monophosphate) is produced and subsequently downstream signalling pathways can be regulated (Nagai *et al.*, 2010). The location of *GNAS1* at the top of multiple signalling cascades would potentially allow it to regulate the functions necessary for EMT/MET and therefore it would be a good candidate as a metastasis-associated gene. In addition, *GNAS1* is located at 20q 13.2-

13.3 – a region which is commonly amplified in many cancers including CRCs. Gene amplification is a means of gain-of-function and it could explain the comparatively low frequency of sequence mutation reported for this gene in CRC (approximately ~2 %) (Yamada *et al.*, 2012; Fecteau *et al.*, 2014).

Finally, Mutations in *GNAS1* occur in 28% of growth-hormone-secreting pituitary tumours and 5% of thyroid adenomas according to the current deep sequencing techniques (Lyons *et al.*, 1990; Landis *et al.*, 1989; Weinstein *et al.*, 1991). In addition, according to these sequencing techniques, *GNAS1* is also mutated in a broad range of other tumour varieties, including hepatocellular carcinoma (2%), colon cancer (4%), parathyroid cancer (3%), pancreatic tumours (12%), lung cancer (1%), endometrial cancers (2%), and cancers of the ovary(3%) (O'Hayre, Degese and Gutkind, 2014). It has been reported that GNAS1 mutation is positively associated with (and therefore potentially collaborating with) *KRASs* and *BRAF* mutation (Sjöblom *et al.*, 2006; Wu *et al.*, 2011) thus further supporting a role for *GNAS1* in CRC.

Based on these facts, it was considered that the *GNAS1* may be involved in the metastatic process. However, the precise role of *GNAS1* in CRC has not yet been fully elucidated and has only been addressed in relatively few publications until now (Walther *et al.*, 2014). Therefore, this study aimed to investigate the functional role of *GNAS* in CRC. Two cell lines, SW620 and RKO, were used and the RNA interference was used to knock down GNAS1 in each of these cell lines. The effect of GNAS1 knockdown on cell proliferation and cell motility was then tested.

5.2 Results

1.1.1 In-vitro analysis of SW620 and RKO Cells

To investigate the potential role of *GNAS1* in the development of metastases, we decided to investigate whether it was involved in the induction of cell motility (which is one of the features which cells must possess in order to become metastatic). The aim was to reduce the amount of *GNAS1* protein by RNA interference and testing the effect of this on a transwell cell migration. We decided to test two different well established cell lines representing different genetic pathways of CRC. The cell line SW620 can be classed as showing chromosomal instability (CIN) and is known to have 20q amplification (unpublished data from our lab). The cell line RKO is characterised by microsatellite instability. Both cell lines were authenticated by STR genotyping using PCR-single locus technology, performed by Eurofins (see appendix). The mutation profile and other features of each cell line are shown in table 2-1 in chapter 2.

1.1.2 Knockdown of *GNAS1* using Specific *GNAS* SiRNA in SW620 and RKO Cells

The role of GNAS1 in cancer is currently under debate and data on the expression of GNAS1 protein in the development of CRC metastasis are sparse. Therefore, this chapter was aimed to investigate the role of GNAS signalling pathway in the regulation of cell motility and cell proliferation in CRC cell lines. To explore this, the knockdown of GNAS expression using siRNA in high expressing cell line SW620 was first optimised (Figure 5-1). GNAS targeting siRNA duplexes were transfected at a concentration range of 15-60 nM together with 10 µl of Lipofectamine 2000 for 48 hours at 37°C. The optimal condition giving the greatest knockdown of GNAS protein expression was by using 60 nM siRNA together with 10 µl of Lipofectamine 2000. This transfection condition was used for future transfection experiments.

Following optimisation, the knockdown of GNAS protein expression using GNAS targeting siRNA duplexes was repeated in SW620 cells. Knockdown of GNAS in SW620 was associated with a decrease in GNAS protein expression compared to cells transfected with luciferase siRNA control. To show that this effect was not only in SW620 cell line, GNAS was also knocked down in RKO, a high expressing GNAS cell line. Consistent with this, there were slight decreases in GNAS expression in RKO cell line when transfected with GNAS targeting siRNA duplexes compared to the luciferase siRNA control (Figure 5-2). This confirms that GNAS was successfully knocked down in both cell lines and the effect of GNAS knockdown on cell motility and cell proliferation was next investigated.

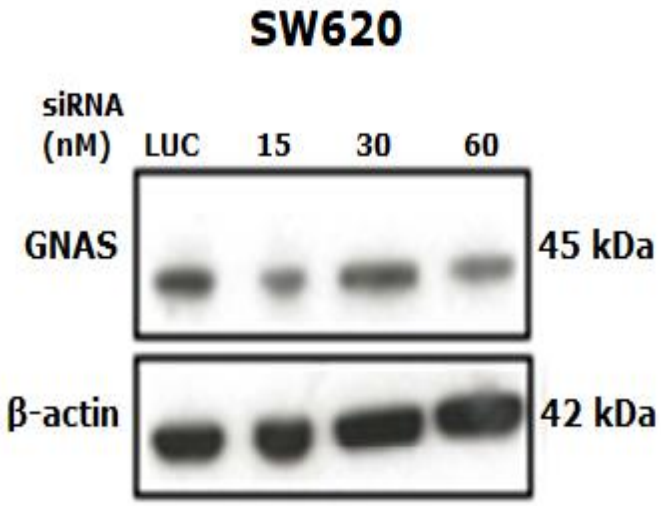

Figure 5-1: *The optimisation of GNAS knockdown. Cells were transfected with a final concentration of 15, 30 or 60 nM of GNAS siRNA together with 10 µl of Lipofectamine 2000. Control cells transfected with 60 nM of Luciferase siRNA (LUC) together with 10 µl of Lipofectamine.*

