Circulating free DNA: a tumour biomarker for early detection and follow-up in colorectal cancer

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Abstract

Background: Circulating cell-free DNA (cfDNA) in plasma is under investigation as a "liquid biopsy" for monitoring cancers as a surrogate for the primary tumour. The aim of the study was to evaluate the potential of cfDNA for early detection and follow up of patients with colorectal cancers (CRC).

Methods: 55 colonic and 29 rectal operable carcinomas were investigated comparing matched tumour tissue and cfDNA. A 69bp ALU repeat qPCR assay was used to quantify fragmented DNA. Allele specific locked nucleic acid/peptide nucleic acid (AS-LNA/PNA qPCR assays were used to detect hot spot mutations in *KRAS*, *BRAF* and *PIK3CA*, optimised using cell lines with known mutation status. Results for cfDNA were compared with clinicopathological features of CRC and overall survival.

Results: Total cfDNA levels correlated with disease stage (p=0.003). Cell lines studies demonstrated a limit of mutant DNA detection of 1 genome equivalent (6pg total DNA). Using this threshold, at least one mutation was detected in cfDNA of 40% of cases of CRC. Mutations in cfDNA were more common in colonic cases than rectal cases (p=0.01) and with significantly higher levels of mutant cfDNA (p=0.02).). In colonic tumours, cfDNA levels correlated with tumour stage (p=0.003), size (p=0.04) and LN status (p<0.005). However, only 6 (86%) with *BRAF*, 6 (45%) with *KRAS* and 3 (20%) with *PIK3CA* matched to the mutations found in the tumour tissue suggesting clonal evolution. *PIK3CA* mutations were detected for the first time in cfDNA in localised primary tumours including early stage of CRC. Potential use of cfDNA total levels to screen for early lymph nodes involvement demonstrated a ROC of 0.76, with a 75% sensitivity and 86% specificity, suggesting early detection of cancer prior to metastasis. No significant survival differences were observed for cohort cancers for mutant cfDNA. However, mutation in cfDNA of colonic cancers was associated with poor overall survival (p<0.05).

Conclusion: This study has shown that cfDNA has a potential for use as a surrogate for the primary tumour in CRC, and for early detection of colonic cancer prior to metastasis. However, further study in a larger cohort is required to validate these findings.

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List of Abbreviations

∆Ct	Delta cycle threshold.
µl	Microliter
ANOVA	**Analysis of variance**
APC	Adenomatous polyposis coli
APR	Abdominoperineal resection
AS	Anterior resection
AS-PCR	Allele specific PCR
ATCC	American type culture collection
AUC	Area under the curve
bp	Base pair
BRAF	v-Raf murine sarcoma viral oncogene homologue B1
Cadherins	Calcium dependant adherins
CCPH	Culture Collections Public Health England – UK
CEA	Carcinoembryonic antigen
cfDNA	Circulating free DNA
ChTh	Chemotherapy
CIMP	CpG islands methylation phenotype
CRC	Colorectal cancer
Ct	Cycle threshold
CT scan	Computed Tomography Scan
ddPCR	Droplet digital PCR
DPX	Dibutyl phthalate and Xylene
EDTA	Ethylene diamine tetra acetic acid
EGFR	Epidermal growth factor receptor
FAM	6-Carboxyfluorescein
FAP	Familial adenomatous polyposis
FFPE	Formalin fixed paraffin embedded
FITs	Faecal immunochemical test
FOBT	Faecal occult blood test
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCP	Good Clinical Practice
GTP	Guanosine triphosphates
H&E	Haematoxylin and Eosin
HG-DNA	Human Genomic DNA
HNPCC	Hereditary non-polyposis colon cancer
HTA	Heath Technology Assessment
IBD	Inflammatory bowel disease

IMS	Industrial methylated spirit
KRAS	Kirsten Rat sarcoma viral oncogene homologue
Lap	Laparoscopic
LN	Lymph node
LNA	Locked nucleic acid
LOH	Loss of heterozygosity
Lt HCt	Left hemicolectomy
MAPK	Mitogen-activated protein kinases
MGB	Minor groove binder
mL	Millilitre
MRI	Magnetic Resonance Imaging
MSI	Microsatellite Instability
ng	Nanogram
NA-G	N-(2-aminoethyl)-glycine
NSCLC	Non-Small Cell Lung Carcinoma
NTC	No template control
OS	Overall survival
P53	Protein 53 or tumour suppressor protein 53
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase gene
PNA	Peptide nucleic acid.
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative real-time polymerase chain reaction
RaTh	Radiotherapy
ROC	Receiver operating characteristics
RT	Room temperature
Rt HCt	Right hemicolectomy
SMAD	Mothers against decapentaplegic homolog
SNPs	Single nucleotide polymorphisms
tDNA	Tumour deoxyribonucleic acid
TEMS	Transanal endoscopic mucosal surgery
TME	Transmission electron microscopy
Wnt	Wnt-signalling pathway

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Chapter 1
Introduction

1.1 The COLON

The large intestine or the colon is the last part of the gastrointestinal tract, it is approximately 150 cm long and is anatomically divided into five regions: caecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum. This can also be divided to the right-sided colon which constitutes of the caecum (and appendix), ascending colon, and proximal two-thirds of the transverse colon [Midgut] and left-sided colon that comprises the distal part of the transverse colon, the sigmoid, rectum, and top two-thirds of the anal canal [Hindgut], (Figure 1-1) (Beck, 2011). The later classification is important regarding the molecular diversity of colonic cancer, as well as treatment and prognosis.