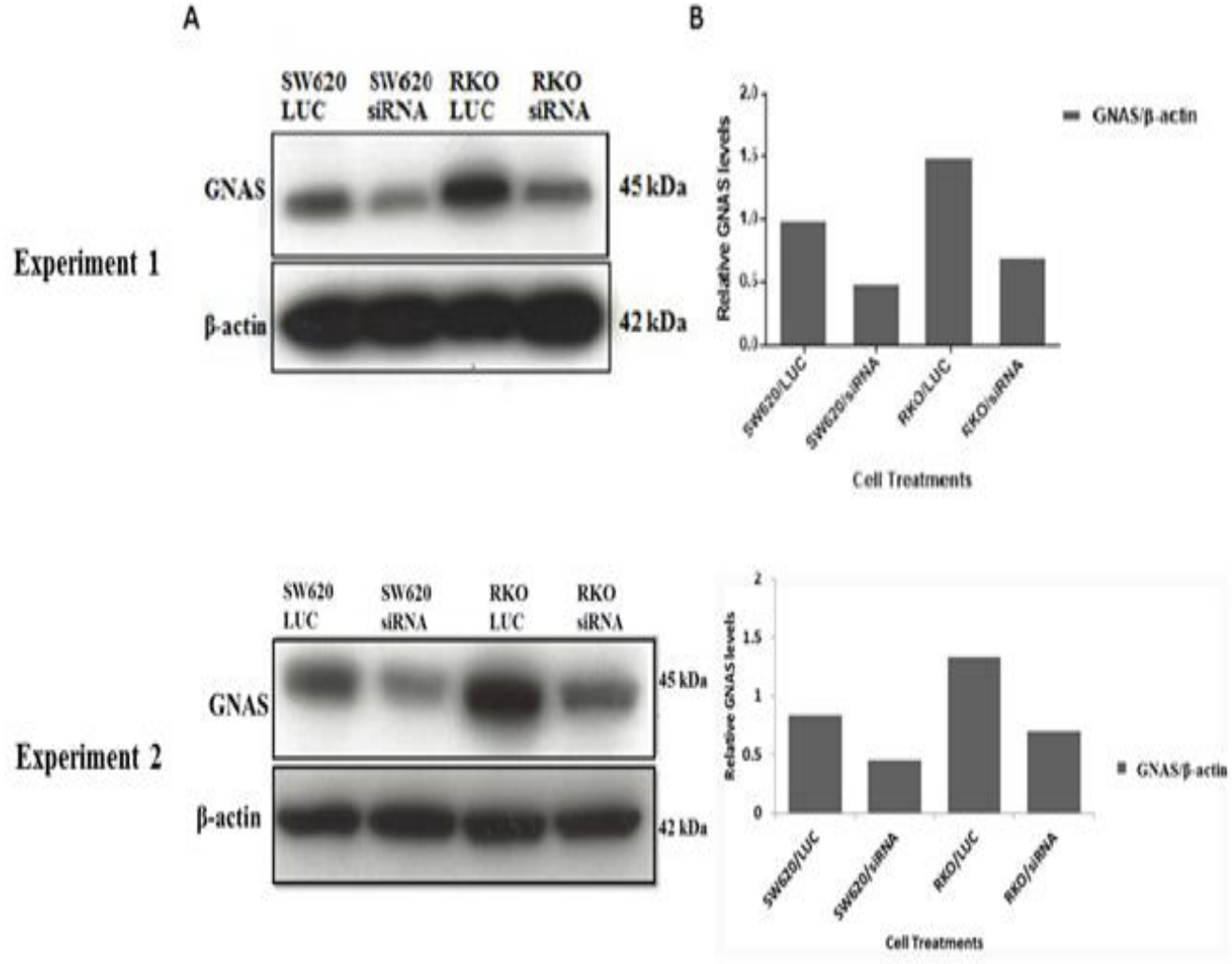

Figure 5-2: The effect of GNAS expression inhibited by GNAS siRNA in SW620 and RKO cell lines.

(A) Immunoblots are shown in the left panel. Molecular weight markers are indicated in kilo Daltons (kDa). (B) The graphs show relative GNAS levels calculated by densitometric analysis of the 45 kDa immunoreactive bands corresponding to soluble GNAS levels in samples (lanes 2 and 4) normalised against the luciferase control in lane 1 and lane 3 and adjusted against β actin loading control. (B) The GNAS/β actin graph shows that there was a small decreases in the expression of GNAS in both cell lines when transfected with GNAS siRNA (lane 2= 0.5 and lane 4= 0.7) compared to the control sample (lane 1=1.0 and lane 3= 1.5) in the first experiment and (lane 2= 0.45 and lane 4= 0.68) compared to the control sample (lane 1=0.83 and lane 3= 1.33) in the second experiment. GNAS knock-down was conducted in two independent experiments (n=2)

5.2.1 The Effects of *GNAS* on Cell Proliferation and Viability in SW620 and RKO Cells

We had wished to investigate the role of *GNAS1* in the regulation of cell motility. However, the evaluation of cell motility can be influenced by changes to cell proliferation. Since *GNAS1* is involved in the regulation of many signalling pathways, it is possible that it may be regulating cell proliferation. To exclude this possibility, the role of GNAS1 in regulating cell proliferation was investigated.

To investigate the effects of *GNAS* knockdown on cell proliferation in CRC, from both cell lines were transfected with GNAS1 siRNA and luciferase siRNA control as described above. Forty-eight hours post transfection, cell proliferation assay was assessed using a PrestoBlue assay which provides a measure of cell metabolic activity and therefore provides a measure of cell viability. The results showed that there were increases in the cell proliferation over the period of the experiment, but there was no significant difference between those transfected with *GNAS* siRNA and the luciferase controls in both cell lines, RKO and SW620 (Figure 5-3 A,B).

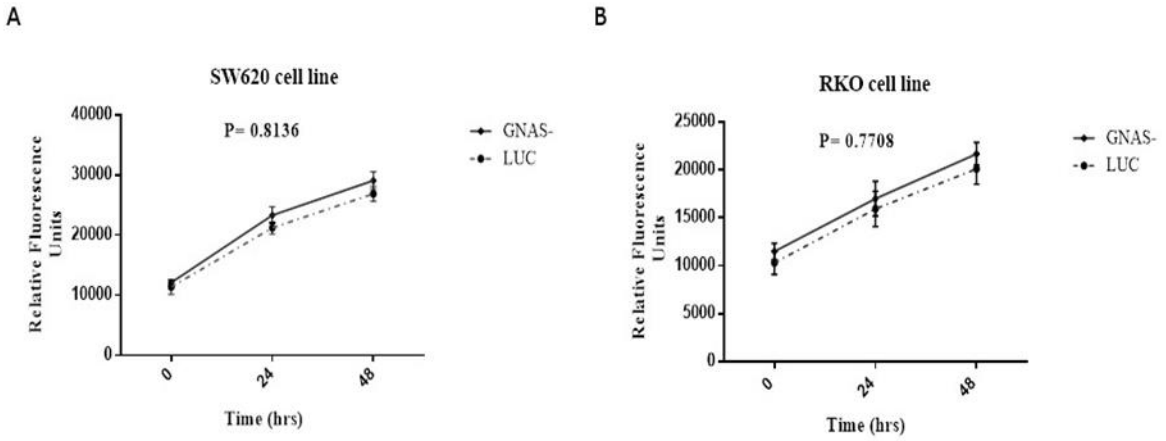

Figure 5-3: *GNAS* does not affect cell proliferation.

(A) In SW620 there was no significant differences in cell proliferation between GNAS siRNA and luciferase siRNA transfected cells (P=0.8136). (B) Similarly, in RKO there was a lack of differences in proliferation between GNAS siRNA and luciferase siRNA control (P=0.7708). The data are measured in triplicate and presented as means ± SD (unpaired 2-tailed t test, n=2).

5.2.2 The Influences of *GNAS* on Cell Motility in CRC Cell Lines, SW620 and RKO

Having shown that there was no effect of GNAS1 knockdown on cell proliferation, we were confident that any effect of served in the transwell motility assay would be a true reflection of the role of GNAS1 on cell motility.

Transwell migration assays were performed on RKO and SW620 cell lines to determine the effect of *GNAS* knockdown on cell migration. There were no significant differences between *GNAS* siRNA and luciferase controls in both cell lines, RKO and SW620 (Figure 5-4 A, B).

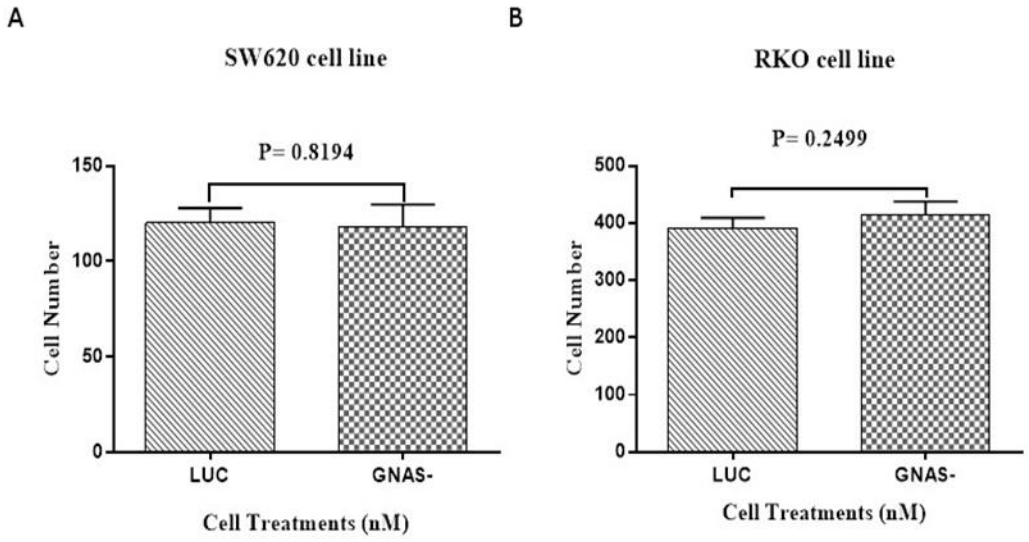

Figure 5-4: *GNAS* knockdown has no effect on cell motility.

(A)Knockdown of GNAS expression in SW620 cells did not cause a significant decrease in cell migration compared to luciferase control, (P=0.8194). (B) Similarly, transfection of RKO cells with GNAS targeting siRNA duplexes did not inhibit cell migration compared to luciferase control, (P=0.2499). The data are measured in triplicate and presented as means ± SD (unpaired 2-tailed t test, n=2).

5.3 Discussion

The GPCR (G-protein-coupled receptor) is the largest family of cell surface receptors involved in signal transduction (Dorsam and Gutkind, 2007), of which, GNAS is a member. The GPCR family has over 800 members, accounting for almost 4% of the encoded human genes (O'Hayre, Degese and Gutkind, 2014). These proteins have vital roles in many physiological events including immune responses, cardiac function, neurotransmission and sensory functions (for example, taste, vision and olfaction); however, some of the most prevalent human diseases are caused by aberrant GPCR expression or activity (Pierce, Premont and Lefkowitz, 2002). Transformations in normal and malignant cell growth, gene transcription and cell survival as well motility occur via the activation of these GPCR-regulated signalling circuits, including GNAS. Given the large number of functions that GNAS1 appears to regulate, it would be an ideal candidate as a gene involved in the development of metastasis. Therefore, this study investigated the role of GNAS1 in the regulation of cell motility a feature which is required for metastasis.

Although this study only used two cell lines, RKO and SW620, they are well-described cell lines found to have a high GNAS protein expression level detectable by western blot. The RKO cell line was found to have a higher GNAS protein expression compared to the SW620 cell line. siRNA was used to knockdown GNAS in the CRC cell lines and firstly cell proliferation was tested in order to exclude the possibility that it may affect the studies on a transwell motility. There was no effect of GNAS1 knockdown on either of the cell lines. Next, the effect of GNAS1 knockdown on cell motility was examined. Although studies have shown that mutations in GNAS can promote cell proliferation and migration in various cancers (O'Hayre, Degese and Gutkind, 2014), our data suggested that the GNAS protein does not induce cell proliferation and motility in CRC cells. However, despite optimisation, the effect of GNAS knockdown on GNAS protein expression, detected by western blot, was consistently weak compared to the luciferase control.

The possible explanation is that might be due poor transfection efficiency. The limitations of using siRNA to knockdown GNAS is that it is only transient as this technique operates at a post-transcriptional level. Moreover, the protein expression on knockdown often is not completely lost and the effect of siRNA knockdown is always inconsistent between experiments (Moore *et al.*, 2010). Although, the knockdown transfection experiments were performed at higher and lower concentrations, data were inconclusive, and it did not appear to increase the transfection efficiency and were eventually abandoned due to time constraints. It is feasible that further optimisation for transfection procedure may increase the knockdown efficiency. It is also possible that these are the maximum efficiencies of the tested siRNA sequence which generated a poor transfection (Jagla *et al.*, 2005; Safari, Barouji and Tamaddon, 2017). To overcome this issue, considering another form or sequence of siRNA, chemically modified, may be useful to reduce the off-target effects (D., 2013; Safari, Barouji and Tamaddon, 2017). Additionally, gene knockout by clustered regularly interspaced short palindromic repeats (CRISPRs)/CRISPR associated 9 (Cas9) system is another alternative technique used for gene editing by offering complete elimination of gene function which may help to resolve the issues with transient siRNA knockdown (Bogdanove and Voytas, 2011; Davis and Ph, 2013). In the light of the findings herein, it has been shown in a recent publication that GNAS mutations were associated with MAPK mutations within the serrated pathway in mucinous adenocarcinoma CRCs (Liu et al., 2017). This may indicate that GNAS mutation may drive the progression of CRC in association with MAPK pathway in mucinous adenocarcinoma CRCs. Furthermore, it has been shown previously that GPCRs and their related proteins are involved in cell proliferation and development in various cancers due to the altered expression and functions of these receptors. Recently, different reports showed that GPCRs are involved in tumour progression and metastasis (O'Hayre, Degese and Gutkind, 2014; Liu et al., 2016). Mutations in genes encoding these receptors have been observed in different types of cancers indicating that certain

GPCRs, including GNAS, can act as oncogenes depending on other genes (O'Hayre, Degese and Gutkind, 2014). It has been previously reported that the cAMP regulatory cascade was implicated in growth control and differentiation in thyroid cells along with these receptors (Dhanasekaran, Heasley and Johnson, 1995; Dorsam and Gutkind, 2007). Consequently, this increases the possibility that activated GNAS mutants can act as oncogenes only in a few types of tissues in which cAMP stimulates proliferation and motility.

In summary, the results obtained in this study indicate that GNAS does not affect cell motility and cell proliferation in CRC cells. These are novel observations not previously reported however further repeats of these experiments using alternative siRNA sequence were not performed due to time constraints and are necessary. Therefore, further validations of this study are required before firm conclusions can be drawn.

6 General Discussion

6.1 Introduction

CRC is a heterogeneous disease derived from different genomic alterations through various molecular mechanisms which are involved in the formation and development of CRC (Armaghany *et al.*, 2012; Colussi *et al.*, 2013). These molecular pathways, namely CIN, MSI, and CIMP, have been established to have a critical role in tumour initiation to metastatic CRC (mCRC) (Pino and Chung, 2010; Geiersbach and Samowitz, 2011; Arends, 2013; Mojarad *et al.*, 2013). However, identifying specific molecular markers involved in mCRC may help to improve the overall survival of patients with this disease. The main aim of this thesis was to study the molecular biomarkers of CRC through screening genetic and epigenetic changes and investigate their role in CRC to expand our understanding of the molecular events involved in tumour metastasis. Molecular Profiling using HRM was used to genotype specific CRC molecular alteration in association with lymphatic and distant metastasis CRC. Furthermore, *ctDNA* obtained from plasma pre- and post-surgically of CRC patients was investigated to determine its potential role for surgical clearance. Finally, regulation of *GNAS* expression was investigated using in vitro models to determine its role in CRC cell proliferation and motility.

6.2 Overview and Main Findings

6.2.1 Mutant *KRAS, PIK3CA, SMAD4, PTEN* and *P16* promoter methylation are associated with Lymph node involvement, local recurrence or distant metastasis CRC

Molecular alterations including mutations, MSI, and promoter methylation play a significant role in CRC progression (McGivern *et al.*, 2004; Poynter *et al.*, 2008). These molecular events are consequential in CRC, but the underlying mechanisms remain unclear. Given the molecular subtypes of these events, these alterations can be useful markers in terms of CRC classification, which may also help to identify tumour stage. It was aimed to provide molecular profiling in CRC using HRM-PCR technology and explore the molecular associations to lymph node metastasis, advanced CRC and distant metastatic.

This was initially performed by QMC-PCR and standard PCR followed by HRM to screen 82 CRCs for the mutational status of panel of genes, *KRAS*, *PIK3CA*, *BRAF*, *PTEN*, *TP53*, and *SMAD4*, MSI status, *MLH1* and *P16* promoter methylation, which are involved in CRC carcinogenesis. Generally, the overall genomic profiling of CRC cases was consistent with published data (Table 3-1). However, the frequency of *TP53* mutations as well as *P16* promoter methylation were relatively low, with 2 fold difference and 3-12 fold difference respectively, in comparison to those in the literature. Additionally, although the frequency of *SMAD4* mutations were in range with published data, but it was detected in only two CRC cases (2%). The low frequency of mutations or methylations within tumour samples may indicate to the failure of the screening methods (QMC-PCR or Standard PCR followed by HRM) used in this study to detect mutation or methylation in tumour samples may thus be referred to as technical artefact resulting from the limit of the detection (LOD) of the methods. It is possible that these tumour samples contained low level of mutation or methylation that cannot be detected by our methods. It has been reported previously that HRM is able to detect 5% of mutant alleles and showed greater