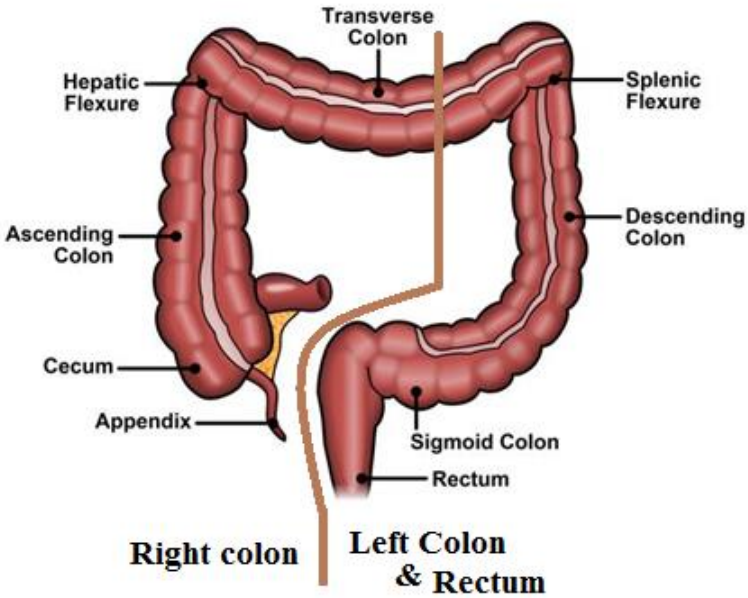

Figure 1-1 Colon anatomy, showing the locations of the caecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum. Also, highlighting the right and left colon and rectum. Adapted from (Beck, 2011).

1.2 Colorectal cancer (CRC)

1.2.1 Overview and Incidence

Colorectal cancer (CRC) is one of the most common malignancies worldwide, (Fearon, 2011), with a high prevalence in Western countries and relatively less prevalence in Asia and Africa. The lifetime risk of being diagnosed with invasive cancer is 4.7% in females and 5.0% in males with mortality being 30% to 40% higher in men (Siegel et al., 2014). In the United Kingdom, CRC is the third most common malignancy in men and women (Atkin et al., 2010) (Chu, 2011). CRC incidence rises with age, and on average half of the patients will survive to 10 years (CRUK, 2015).

Bowel Cancer (including Colorectal cancer) together with lung, prostate, breast cancers accounts for over half (53%) of all new cases of cancer in the UK (2013). Bowel Cancer affects both genders with a male: female ratio 13:10. In 2014 Cancer Research UK published the latest statistics indicated that around 1 in 14 men and 1 in 19 women in the UK will acquire the disease in their lifetime (Figure 1-2) (CRUK, 2015). CRC has been diagnosed in over 37,000 people per year and killing more than 16,000 patients annually (Swinson and Seymour, 2012). The majority (90%) of cases are over 50 because of the age-associated risk factors. Nonetheless, there has been a slight decrease in the mortality as a result of many factors including improvement in early detection and treatment strategies (Chu, 2011).

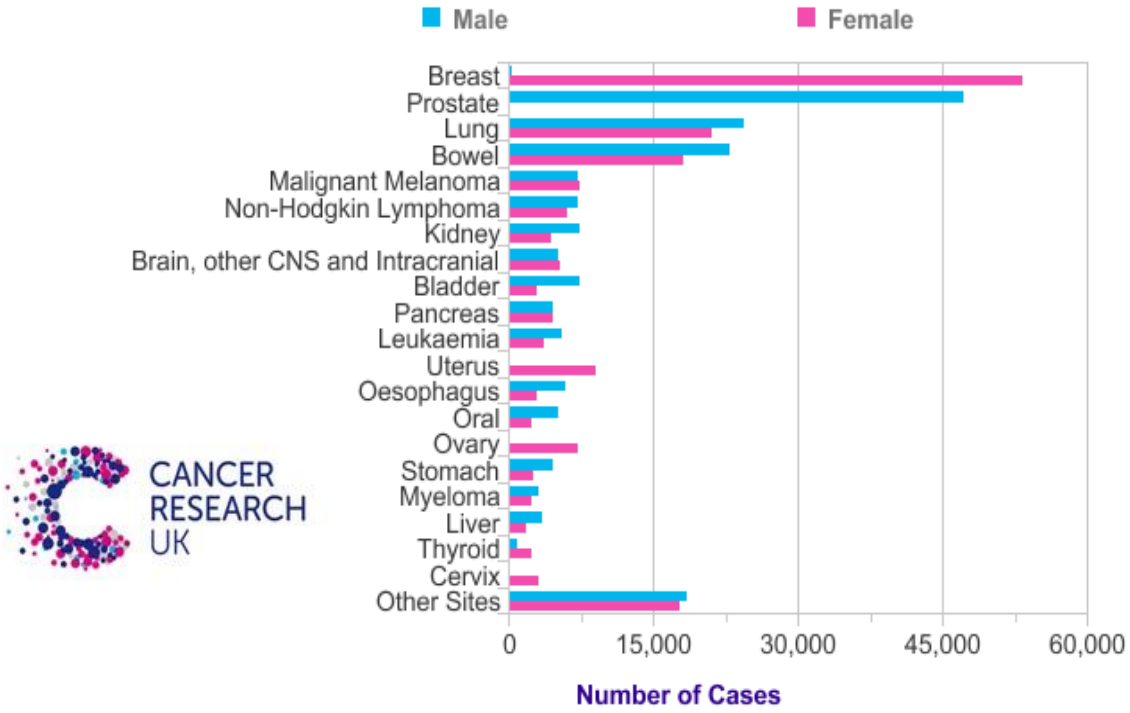

Figure 1-2: The incidence of most common cancers in the UK represented by gender-adapted from (CRUK, 2015).

1.2.2 **Risk factors**

1.2.2.1 **Environmental Factors and Lifestyle**

Globally, the incidence of CRC is variable by at least 25 fold. The majority of cases lie within the developed countries, such as Western Europe, North America, New Zealand and Australia, while low incidence rates are noticed in Asia, Africa and South America (Swinson and Seymour, 2012) (Norat et al., 2005). However, there is a rising incidence of CRC in low-risk populations in countries in economic transition including changes in their lifestyle and diet. For instance, the first generation of migrant Japanese born in the USA acquired a rising risk more than the local white population (Labianca et al., 2010a) (Tomatis, 1991).

An increased risk of CRC has also been linked to tobacco smoking, alcohol consumption and obesity. Long-term exposure to tobacco has a significant effect on CRC carcinogenesis over a period of 3 to 4 decades between the time of toxic exposure and cancer diagnosis. In the USA 1 in 5 CRC cases have been potentially linked to smoking tobacco (Giovannucci, 2001).

Alcohol consumption of two or three drinks (30-45g /day) per day raises CRC risk by 16% up to 40% (Norat et al., 2005). Obesity has also been associated with elevated risk of CRC. A study conducted by Moore et al., (2004) suggested that people with a Body Mass Index (BMI) > 30 have a 1.5 to 2.4 times heightened risk of CRC, and central obesity is also a strong contributor to distal and proximal colon cancer (Moore et al., 2004).