sensitivity in comparison to direct sequencing (Ibrahem *et al.*, 2010; Fadhil *et al.*, 2012). This may agree with the possibility of missing some variant genes presented at low levels in tumour samples. Therefore, further validation with more sensitive methods, such as NGS, for gene mutation and methylation screening is suggested in order to confirm the presence or absence of mutations and promoter methylation within the tumour samples. Furthermore, as expected, *BRAF* and *KRAS* mutations were exclusive, whereas *KRAS* and *PIK3CA* mutations coexisted. The primary objective of this study was to investigate the association between these molecular alterations and clinicopathological features of the patients. The statistical analysis showed significant association between mutant *KRAS*, *PIK3CA*, *PTEN* and *SMAD4* genes, and *P16* promoter methylation and clinicopathological parameters including lymph node metastasis, advanced disease, local recurrence and distant metastasis. It was observed a significant association between *PIK3CA* mutations and local recurrent CRC. Similar data have previously reported showing a significant association between *PIK3CA* mutations and increased local recurrence rate (He *et al.*, 2009). This may support the finding obtained in this study indicating that CRC exhibiting *PIK3CA* mutation are more likely to develop local recurrence after tumour removal. However, our study is limited by low number of cases with local recurrences (n=5/82, 6%) in comparison to those included in published data and the conclusion for this association need to be validated in larger number of samples (Kato *et al.*, 2007; Abubaker *et al.*, 2008; He *et al.*, 2009; Ogino, Nosho, Gregory J Kirkner, *et al.*, 2009; Fariña Sarasqueta *et al.*, 2011; Liao *et al.*, 2012; Day *et al.*, 2013; Manceau *et al.*, 2015). *KRAS* mutations, on the other hand, were frequently detected in patients developed distant metastasis. This association have been previously identified in different studies (Li *et al.*, 2011; Modest *et al.*, 2011; Mannan and Hahn-Strömberg, 2012; Boutin *et al.*, 2017). Several reports showed that *KRAS* mutations are associated with metastatic CRC and revealed that mutant *KRAS* in primary CRC was concordant with metastatic CRC of same patients (Zauber *et al.*, 2003; Artale *et al.*, 2008; Pritchard and Grady, 2011). This is indicative that *KRAS* mutations are maintained throughout

CRC carcinogenesis and primary cells with mutant *KRAS* may metastasize to distant tissues. The findings suggest that *KRAS* mutations may be potential predictive and prognostic biomarker for CRC patients. Although S*MAD4* mutations were detected in only 2 CRC cases, both cases found to have developed distant metastasis. The correlation between S*MAD4* mutations and metastatic CRC have been observed previously and published data showed S*MAD4* mutations were identified in both primary CRC and distant metastases in the same patients (Miyaki *et al.*, 1999). This may suggest that primary CRCs acquiring S*MAD4* mutations may develop to distant metastasis. With that assumption, mutant S*MAD4* in primary tumours may be used as predictive and prognostic biomarker for metastatic CRC. However, due to the low frequency of S*MAD4* mutations identified in this current study further validation is required to confirm the presence of S*MAD4* mutations in distant metastasis in CRC patients. Furthermore, mutant *PTEN* was significantly correlated with involvement of the lymph node and distant metastatic, which indicates that *PTEN* mutations may be involved in the transformation to metastasis stage in CRC. Other studies have shown that *PTEN* gene mutations are significantly associated with locally advanced or metastatic CRCs (Sawai *et al.*, 2008; X. Li *et al.*, 2009; Lin *et al.*, 2011; Molinari and Frattini, 2014). Mutant *PTEN* has been also associated with the absence or low expression of *PTEN* in advanced stage tumours, suggesting a link between *PTEN* dysregulation and CRC aggressive phenotype (X. Li *et al.*, 2009; Lin *et al.*, 2011; Molinari and Frattini, 2014). However, the precise relationship between *PTEN* mutations and their expression in CRC has not been fully investigated and requires further validation.

P16 promoter methylation is one of the main elements of a classic panel for the detection of CIMP in CRC (Lee *et al.*, 2008; Mitomi *et al.*, 2010; Mojarad *et al.*, 2013). Loss in expression of *P16* tumour suppressor gene has been reported to be a result of *P16* promoter methylation, which possibly contributes to the development, progression and invasion in CRC tumours. Additionally, aberrant activation of *P16* by methylation has

been found associated with advanced tumours and poor survival (Bai *et al.*, 2004; Karamitopoulou *et al.*, 2010; Mitomi *et al.*, 2010; Nakayama *et al.*, 2011). The aim of this analysis was to investigate the association between *P16* promoter methylation and metastatic CRC. HRM-PCR analysis showed aberrant methylation of the *P16* promoter region is only detected in 4% of CRC tumours. Aberrant *P16* methylation was significantly associated with Dukes' staging, most were detected in Dukes' stage C and D tumours; similar findings have been previously reported (Yi *et al.*, 2001). Similarly, aberrant *P16* methylations were associated with other mutant genes, *BRAF* and *PTEN*, and both were also associated with Dukes' staging C and D. It has been shown previously that CRC patients with CIMP-positive driven by *BRAF* mutations demonstrated the worse outcome which may indicate that this molecular interaction may be implicated in advanced disease (Samowitz *et al.*, 2005; Kim *et al.*, 2009; Ogino, Nosho, Gregory J. Kirkner, *et al.*, 2009). Moreover, both *P16* promoter methylation and *PTEN* mutations have been found to be associated with advanced tumours, it may suggest that these molecular events may contribute to metastatic stage. The association between these molecular alterations (i.e. *PTEN* mutations with *P16* promoter methylation) and their role into the development to metastatic CRC need to be further confirmed by analysis approaches, such as Hofree-NBS, ccpwModel, and xGeneModel, to identify genetic sub-types within cancer type and relate the sub-types to clinical outcomes and pathologic features of patients (Hofree *et al.*, 2013; Kim, Sael and Yu, 2015; Zhang, Flemington and Zhang, 2018).

MSI status was tested in CRC cases to determine the mutational frequency and potential correlation with *BRAF* mutations and *MLH1* promoter methylation in CRCs. MSI-H is found more frequently in LS tumours than in sporadic CRC tumours (Grady and Carethers, 2008; Sinicrope and Sargent, 2009). In sporadic CRC, MSI arises from promoter methylation of one of MMR genes, such as *MLH1*, and found to be strongly correlated with mutant *BRAF* (McGivern *et al.*, 2004; Poynter *et al.*, 2008). Furthermore,

the *BRAF* mutation is found as an early step in CIMP tumours, which are strongly associated with *MLH1* hypermethylation (Parsons *et al.*, 2012). *MLH1* hypermethylation is commonly found in sporadic CRC with MSI, rather than HPNNC MSI, with loss of expression and no aberrant mutation detected in MMR genes (Raedle *et al.*, 2001; Li *et al.*, 2013). Thus, it was of interest to screen for MSI-H status together with *BRAF* mutation and *MLH1* promoter methylation analysis in order to differentiate between Lynch syndrome and sporadic CRC. This study revealed 13% of CRC patients showed MSI-H. Statistical analysis showed that the *BRAF* mutation is significantly associated with MSI-H, which is consistent with previously published data (Jensen *et al.*, 2008; Capper *et al.*, 2013; Thiel *et al.*, 2013).. This data also agrees with findings obtained by our collaborator group, Edinburgh Molecular Pathology Node, showing significant association of MSI with *BRAF* mutations (Susanti *et al*., 2018). *MLH1* promoter methylation was analysed by HRM-PCR methods to identify the promoter status in 82 sporadic CRC tumours. This was performed by melting analysis of DNA products previously amplified by PCR. The methylation status was detected by spike-in methylated DNA into unmethylated DNA (commercially available controls, EpiTech Qiagen) to obtain ratios of 0%, 10%, 50%, and 100% methylations and samples with 50% or higher methylation were regarded as methylated tumours. *MLH1* promoter methylation was detected in 19% of total CRC cases, 45% of MSI-H CRCs and 15% of MSS tumours. The possible explanations for MSI in the group with no detected *MLH1* methylation are that might be a result of germline mutation in one of the MMR genes or partial methylation (Methylation around 50%) of *MLH1* promoter. In addition, *MLH1* methylation was correlated with MSI-H CRCs exhibiting *BRAF* mutations than MSI-H CRCs with *BRAF* wild-type. This study suggests that the frequency of *MLH1* promoter methylation was significantly associated with MSI-H CRC with *BRAF* mutations which confirm other data that tumours with this subtype are sporadic CRC.