Red and processed meat are also linked to increased risk of acquiring CRC by 30% among individuals who consume meat compared to vegetarians (Norat et al., 2005), due to heterocyclic amines in well-done meat. In contrast, a high fibre diet and consuming vegetables, fruit and chicken or fish are more protective against the disease (Norat et al., 2005). Nevertheless, there is poor evidence that high dietary fibre can reduce the risk of CRC (Park et al., 2005).

Physical activity plays a supportive role in decreasing the incidence of colorectal cancer by 20 to 40% as illustrated by a recent Swedish study that increased amount of leisure time lowers the risk of acquiring the CRC. This positive factor is stronger in men than in women (Larsson et al., 2006).

These lifestyle factors are modifiable and a recent estimation suggest that CRC incidence could be reduced by 70% through enhancing the lifestyle in developed nations (Willett, 2002). Consuming foods with high fibre, vitamins and calcium and less fat and red meat, combined with physical fitness and avoid smoking and alcohol consumption could potentially save millions of lives across the world.

1.2.2.2 **Genetic factors**

The majority of CRCs are sporadic disease due to aging (Figure 1-3). However, approximately 35% of cases are associated with genetic alterations as illustrated by twin studies (Lichtenstein et al., 2000). Familial adenomatous polyposis (FAP) is a inheritied autosomal dominant disease, which is characterised by developing hundreds to thousands of adenomas in the bowel. The affected patients are usually in their middle age 30s and 40s (Half et al., 2009, Fearon, 2011)) (Fearon, 2011). This syndrome affects nearly 1 in 12,000 people and comprises <1% of all colorectal cancers (Rustgi, 2007). The lifetime incidence of CRC in untreated individuals approaches ≈100%, with the earliest time of diagnosis around 35 years. Prophylactic surgical removal of the colon

remains the primary treatment for FAP in early adulthood, ideally total proctocolectomy with ileoanal anastomosis (Fearon, 2011) (Galiatsatos and Foulkes, 2006).

FAP is caused by germ-line mutations in the *adenomatous polyposis Coli* (*APC*) gene located on chromosome 5q21. Mutant *APC* gene comprises the primary step in the evolution of CRC in FAP and leads to a decline in degradation *of β*-catenin, leading to nuclear accumulaiton and transcriptional changes (Swinson and Seymour, 2012) (Fearon, 2011).The identification of the gene has led to dramatic improvements in the management of FAP, which has been achieved by screening for FAP by genetic testing for *APC* mutations, especially for the 1st degree relatives of patients so affected individuals can be treated successfully and early (Galiatsatos and Foulkes, 2006) (Young. et al., 2011).

Hereditary Non-polyposis CRC (HNPCC) or Lynch Syndrome (LS) is also an autosomal dominant disorder and was first described in 1913 (WARTHIN, 1913). It is marked by Microsatellite Instability (MSI) that increase the risk of CRC and extra-colon tumours such as gynaecological, gastrointestinal and pancreaticobiliary carcinomas (Young. et al., 2011).

More than 90% of LS cases that harbour germline mutations in MMR genes mainly *MLH1* and *MSH2*, those patients have a lifetime risk of bowel tumour ranging from 50% to 100% and constitute 2.2% - 4% of all CRC (Young. et al., 2011). LS individuals develop CRC in their middle age, and 70% of cancer arise in the right side of the colon (Swinson and Seymour, 2012). The disease is diagnosed by family history as well as its occurrence in the bowel. Nevertheless, the diagnosis is proved by a molecular test for the affected genes (Young. et al., 2011). Screening is the best method to reduce the CRC mortality related to LS and decreased CRC mortality (Swinson and Seymour, 2012).

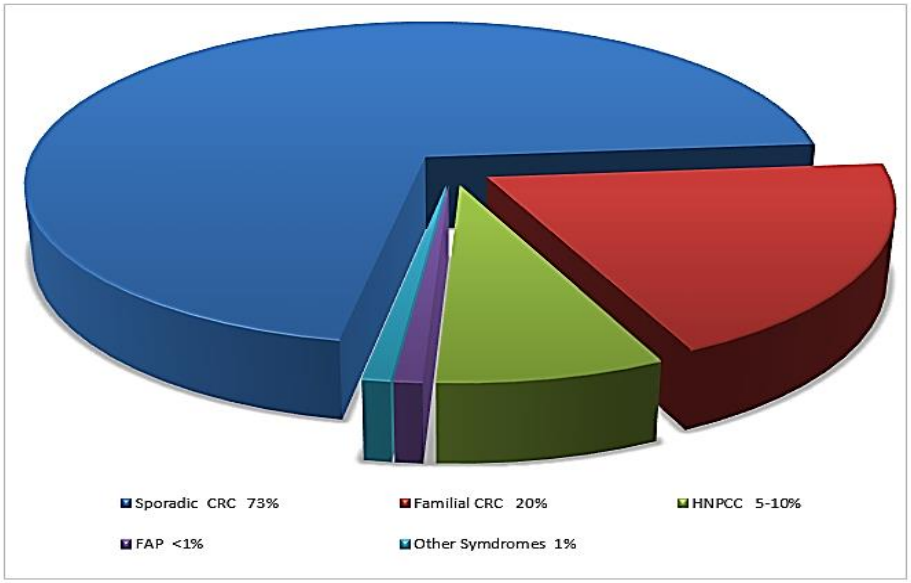

Figure 1-3 The distribution of the three leading causes of CRC. The vast majority are sporadic by (73%), followed by familial CRC (low penetrance genes) (20%) and the hereditary CRC syndromes (< 10%) adapted from (Chu, 2011).

1.2.2.3 **Family History of Colorectal Cancer**

A strong family history of CRC rises the probability of acquiring the disease from 1.7 to 8 fold among first-degree relatives. It accounts for approximately 20% of CRC (Figure 1-3) and is more likely to affect individuals aged less than 45 years. However, many individuals have no genetically inherited disorders such as familial adenomatous polyposis (FAP) or Lynch syndrome (Fuchs et al., 1994, Chu, 2011). Therefore, knowing this association led to more focus on a precise registry of family history and raising the importance of endoscopic screening for individuals at risk (Church and McGannon, 2000).

1.2.2.4 **History of Inflammatory bowel disease:**

Long-existing inflammatory bowel disease IBD (Ulcerative colitis UC and Crohn's disease CD) plays a major role in the development of CRC. There are many factors that can initiate CRC carcinogenesis in IBD such as: duration of colitis, a cumulative risk for cancer. For example, the cumulative risk for UC is 2% at ten years, 8% at 20 years and 18% at 30 years, the greater the cumulative risk, the higher chance of acquiring cancer. Extent of colitis is another significant contributor; CRC risk increases with the

larger extent of colonic surface involved (Itzkowitz and Harpaz, 2004). Furthermore, family history of CRC, IBD with sclerosing cholangitis and younger age onset of the disease carries a greater risk of developing colorectal cancer (Itzkowitz and Harpaz, 2004).

Colonoscopic screening is still a primary tool to detect any early mucosal dysplasia, and total proctocolectomy is necessary, and chemoprevention is recommended if the dysplastic changes are confirmed (Potack and Itzkowitz, 2008).

1.2.3 **Mortality and survival**

Bowel cancer (including colorectal cancer) is the second dominant cause of death in the United Kingdom (2011) and constitutes a 10% of cancer mortality. The number of deaths in 2014 from CRC was about 15,900. Deaths from colon comprise 62% of cases and rectum tumour cause death in 38% (CRUK, 2015).

Overall 5-year survival for CRC is excellent for tumour confined to the colon (90.3% for stage I and 82% for stage II). However, this declines to 70.4% for individuals with regional metastasis to the lymph nodes (stage III) and patients with distant metastases (stage IV) have the poorest survival 12.5% (Siegel et al., 2014) (Parkin et al., 2005).

Less than 40% of CRC patients are diagnosed with a stage I or II cancer, for which surgical resection alone can be curative. Individuals with stage III cancer (36–54%) are candidates for adjuvant therapy after surgery. Patients with stage IV cancer (20–31%) are usually subjected to first-line chemotherapy, potentially followed by surgical resection (Maringe et al., 2013) (Siegel et al., 2014).

Over last four decades, mortality rates of CRC have declined in the UK. The European death rate decline by 38% in men and by 51% in women between 1971 and 2011. More interestingly, the mortality rates have decreased over the first decade of this century by 15% in men and 13% of women, which is likely due to improvement in treatment strategies and earlier detection from intensive screening (Labianca et al., 2010a) (ONS, 2013). Age has been highly connected to the mortality rate, in the UK high mortality rates were recorded in the population aged over 75 years by 57% of all CRC deaths in both males and females (Chu, 2011) (CRUK, 2015).

1.2.4 Screening for colorectal cancers

Colorectal cancer comply with the criteria established by the World Health Organisation as a condition worthy of screening. The expectation of decreasing the mortality and rising detection of early disease has encouraged the establishment of national screening strategies for CRC in several countries.

Charactersitics of good screening test:

The aim of screening for disorder or disease is to detect those among the seemingly well who are actually affected by the disease. Those individuals can be treated and, if the disease is transferable, measures should be taken to prevent it from being a risk for the rest of the community. Theoretically, screening is a worthwhile technique of tackling diseases, since it should help discover it in its early stages and allow it to be treated appropriately before it gets into advanced incurable stages (Wilson et al., 1968). Screening markers should also comply with WHO standards (Pesch et al., 2014). Therefore, the screening tool should have the following characteristics:

Validity: the capability of the tool to discriminate those who have the disease from those who do not. I.e., the test should have good sensitivity and specificity (Wilson et al., 1968).

Reliability: Reliability indicates the degree to which a measurement method can be reproduced. The absence of reliability may result from differences between observation, or instruments of measurements, or instability of the feature being measured (Kadir et al., 1994).

Yield: The yield of a screening test can affect the feasibility of a screening program i.e., the number of cases detected. This can be determined from the positive predictive value. Sensitivity and specificity are features of the test and are only affected by the test characteristics and the criterion of positivity that is chosen. On the other hand, the positive predictive value of a test, or the yield, is very reliant on the prevalence of the disease in the group of people being screened. The greater the prevalence of a disease, the greater the positive predictive values (and the yield).

As a consequence, the initial means of increasing the yield of a screening program is to aim the test to groups of people who are at higher chance of acquiring the disease**.** (Wilson et al., 1968).

Acceptance and Cost: A screening test should fulfil all criteria of having accepted treatment and to manage the disease adequately, when detected, is properly the most

important. And also to avoiding harm to the patient at all costs with scientifically proved value (Lansdorp-Vogelaar et al., 2010).

Recently, a national CRC screening strategy has been applied to reduce the risk of CRC and mortality. Identifying and removing the pre-cancerous polyps with improved survival rates that linked to early detection of CRC would signify the screening of colorectal cancer especially among people who are at high risk (Weitz et al., 2005) (Young. et al., 2011).

In England the NHS Bowel Cancer Screening Programme (NHS-BCSP) was initiated in 2006 and implemented nationwide by 2010 after a successful pilot study. The programme targets people aged 60-75, who are at high risk of acquiring the CRC (Anwar, 2006). It is carried out every two years and successfully detects CRC or adenomas in up to 50% of the screened cohort (Labianca et al., 2010a) (Young. et al., 2011) (Table 1-1). Faecal Occult Blood Test (FOBt) is used to screen individuals at risk, then postive cases are subjected to further investigation by colonoscopy. FOBt has been have been tested in randomised pilot studies and contributed to the reduction in the mortality of the disease up to 25 % (Labianca et al., 2010a) (Bretthauer, 2010) (Young. et al., 2011).