The next step was to design an assay to test for *BRAF* mutation and MSI to help identify sporadic tumours. This test was performed in Edinburgh cohort (88 CRC cases) which is previously tested MSI and MSS by IHC. MSI test was performed by screening 5 markers using close-tube test in a single run PCR. All MSI or MSS cases were categorised correctly by HRM-PCR single-tube analysis. *BRAF* gene mutational status was analysed to further confirm the association with MSI-H CRCs. The findings confirmed association between *BRAF* mutations and MSI with loss of expression in one of *MLH1*, MSH2, PMS2 or MSH6, which are concordant with published data (Seppälä *et al.*, 2015). It has been showed previously that *BRAF* mutation may arise from loss of expression or promoter methylation in one of MMR genes in sporadic CRC (Vilkin *et al.*, 2009; Li *et al.*, 2013). This suggests that all cases in both cohorts with mutant *BRAF* and MSI-H are sporadic CRCs. MSI testing includes a panel of 5 PCRs which are performed in a closed-tube run using single cycling program and HRM is performed at the end of the run. This method is simple and fast as well as eliminates the risk of laboratory contamination with PCR products. Therefore, implying this test would be useful tool in diagnostic pathology lab for Lynch syndrome and sporadic CRC to categorise patients into groups suitable for treatment with 5-Fluorouracil based therapy or immunotherapy (Bertagnolli *et al.*, 2009; Des Guetz, Schischmanoff, *et al.*, 2009; Roth *et al.*, 2010; Llosa *et al.*, 2015).

In conclusion, the findings in this study are mostly consistent with previous published data. This study have identified some molecular alterations (*KRAS*, *PIK3CA*, *PTEN*, or *SMAD4* mutations and *P16* promoter methylation) in association with lymph node metastasis, advanced CRC, and metastasis recurrence. These findings may indicate that primary CRCs acquiring mutant *KRAS*, *PIK3CA*, *PTEN*, *SMAD4* or *P16* promoter methylation may develop into metastatic CRC. *P16* promoter methylation and *PTEN* mutations were significantly correlated and associated with a late stage of CRC tumours according to Dukes' staging and lymph node involvement. Therefore, further investigations of these molecular associations are required to fully understand their role

in metastatic CRC cells. Furthermore, this study confirms that *MLH1* promoter methylation is significantly associated with MSI-H CRCs with *BRAF* mutation and this molecular subgroup can be used as a marker to differentiate between LS and sporadic CRC tumours.

6.2.2 Analysis of *CfDNA/CfmiRNA* May Be a Useful Biomarker in CRC Patients as Surgical Clearance

During metastasis process, tumour cells, also called *cfDNA,* are detached and released into the blood circulation to migrate forming secondary tumor at distant site or organ. These cells have potential biomarkers for tumor screening since are released circulatory during necrosis or apoptosis. *cfDNA* is a reflection of many genetic events occurring in tumours. Considering tumour tissue or biopsies for molecular investigation have been challenging and may be limited in many studies. Performing biopsies or resection for patients is considered painful and invasive, which may result in complications for the patients (Coppedè, Lopomo, *et al.*, 2014). Heterogeneity is also one of the main limitations for analysing biopsies for patients due to the amount of tumour cells present in each biopsy and may be dependent on the quantity of tumour which may affect the molecular analysis (Gerlinger *et al.*, 2012; Tellez-Gabriel *et al.*, 2016) However, it is beneficial to sample the blood circulation for a fresh source of DNA. Recently, the analysis of *cfDNA* in blood has been developed as a non-invasive procedure for the diagnosis and prognosis in many cancers (Diaz and Bardelli, 2014; Rapisuwon, Vietsch and Wellstein, 2016). So, it was hypothesised that such a non-invasive and sensitive method may overcome these limitations and provides more precise biomarker for surgical clearance and residual tumour.

The objective was to introduce a sensitive method to use mutant tumour-derived *cfDNA* as a non-invasive diagnostic marker for colorectal cancer and investigate the use of *cfDNA*/*cfmiRNA* as markers for surgical clearance. *CfDNA* has been shown to carry a wide range of variant alleles which are low in frequency in the majority of cancer patients (Mouliere et al., 2013b). It has been shown previously that *cfDNA* variant alleles can be present at very low percentages (0.01%) in a proportion of patients, which is challenging for *cfDNA* analysis (Chaudhuri *et al.*, 2015). It is believed that metastasis and large tumour size may contribute to increased *cfDNA* levels in plasma due to increases in

tumour cell death rate at late stages of the disease, thereby increasing the variant allele percentage to a large degree as well as the quantity of *cfDNA* that can be extracted (Qin *et al.*, 2016). This study revealed important information about the characteristics of *cfDNA* and successful methods for extraction and analysis. In this study, 1 ml of plasma was processed for *cfDNA* extraction per patient using Qiagen circulating nucleic acid kit (CNAK). CNAK generated sufficient, high quality *cfDNA* in comparison to Qiagen Blood mini kit which allows lower volume of plasma for extraction (200µl of plasam). This indicates that the use of a higher plasma volume would generate a higher total *cfDNA* yield for analysis.

This study was performed using nested full-COLD-PCR for the detection of mutant *KRAS* and *BRAF* in *cfDNA*. COLD-PCR followed by HRM is sensitive and simple method to detect low variant alleles in comparison to many available techniques. The optimised protocol achieved a LOD of 0.75% of variant alleles. In this study, using full-COLD-PCR for *cfDNA* analysis achieved 77% sensitivity and 87% specificity for *KRAS* mutations and 100% sensitivity and specificity for *BRAF* mutations, when compared to biopsy (FFPE) tumour mutation analysis. Although this method is sufficient for diagnostic research, it was expected to have a greater specificity and sensitivity regarding the high accuracy of the test. Various studies have reported inconsistent results for the concordance between *cfDNA* and FFPE tissues using different platforms commonly used for plasma analysis such as PCR-based methods including Digital-PCR, ARMS, and NGS (Diaz and Bardelli, 2014; Kuo *et al.*, 2014; Perrone *et al.*, 2014; Thierry *et al.*, 2014; Thress *et al.*, 2015; Hao *et al.*, 2017; Xu *et al.*, 2017). A few considerations regarding the plasma volume, low variant *KRAS* mutations in *cfDNA*/FFPE samples, metastasis/unknown primary tumour harbouring same mutations may be the possible causes of these discrepancies between both methods. Increasing the volume of plasma to 10ml would generate higher *cfDNA* yield and, therefore, increases the mutant copy number in extracted *cfDNA*, allowing higher concentration of *cfDNA* template in PCR reactions which would increase

the sensitivity of the method. Moreover, it is possible that is a failure of the method to detect low variant *KRAS* mutations in either *cfDNA* or FFPE tissues. Using more sensitive techniques such as ARMS, Digital PCR or NGS would be suggested to confirm the concordance between *cfDNA* or FFPE tissues. Furthermore, this study is lack of survival rate as because the follow up analysis was only a maximum of six days after surgery. Including 6-month to 5 years follow-up for the patients is required to confirm recurrence rates and these results. Furthermore, pre-and post-surgical analysis revealed that the mutation disappeared after surgery in more than half of the patients. This may be indicative of the potential role of utilising *cfDNA* as biomarker for surgical clearance. However, it is difficult to make a conclusion based on the small number of patients and markers, so further analysis at a 6-month follow up, larger number of patients and panel of targeted genes is recommended to confirm the potential of *cfDNA* as a biomarker.