Test	*Advantages*	*Disadvantages*
Colonoscopy	• CRC lesions are highly and effectively detected. • Visualise the whole colon. • Can excise polyps. • Biopsies can be obtained.	• Some cancer and polyps lesions can be missed. • Requires Anaesthesia. • Bowel preparation is essential. • Perforation may happen. (Gatto et al., 2003)
Flexible sigmoidoscopy	• Cancerous lesions are effectively detected in the sigmoid and rectum. • Does not need anaesthesia. • Biopsies can be obtained. • Can excise polyps. • Less Bowel preparation.	• Lesions in upper part of the colon cannot be detected. • The risk of perforation or tearing is very small. • (Gatto et al., 2003) (Fletcher, 2003) (Anwar, 2006)
Computer tomographic colonography	• High performance for CRC detection. • Visualise the whole colon. • Non-invasive and does not need anaesthesia	• May not detect some lesions • Bowel preparation is needed • Subsequent polypectomy by colonoscopy might be required. (Johnson et al., 2008) (Pickhardt et al., 2003) (Summerton et al., 2008) •
Faecal blood test	• Intermediate performance for CRC detection. • Non-invasive • Bowel preparation is not needed and cheap. • Used for screening/early detection.	• May not detect some lesions Bowel preparation is needed • Subsequent polypectomy by colonoscopy might be required. • Sensitivity is low. (Burch et al., 2007) (Ouyang et al., 2005) •
Stool DNA test	• Intermediate performance for CRC detection. • Non-invasive • Bowel preparation is not needed. • Used for screening/early detection.	• Fails to detect most cancers and adenomatous lesions. • May requires dietary restrictions • False-positive results may be obtained. • Positive results need to be confirmed by colonoscopy (Burch et al., 2007) (Ouyang et al., 2005)

Table 1-1 Current techniques used for CRC screening, diagnosis and early detected of the disease

1.2.4.1 **Faecal Occult Blood Test (FOBt):**

FOBt is the oldest and most common test used for CRC screening in the UK and Europe. It is a cheap, non-invasive and it works by detecting haematin produced from broken red blood cells in the form of occult blood, which released into the bowel (Bretthauer, 2010) (Table 1-1). There are two types of FOBt; one is guaiac FOBt (gFOBt), it works by detecting peroxidase–like activity haematin in patient's faeces, the activity of the enzyme reduced as the haemoglobin passes through the gastrointestinal tract. False-positive results are expected because of haemoglobin consumed from animal or plant source and false-negatives can occur due to the nature of irregular bleeding of the tumour. Therefore, the sensitivity of gFOBt is low (Anwar, 2006) (Morikawa et al., 2005) (Table 1-1)

The second type is immunochemical FOBt (iFOBt), which is more specific for human blood as it detects only human globin, no dietary restrictions, it is more expensive and performs better than gFOBt (van Rossum et al., 2008). The sensitivity of FOBt ranges from 50-60% but it varies according to the manufacturer (Bretthauer, 2010).

Individuals with positive results have to repeat the test at least once, if still positive (10% of cases), they then undergo colonoscopy. Overall, results show 50% of cases have adenomas and CRC; one-third is normal and the rest have other benign lesions such as diverticulosis and haemorrhoids (Young. et al., 2011).

1.2.4.2 **Flexible Sigmoidoscopy**

Flexible Sigmoidoscopy (FS) is used to examine the rectum and sigmoid colon as they harbour around 50% of adenomas and cancers. The technique has a considerable sensitivity and specificity for both adenomas and CRC in distal colon (Young. et al., 2011) (Table 1-1). The technique can detect two-third of CRC in the area examined, it is more cost effective and accepted by patients than total colonoscopy (Anwar, 2006) (Fletcher, 2003).

Many trials have studied the effectiveness of the FS; the studies showed that the procedure has contributed to the reduction of the CRC mortality by 32% as a whole colon cancer and by 50% for the left side CRC (Atkin et al., 2010) (Neugut and Lebwohl, 2010). The test is implemented by giving the patient self-administrated enema for cleansing; most patients have their adenomas excised during their screening (Atkin

et al., 2010). However, the technique has drawbacks such as the need for proper colonic cleansing, inability to visualise the entire colon, its invasiveness and highly demanding as compared to Faecal Occult Blood Testing (Anwar, 2006).

Colonoscopy and CT colonography are potential methods in the screening programme of colorectal cancer, yet they have not been investigated in randomised trials, they are expensive and not suitable for a high-risk population, so they are not recommended as part of screening programme unless indicated (Anwar, 2006) (Young. et al., 2011).

1.2.5 **Disease staging**

Cancer staging is an estimation of tumour size and its amount of penetration into surrounding tissue and organs. Tumour stage is a potent predictor of survival and prognosis in patients with colorectal cancer. Precise staging is crucial for proper patient management and required for accurate evaluation of treatment strategies and outcome (Compton and Greene, 2004). There are several details such as age, gender of the individuals; site, grade and the vascular invasion of the cancer are required before deciding on the treatment or predicting the outcome (Davis et al., 1984).

There are two common systems for colorectal staging: TNM and the Duke's system. Duke's staging was firstly reported in 1932 by Cuthbert Dukes is based on a pathological study of the tumour specimens and it does not need input from clinicians (Davis et al., 1984). The tumour stages are described in A, B, C and stage D (Kyriakos, 1985), a comparison of TNM and Dukes' Classification is shown in Table 1-2.

Tumour stage has good indication of prognosis, the more advanced the stage of a tumour the poorer prognosis. The second staging system is TNM, which is the most common system used in the UK (CRUK, 2015) and defined by American Joint Committee on Cancer. This staging system identifies tumour size tumour local invasion and depth (T), any lymph nodes invasion (N), and whether cancer has metastasized to somewhere else in the body (M), (Table 1-2) (Compton and Greene, 2004) (Greene, 2002).

TNM classification				Duke's Stages	Prognosis
AJCC stages	T	N	M	Stages	
Stage 0	Tis	N0	M0		
Stage I	T1	N0	M0	A	5 years survival >90%
	T2	N0	M0	B1	5 years survival > 70-85%
Stage II	T3	N0	M0	B2	
	T4	N0	M0	B2	
Stage III	T1, T2	N1 or N2	M0	C1	5 years survival >55-65%
	T3, T4	N1 or N2	M0	C2	5 years survival >20-30%
Stage IV	Any T	Any N	M1	D	5 years survival <5%

Table 1-2: A comparison of TNM and Dukes' from (*American Joint Commission on Cancer- AJCC*) Classification adapted from (Kin et al., 2013) and (Blanke and Faigel, 2011)

1.2.6 **Treatment Strategies**

Tumour staging is essential in creating the proper option and course of therapy for each CRC case who needs a personal therapeutic strategy (Young and Rhea, 2000). Many factors such as tumour (stage, grade, tumour size and location), age and general health of the patient can determine the type of treatment. Chemotherapy and radiotherapy are the most broadly treatment for CRC with or without surgical resection.