Furthermore, in this study we have also devised a protocol for *cfmiRNA* analysis using Q-PCR by analysing the expression of a group of miRNAs (miR-20a, miR-21, miR-29a, miR-31, and miR-92a) in patients' plasma. Investigating *cfmiRNA* signatures have been of interest in clinical research due to its stable state and easy excess into the blood circulation (Kanaan *et al.*, 2012; Ogata-Kawata *et al.*, 2014; Ristau *et al.*, 2014). Recent studies have explored the role of *cfmiRNA* as predictive marker and showed a significant decrease of some miRNAs expression post-operatively (Ogata-Kawata *et al.*, 2014; Ristau *et al.*, 2014; J. Li *et al.*, 2015; Jo *et al.*, 2017). However, it was of interest to include *cfmiRNA* analysis for pre- and post-operative samples in this study to investigate the potential role of *cfmiRNA* for surgical clearance. The overall analysis revealed that more than half the patients (70%) showed over-expression of one or more of candidate miRNAs in pre-surgical samples and almost half (42%) of these showed a decrease in the expression of *cfmiRNAs* in post-operative samples. The fluctuation in expression levels in some samples after surgery may be a result of inflammation occurring after surgery or impact of a mixture of cancer-specific miRNAs derived from other organs

(Bravo-Egana *et al.*, 2012; Penman, Kaufman and Daniels, 2014; Poon *et al.*, 2017). Overall, this study suggests that *cfmiRNA* may be a potential biomarker in CRC patients; however, a larger study with more miRNAs is required.

In summary, the *KRAS* mutation signal was observed in more than half of the patients tested in after surgery, indicating that analysing *cfDNA*/*cfmiRNA* for surgical clearance in CRC may be is an useful diagnostic tool for prognostic use. This should be further investigated using a larger cohort and more markers.

6.2.3 *GNAS* does not affect Cell Proliferation and Motility in CRC

GNAS1 is a member of the GPCR (G-protein-coupled receptor) family which is involved in many physiological events (Dorsam and Gutkind, 2007; O'Hayre, Degese and Gutkind, 2014). *GNAS* mutations have been detected in many cancers and found to promote cell proliferation and migration (Lyons *et al.*, 1990; Landis *et al.*, 1989; Weinstein *et al.*, 1991). The precise role of *GNAS1* in CRC has not yet been fully investigated and has only been addressed in relatively few publications until now (Walther et al., 2014). Therefore, this study investigated the role of *GNAS1* in the regulation of cell motility in CRC cell lines. *GNAS* knockdown was assessed using siRNA to investigate the role of *GNAS* in regulating cell proliferation and motility in CRC cell lines, RKO and SW620 (high *GNAS* expressing cell lines). The findings herein showed that *GNAS* knockdown has no effect on either cell proliferation or motility in CRC. However, it is difficult to withdraw a conclusion from data obtained in this study since the GNAS protein expression on knockdown was not completely lost. Further validations of this data by using different siRNA sequence or alternative technique such as CRISPR/Cas 9 system may help to reduce the off-target effects and eliminate the function of protein expression. Based on findings obtained herein, *GNAS* knockdown has no effect on either cell proliferation or motility in CRC. It has been shown recently that proportion of mucinous adenocarcinoma significantly associated with *GNAS* mutant CRC in correlation with MAPK pathway and serrated morphology (Liu *et al.*, 2017). This may suggest that *GNAS* mutations in interaction with MAPK pathway may drive progression in CRCs. Moreover, it has been shown previously that altered expression and functions of GPCRs and genes encoding these receptors are involved in tumour progression and metastasis of various cancers, indicating that certain GPCRs, including *GNAS*, can act as oncogenes depending on other genes (O'Hayre, Degese and Gutkind, 2014; Liu *et al.*, 2016). Moreover, the cAMP regulatory cascade has been shown to be involved in growth control and differentiation in thyroid cells along with these receptors (Dhanasekaran, Heasley and Johnson, 1995;

Dorsam and Gutkind, 2007). Therefore, *GNAS* activity may be dependent on other genes in order to serve as an oncogene in certain tissues.

Taken together, these results showed that *GNAS* protein does not induce cell proliferation and motility in CRC. However, this finding should be further investigated to ensure that this is not due to poor transfection efficiency and thus confirming the role of GNAS signalling in CRC cells.

6.2.4 Conclusion

This work addressed the molecular alterations occurring during CRC development and their role in metastatic CRC. It has been shown previously that these genomic changes can influence CRC progression and may be useful for diagnosis and prognosis. This study has identified several molecular alterations, *KRAS, PIK3CA, PTEN, SMAD4* mutations and *P16* promoter methylations, in association with advanced tumours and metastatic CRC. Further analysis for these associations in larger cohort and validation with more sensitive methods is required to confirm these results and their role in metastatic CRC. It was shown that *P16* methylation and *PTEN* mutation may coexist in advanced local tumours, but their underlying mechanisms in CRC progression remain unclear and require further investigation. This study also confirmed that MSI screening coupled with *BRAF* detection and *MLH1* promoter methylation analysis is a sensitive and simple method to differentiate between LS and sporadic CRC. Furthermore, due to the lack of sensitive procedure, such as tissue sampling and screening technique, the precise value of these events in clinical practice remain challenging. However, *cfDNA* and *cfmiRNA* were successfully detected in plasma, demonstrating their possible use as potential biomarkers for surgical clearance. The sensitivity and specificity of *cfDNA* analysis may be sufficient for clinical analysis, but 100% or nearer of sensitivity and specificity should be achieved. Further investigation to increase the sensitivity and specificity of the method is required to confirm the potential role of *cfDNA* in clinical setting. Additionally, the *GNAS* mutation has been detected in various cancers, but the expression of *GNAS* is rarely investigated in the context of CRCs and its role in CRC still unclear. It has been shown that *GNAS* has no influence on CRC cell proliferation and motility in RKO and SW620 cell lines. However, these data are based on 50% knock-down, which may not be accurately representing the role of *GNAS* in CRC. Therefore, further investigation with complete knock-down for *GNAS* expression is required to confirm whether *GNAS* can induce cell proliferation and motility in CRC cell lines.

6.2.5 Future Perspectives

This study revealed potential markers can be used in the diagnostic screening in pathological labs. Further validation of these markers in larger cohort and different tumour types, such as breast, lung and pancreatic cancers, are required.

The present study has identified genetic alterations (*KRAS, PIK3CA, PTEN, SMAD4* mutations and *P16* promoter methylations) in association with lymphatic metastasis, advanced disease or metastatic CRC. This work also revealed significant association between mutant *PTEN* and *P16* promoter methylation in correlation with lymphatic metastasis or advanced CRC. This should be confirmed by more sensitive method for mutation analysis and bioinformatics of identified markers, such as *PTEN* and *P16* promoter methylation, in metastatic disease to investigate their functional role in CRC progression. Furthermore, this study has also confirmed the significance of screening MSI, *BRAF* and *MLH1* promoter methylation in CRC classifications. Larger study including bigger number of samples and genes are suggested to further validate this result. It would be also of interest to investigate these findings in different types of CRC tumours, such Lynch Syndrome, Primary and Metastatic CRCs, to gain deeper understanding of this molecular alteration throughout CRC developments.

The investigation of *cfDNA*/*ctmiRNA* in CRC patients revealed potential biomarkers for surgical clearance. This should be validated with more sensitive PCR-based assays used for plasma analysis, such as ARMS, to increase the sensitivity and specificity of the method. Furthermore, larger study with bigger number of molecular markers in CRC is required to confirm the potential role of *cfDNA* for surgical clearance in CRC patients. Moreover, circulating tumour cells (CTC) may be defined as metastasis-derived cells that are present in blood circulations. Identifying these cells may be useful adjunct to therapeutic assessment, including surgical removal, of tumour response to therapy.

The present study has demonstrated the role of *GNAS* in CRC cells proliferation and motility. This could be artefact results obtained from poor transfection which effected *GNAS* expression in both cell lines. Further optimisation for the transfection experiment with gene knock-out methods (CRISPR) is required to eliminate the function of *GNAS* expression. These investigations were established in two cell lines and confirmation should include more CRC cell lines to further validate *GNAS* effect on CRC progression.