Initially, the majority of CRC patients will be surgically operated to remove the primary tumours, then, the therapeutic plan is mainly dependent on the stage of the tumour (McArdle, 2000). The treatment for stage 0 is resection of the local lesion, and complete endoscopic polypectomy is recommended whenever the shape of the polyp permits

(Sobin LH, 2002). The subsequent decision will be made according to a histopathological examination of the excised polyp, resection of the colon is recommended if there are any neoplastic features, but the large lesions should be surgically resected. Individuals with a stage II tumour which displays features considered to be a high risk (≥12 lymph nodes are affected, poorly differentiated, colon obstruction, vascular or lymphatic invasion by the tumour) and stage III tumours, will also be given neoadjuvant therapy (Labianca et al., 2010b). Importantly, the aim of surgery is an extensive resection of the colon or the rectum with its lymphatic drainage (Labianca et al., 2010a) (Wils, 2007).

The current combination of chemotherapeutic treatment that is referred to as FOLFOX is Oxaliplatin and 5-FU, plus folinic acid (Leucovorin, LV). In cases where it is not possible to administer oxaliplatin due to undesirable neuropathic adverse effects, then 5-FU and LV only, or capecitabine (an oral fluoropyrimidine) will be given. Before contemplating the chemotherapy, another factor such as the MSI status should be taken into account because cases with positive MSI are unlikely to benefit from 5-FU chemotherapy (Jover et al., 2011). Node–positive cases (stage III) should receive adjuvant fluorouracil, which has reduced the mortality by 30% (Marsoni, 1995). The alternatives could be oral fluoropyrimidine, or capecitabine, which they have the same effect as fluorouracil alone (Twelves et al., 2005).

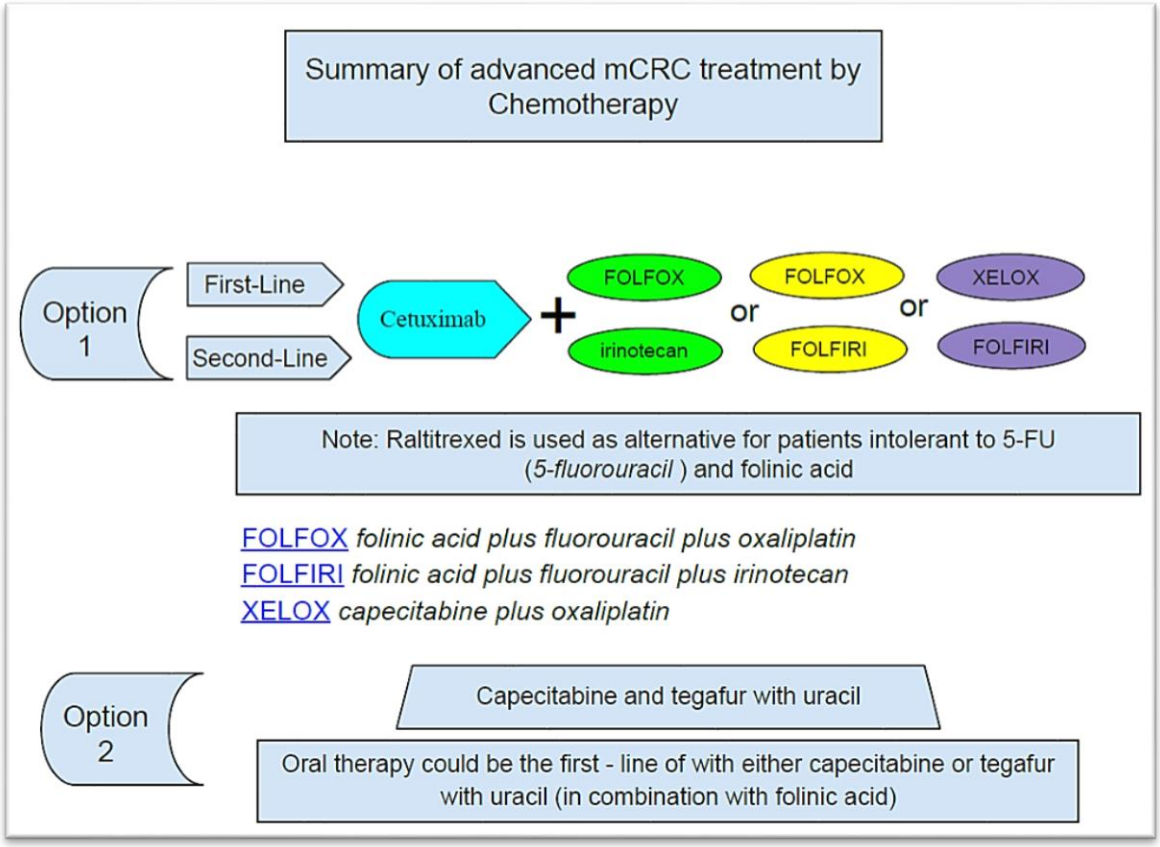

Figure 1-4 Shows chemotherapeutic options in the treatment of advanced mCRC (NICE, 2016).

In metastatic CRC, liver is usually the first distant tissue to be involved; surgical resection is the best decision for small lesions, as the remaining tissue will compensate the functional deficit. However, with large metastatic lesions, neoadjuvant treatment should be given before surgery to downsize the tumour and possibly to eliminate the micrometastases (Allen et al., 2003). However, neoadjuvant treatment is costly, hepatotoxic and causes poor visualisation of the tumour through the surgery (Benoist et al., 2006). Post-operative relapse accounts for 30-50% of the patients within 5 years. Micrometastases are hard to see during the operation and could be responsible for cancer relapse (Fong et al., 1999) (Benoist et al., 2006) (Figure 1-4).

In rectal tumours, Radiotherapy is used pre-surgically as palliative treatment especially to relieve the symptoms and arrest the tumour growth. However, the general health of the patients should be considered before making the decision to weigh the outcomes from the adverse effects (Cunningham et al., 2004).

Anti-epidermal growth factor receptor (*EGFR)* antibodies

The EGFR is a membrane receptor with intrinsic tyrosine kinase activity, activation of these receptors stimulates intracellular signalling cascades, which eventually leads to cells proliferation. The Anti-EGFR, also called monoclonal antibodies (mAb) therapy, consisted of Cetuximab, Bevacizumab and Panitumumab and used after first-line chemotherapy as shown in the Figure 1-4. EGFR signalling is transmitted through cells via two major mechanisms, *PIK3CA* and the *RAS/RAF/ERK* pathways (Bardelli and Jänne, 2012). Many studies and numerous trials have illustrated that mutant *KRAS* patients do not benefit from anti-EGFR therapy (Bokemeyer et al., 2011) (Van Cutsem et al., 2011). Therefore, *KRAS* mutations testing has included as pretest before the initiation of anti-EGFR treatment to avoid such resistance (De Roock et al., 2010a) (Amado et al., 2008) (Diaz Jr et al., 2012).