7 References

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8 Appendix

Table 8–1: List of Primers

Gene	*Primer Sequence*	*Amplicon size (bp)*
KRAS Exon2 OUTER	*Forward 5'-GCCTGCTGAAAATGACTGAA-`3*	*114*
	Reverse 5'-TTGGATCATATTCGTCCACAA-3'	
KRAS Exon2 INNER	*Forward 5'-CCTGCTGAAAATGACTGAATATAA-3'*	*112*
	Reverse 5'-TGGATCATATTCGTCCACAAAA-3'	
KRAS Exon3 OUTER	*Forward 5'-CCAGACTGTGTTTCTCCCTTC-3'*	*152*
	Reverse 5'-AAAGAAAGCCCTCCCCAGT-3'	
KRAS Exon3 INNER	*Forward 5'-TGTGTTTCTCCCTTCTGAGGA-3'*	*145*
	Reverse 5'-AAGAAAGCCCTCCCCAGT-3'	
KRAS Exon 4 OUTER	*Forward 5'-AGACACAAAACAGGCTCAGGA-3'*	*160*
	Reverse 5'-TTGAGAGAAAAACTGATATATTAAATGAC-3'	
KRAS Exon 4 INNER	*Forward 5'-GACACAAAACAGGCTCAGGACT-3'*	*145*
	Reverse 5'-CAGATCTGTATTTATTTCAGTGTTA-3'	
BRAF Exon11 OUTER	*Forward 5'-TGTTTGGCTTGACTTGACTTT-3'*	*189*
	Reverse 5'-CTTGTCACAATGTCACCACATTACATA-3'	
BRAF Exon11 INNER	*Forward 5'-GACGGGACTCGAGTGATGAT-3'*	*135*
	Reverse 5'-TGTCACAATGTCACCACATTACA-3'	
BRAF Exon15 OUTER	*Forward 5'-ATCTACTGTTTTCCTTTACTTACTACAC-3'*	*205*
	Reverse 5'-CAGCATCTCAGGGCCAA-3'	
BRAF Exon15 INNER	*Forward 5'-TGTTTTCCTTTACTTACTACACCTCA-3'*	*143*
	Reverse 5'-CCACAAAATGGATCCAGACA-3'	
SMAD4 Exon9 OUTER	*Forward 5'-GCTCCTGAGTATTGGTGTTCC-'3*	*183*
	Reverse 5'- TGCTCTCTCAATGGCTTCTG-'3	
SMAD4 Exon9 INNER	*Forward 5'- TCCTTCAAGCTGCCCTATTG-'3*	*108*
	Reverse 5'- TCAATGGCTTCTGTCCTGTG-'3	
PTEN Exon 3 OUTER	*Forward 5`- TCATTTTTGTTAATGGTGGCTTT-`3*	*182*
	Reverse 5`- AACTCTACCTCACTCTAACAAGCAGA-`3	
PTEN Exon 3 INNER	*Forward 5`- GGCTTTTTGTTTGTTTGTTTTG-`3*	*158*
	Reverse 5`- CCTCACTCTAACAAGCAGATAACTTTC-`3	
PTEN Exon 8 OUTER	*Forward 5`- GCGTGCAGATAATGACAAGG `-3*	*201*
	Reverse 5`- TCAAGCAAGTTCTTCATCAGC -`3	
PTEN Exon 8 INNER	*Forward 5' CGTGCAGATAATGACAAGGAA-'3*	*100*
	Reverse 5'- AATTTGGAGAAAAGTATCGGTTG-'3	
PIK3CA Exon 20 OUTER	*Forward 5'-TGAGCAAGAGGCTTTGGAGT-3'*	*201*
	Reverse 5'-CCTATGCAATCGGTCTTTGC-3'	
PIK3CA Exon 20 INNER	*Forward 5'-GCAAGAGGCTTTGGAGTATTTC-3'*	*115*
	Reverse 5'-TTTTCAGTTCAATGCATGCTG-3'	
PIK3CA Exon 9 OUTTER	*Forward 5'-CTGTGAATCCAGAGGGGAAA-3'*	*197*
	Reverse 5'-GCACTTACCTGTGACTCCATAGAA-3'	
PIK3CA Exon 9 INNER	*Forward 5'-AAGGGAAAATGACAAAGAACAG-3'*	*103*
	Reverse 5'-CACTTACCTGTGACTCCATAGAAA-3'	
TP53 Exon6 outer	*Forward 5'- AGGCCTCTGATTCCTCACTGAT-3'*	*187*
	Reverse 5'- ACCCTTAACCCCTCCTCCCA-`3	
TP53 Exon6 Inner	*Forward 5'-CCTCTGATTCCTCACTGATTGC-3'*	*181*
	Reverse 5'-CTTAACCCCTCCTCCCAGAG-3'	
TP53 Exon8 Outer	*Forward 5'-CTCTTGCTTCTCTTTTCCTATCC-3'*	*192*
	Reverse 5'-ACCGCTTCTTGTCCTGCTTG-3'	
TP53 Exon8 Inner	*Forward 5'-TTGCTTCTCTTTTCCTATCCTGA-3'*	*186*
	Reverse 5`- GCTTCTTGTCCTGCTTGCTT-3'	

Table 8–2: List of sequences of MSI markers

Marker	*Sequence*	*Amplicon size (bp)*
BAT25	*F:5′-TCGCCTCCAAGAATGTAAGTG-3′* *R:5′-TGGTTACCACACTTCAAAATGAC-3′*	*149*
BAT25 (New)	*F: 5′-gtgattctctaaagagttttgtgttttgt-3′* *R: 5′-tggctctaaaatgctctgttctcaa-3′*	*85*
BAT26	*F:5′-TTGGATATTGCAGCAGTCAGAG-3′* *R:5′-TTTAGCTCCTTTATAAGCTTCTTC-3′*	*140*
BAT26 *(New)*	*F: 5′-gcccttaacctttttcaggtaa-3′* *R: 5′-catttttttaaccattcaacatttttaa-3′*	*79*
BCAT25	*F:5′-TCTGTAATGGTACTGACTTTGCT-3′* *R:5′-AACTTAACACTACGAGAGACTTAAAA-3′*	*102*
NR21	*F:5′-TCGCTGGCACAGTTCTATTTT-3′* *R:5′-CCGCATTCACACTTTCTGGT-3′*	*122*
NR22	*F:5′-TTCGCACTGAGCACATCAC-3′* *R:5′-CCAAGACAAAACTTCCAGACAA-3′*	*120*
NR24	*F:5′-CCTCCTGACTCCAAAAACTCT-3′* *R:5′-AGATTGTGCCATTGCATTCC-3′*	*119*
MYB *(New)*	*F: 5′-cttacacctctgggctttatagctt-3′* *R: 5′-caaaaatgtaaaacacgatatgataaagca-3′*	*75*
EWSR1 *(New)*	*F: 5′-taacaatgttcatggttgtgatgt-3′* *R: 5′-tgactctttataaacatttggaatttta-3′*	*70*

Table 8–3: List of primers sequence of *MLH1* and *P16* promoter methylation genes

**Both primers were published previously in (Li et al., 2014)*

Promoter Gene	*Sequence*	*Number of CpG-sites/Amplicon size (bp)*
*MLH1**	*Forward:5'-TTTTTTTAGGAGTGAAGGAGG-3'* *Reverse: 5'- AACRCCACTACRAAACTAAA-3'*	*15/110*
*P16**	*Forward:5'-GGAGTTTTCGGTTGATTGGTTGGTT-3'* *Reverse:5'- AACAACGCCCGCACCTCCTCTA-3'*	*5/69*