Mutant *BRAF* has also been linked to anti-EGFR antibody resistance in *KRAS* WT patients with CRC (De Roock et al., 2010a), (Di Nicolantonio et al., 2008). Anti-EGFR resistance also developed in patients with *PIK3CA* gene mutations (Mao et al., 2011) (De Roock et al., 2010a), these mutations can exist separately, or synchronously, with mutations in the *RAS/RAF/ERK* pathway. Hence, studying both cascades offers a remarkable approach to determining individuals who inherented an anti-EGFR therapy resistance (Cunningham et al., 2004). Interestingly, a study by Sartore-Bianchi *et al*, found oncogenic alterations in *KRAS*, *PTEN*, and *PIK3CA* in 70% of metastatic CRC (mCRC) cases who did not respond to monoclonal antibodies (mAb) therapy (Sartore-Bianchi et al., 2009).

Therefore, this study aimed to establish a convenient approach to identify the mutational status of the CRC patients, the test that can be carried out at any stage of the disease and to anticipate the outcomes.

1.3 Colorectal carcinogenesis and molecular profiling

1.3.1 Histological adenoma-carcinoma sequence

Most sporadic CRC develops from normal epithelium in relatively coordinated histological succession. The earliest event in Adenoma evolution is a small polyp formation. Histologically, the lesion appears in a tubular shape, apart from atypical features such as "large nuclei", it still resembles normal epithelium, these manifestations are termed dysplasia, which is a low grade in this stage (Young. et al., 2011). As the adenoma grows, its histology become more complicated, and it either forms stalk or stays sessile, its shape becomes tubulovillous structure with a high grade of dysplasia "late Adenoma". Eventually, adenomatous polyps will progress to malignant stage and metastases the surrounding tissue then it manifests as ulcerated mass (Figure 1-5). The progression from adenoma to the malignant tumour is believed to take from 10 years to 40 years (Jass, 2007).

Recently, 5 to 10% of colorectal cancers were identified with different histological features, which called the serrated neoplasia sequence. In this particular pathway two distinctive premalignant lesions can be seen such as traditional serrated adenomas or sessile serrated adenomas. Nevertheless, the real risk of development of adenoma into adenocarcinoma stays unknown, and it is under investigation (Jass, 2007). The serrated neoplasia sequence is linked to different genetic and epigenetic changes from the traditional adenoma-carcinoma sequence. DNA mismatch repair deficient sporadic CRC could be responsible for this kind of sequence (Young. et al., 2011) (Markowitz and Bertagnolli, 2009a).

1.3.2 Molecular Genetics of colorectal cancer

Bowel benign tumours are diverse, but some local tumours that protrude from the bowel mucosa are usually named a polyp. Most colorectal polyps especially the ones > 5mm in size are described as hyperplastic lesions and most studies suggested that those polyps are the main precursor to colorectal tumours (Jass, 2007). On the other hand, the adenomatous polyps are potentially the main precursor to adenocarcinoma (Jass, 2007) (Robbins et al., 2013). Adenoma evolve from the glandular epithelium; it has characteristics of epithelial dysplasia, transformed differentiation, and it varies from

small with stalk, which called pedunculated polyp, to large without stalk, which called sessile polyp. There are residues of adenoma tissue in specimens of CRC and vice versa (Fearon, 2011). However, there are only small set of adenomas that progress to cancer over the years, for example, adenoma sized 1cm has nearly 10 to 15% chance to become carcinoma over ten years (Stryker et al., 1987).

The adenoma-carcinoma sequence is a progressive multistep process that needs an aggregation of genetic events in a normal cell to transform into a malignant tumour cell (Young. et al., 2011). This process requires genetic mutations and epigenetic changes in the genome that cause deactivation of tumour suppressor genes or activation of oncogenes in different stages with different gene involvement (Fearon, 2011) (Figure 1-5).

Latest advances in molecular genetics have revealed more details about the adenoma-carcinoma sequence events (Fearon, 2011) (Segditsas and Tomlinson, 2006). This event starts by biallelic mutations in the gene *APC* (adenomatous polyposis coil) and accounts for 70% of microadenomas early changes. *APC* mutations activate Wnt pathway that plays a significant role in stem cells repair (Figure 1-5), differentiation and progression of colonic epithelial cells (Polakis, 2007) (Segditsas and Tomlinson, 2006) (Arnold et al., 2005).

Even though *APC* mutations are needed to initiate CRC tumourigenesis, involvement of further genetic alterations are required for adenoma growth and progression (Young. et al., 2011). Activating mutations in *KRAS* oncogene, which mediates the mitogen-activated protein kinase (MAPK) pathway are observed in the transformation from an early to an intermediate stage of adenoma in around 40% of cases (Fearon, 2011) (Markowitz and Bertagnolli, 2009a) (Pretlow and Pretlow, 2005).

The development of adenoma from an intermediate to the late stage is related to loss of chromosome 18q (*DCC*) (Labianca et al., 2010a) and it accounts for around 70 % of advanced or late adenoma (Vogelstein et al., 1988). This loss also affects *SMAD2* and *SMAD4,* which are genes encode proteins that act on TGF-β receptors complex (Figure 1-5) and playing a vital role in cell growth, differentiation and apoptosis (Dijke and Hill, 2004) (Cancer Genome Atlas, 2012) (Fleming et al., 2013) .