Table 8–4: List of primers used in cfDNA analysis

KRAS exon 2 Outer primers (92bp) (Mack et al. 2016)	
Forward	*5' - TGAATTAGCTGTATCGTCAAGGCACT- 3'*
Reverse	*5' - TTATAAGGCCTGCTGAAAATGACTGAA- 3'*
KRAS exon 2 Inner primers (62bp)	
Forward	*5' - TGA ATA TAA ACT TGT GGT AGT TGG - 3'*
Reverse	*5' - GCT GTA TCG TCA AGG CAC TCT - 3'*
BRAF exon15 outer primers (105bp)	
Forward	*5′ TTCATGAAGACCTCACAGTAAA 3′*
Reverse	*5′ACAAAATGGATCCAGACAACT 3′*
BRAF exon15 inner primers (70bp)	
Forward	*TTCATGAAGACCTCACAGTAAA*
Reverse	*GACCCACTCCATCGAGAT*

Table 8–5: Details of primers for the squirrel primers

Target	*Primer sequence*	*Amplicon length*	*Estimated primer Tm (F/R)*	*Primer Length (F/R)*
KRAS exon 2 seq	*F: 5′ AACTGACTAAACTAGGTGCCACGTCGTGAAAGTCTGACAATGAATATAAACTTGTGGTAGTTGG 3′* *R: 5′ TAGACTCCTGATCCCTTCATTGCCCTGCATCTGACACGCAGCTGTATCGTCAAGGCACTCT 3′*	*142bp*	*79.2/84.6*	*64/61*
Useq	*F: 5′ CTGACTAAACTAGGTGCCACGT 3′* *R: 5′ TAGACTCCTGATCCCTTCAT 3′*	*N/A*	*63.6/58.0*	*22/20*

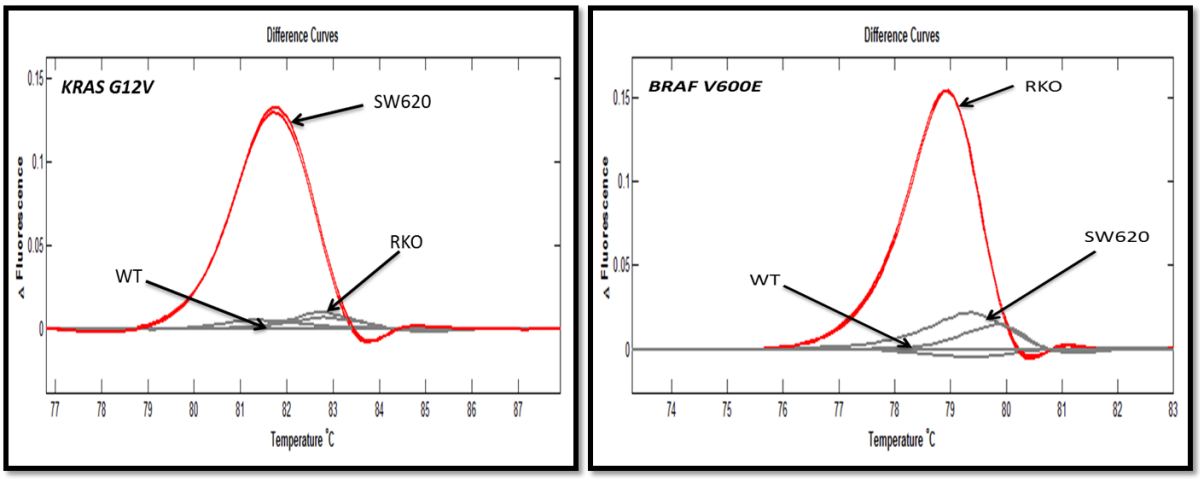

Figure 8-1: HRM-PCR screening analysis for specific mutations for Cell lines identification.

School of Medicine
Division of Cancer and Stem Cell
Teresa Pereira Raposo
D Floor, West Block QMC
Nottingham, NG7 2UH
UK

06.02.2017

Certificate

Order
By order of Teresa Pereira Raposo (School of Medicine) we were requested to perform a cell line authentication test. Following samples were examined:

Our sample number	Client sample name	
CL170130_006	SW480	
CL170130_007	DLD1	
CL170130_008	SW620 (A)	A - OLD, SW620 Cten KO
CL170130_009	HCT116	
CL170130_010	RKO	
CL170130_011	SW620 (B)	B - SW620 WT

Method:
Genetic characteristics were determined by PCR-single-locus-technology. 21 independent PCR-systems Amelogenin, D3S1358, D1S1656, D6S1043, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11, D7S820, D5S818, TPOX, D8S1179, D12S391, D19S433 and FGA were investigated (Promega, PowerPlex 21 PCR Kit).
In parallel, positive and negative controls were carried out yielding correct results.

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Steuernummer 114/116/00159

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DAkkS ISO 17025:2005 accredited

Results:

DNA-System	DNA-criteria SW480 CL170130_006	DNA-criteria DLD1 CL170130_007	DNA-criteria SW620 (A) CL170130_008	DNA-criteria HCT116 CL170130_009	DNA-criteria RKO CL170130_010
AM	X, X	X, Y	X, X	X, X	X, X
D3S1358	15, 15	17, 17	16, 16	12, 16, 17, 18, 19	12, 16, 17, 18, 19
D1S1656	13, 14	17.3, 19.3	13, 14	12, 13, 14, 15	12, 13, 14, 16.3
D6S1043	11, 12	11, 13	11, 12	12, 13, 14	12, 13, 14.1, 20
D13S317	12, 12	8, 11	12, 12	10, 12, 13	8, 10, 11, 12
Penta E	10, 10	7, 14	10, 10	13, 14	10, 13, 14
D16S539	13, 13	12, 13	9, 13	11, 12, 13, 14	11, 12, 13
D18S51	13, 13	11, 17	13, 13	16, 17, 18	11, 12, 16, 17
D2S1338	17, 24	17, 25	17, 24	16, 16	16, 16
CSF1PO	13, 14	11, 12	13, 14	7, 10	7, 8, 10
Penta D	9, 15	9, 14	9, 15	9, 13	9, 10, 13
TH01	8, 8	7, 9.3	8, 8	8, 9	8, 9, 10
vWA	16, 16	18, 19	16, 16	17, 22, 23	17, 21, 22, 23
D21S11	30, 30.2	29, 32.2	30, 30.2	29, 30	29, 30
D7S820	8, 8	10, 12	8, 9	11, 12	11, 12
D5S818	13, 13	13, 13	13, 13	10, 11	10, 11
TPOX	11, 11	8, 11	11, 11	8, 8	8, 8
D8S1179	13, 13	15, 15	13, 13	12, 13, 14	9, 12, 13, 14
D12S391	17, 17	19, 22	17, 17	17, 18, 20, 21, 22	15, 17, 18, 20, 21, 22
D19S433	13, 13	14, 16	13, 13	12, 13	12, 13, 14, 18.2
FGA	24, 24	22, 22	24, 24	18, 19, 21, 22, 23	18, 19, 22, 23

DNA-System	DNA-criteria SW620 (B) CL170130_011
AM	X, X
D3S1358	16, 16
D1S1656	13, 14
D6S1043	11, 12
D13S317	12, 12
Penta E	10, 10
D16S539	9, 13
D18S51	13, 13
D2S1338	17, 24
CSF1PO	13, 14
Penta D	9, 15
TH01	8, 8
vWA	16, 16
D21S11	30, 30.2
D7S820	8, 9
D5S818	13, 13
TPOX	11, 11
D8S1179	13, 13
D12S391	17, 17
D19S433	13, 13
FGA	24, 24

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Steuernummer 114/116/00159

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Summary:
The following cell lines could be detected in the online database of the DSMZ (http://www.dsmz.de/de/service/services-human-and-animal-cell-lines/online-str-analysis.html):

Our sample number	Client sample name	DSMZ name
CL170130_006	SW480	SW-480
CL170130_007	DLD1	DLD-1
CL170130_008	SW620 (A)	SW620 [SW-620]
CL170130_009	HCT116	HCT-116
CL170130_010	RKO	RKO can be present in the mixture but the main component is HCT-116
CL170130_011	SW620 (B)	SW620 [SW-620]

Dr. Burkhard Rolf
Director Forensic Services

Dr. Michaela Bosch
Project Manager DNA-Forensics

Cell_line-certificate_eng_V03_141211

Vorlage_Verwandtschaftsanalyse_EUROFINS_v02_121127

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