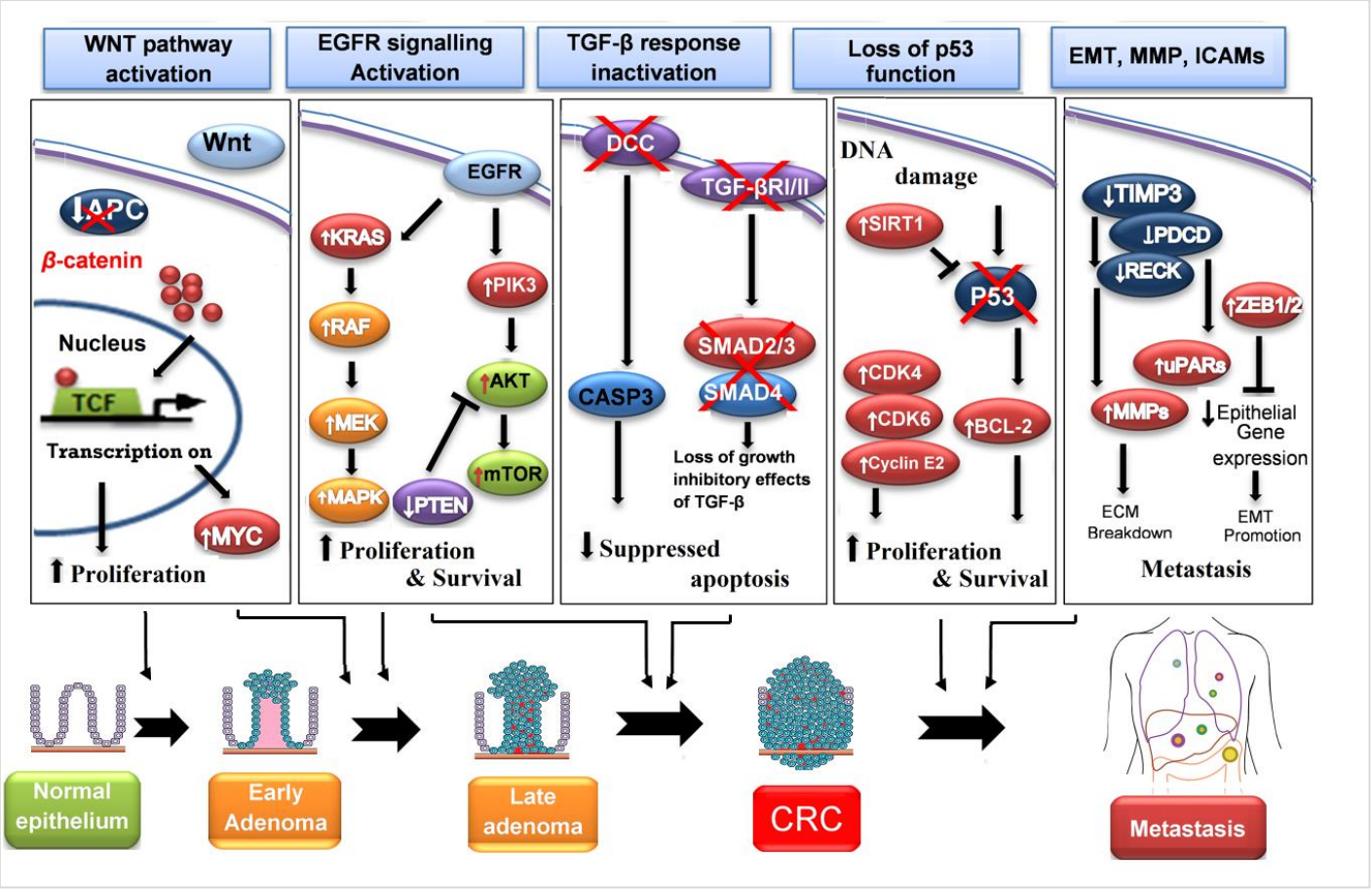

Figure 1-5 Illustration of key molecular and histological changes in the adenoma-carcinoma sequence {adapted from (Markowitz and Bertagnolli, 2009a) and (Fearon, 2011).

The transition from advanced adenoma to adenocarcinoma frequently occurs by loss of chromosome 17q and 50% of CRC cases harbour this loss. The TP53 gene tumour protein p53) is a significant tumour suppressor gene, which regulates the cell-cycle arrest, apoptosis and anti-angiogenic pathway. The gene is a target of 17q LOH, and its mutation contributes to uncontrollable growth that associated with late event in the adenoma-adenocarcinoma sequence (Baker et al., 1990, Vousden and Prives, 2009, Markowitz and Bertagnolli, 2009b).

There is an alternative genetic mechanism of tumourigenesis, which characterised by inactivation of genes required for DNA mismatch repair (MMR) function (Bronner et al., 1994). This disorder is often caused by hypermethylation of *MHL1* (mutL homolog 1 promoter) that leads to silencing of transcription of this DNA MMR gene (Markowitz

and Bertagnolli, 2009b). This defect can be inherited as Lynch Syndrome in addition to the somatic mutations (Lynch et al., 2008).

MMR deficiency is associated with hypermethylation, and leads to microsatellite instability (MSI), due to the lack of ability to repair the short repeat (1-6 base-pair) of DNA sequence (Boland et al., 2008). DNA MMR-deficient tumours can be derived from adenomas with active point mutations in *BRAF* oncogene, which drive the CpG island methylator phenotype (CIMP) and hypermethylation of promoter-specific sites of the genome, these tumours characterised by normal chromosomal karyotype and associated with exclusive microsatellite instability (Figure 1-5) (Ogino et al., 2009).

1.3.3 **Mutational inactivation of tumour-Suppressor Genes**

1.3.3.1 **Adenomatous Polyposis Coli (*APC*):**

APC is a tumour suppressor gene and localised on chromosome band 5q21-q22, it was firstly localised in 1987 and then cloned in 1991 (Galiatsatos and Foulkes, 2006). It expressed in colorectal epithelium and encodes a multifunctional 312-KDa protein (van Es et al., 2001), it regulates the cell growth and plays an important role in cell division and cell adhesion. Moreover, the APC protein has an impact on cell migration, chromosomal segregation and microtubular cytoskeleton assembly (Fearon and Bommer, 2008, Smith et al., 1994). Biallelic somatic mutations and deletions are responsible for initiation of two third of sporadic CRC as a result of *APC* mutations in small adenoma, late-stage adenoma and the carcinoma (Polakis, 2007). Moreover, the gene mutations are found in early microscopic adenoma with some dysplastic changes (Kinzler and Vogelstein, 1996).

Although all critical functions that APC protein do, its main intracellular function is to regulate the cytoplasmic level of β-catenin and controlling the Wnt signalling pathway (Figure 1-5) (Aoki and Taketo, 2007). Wnt signalling pathway plays a significant role in cell proliferation and cell differentiation, and it is mainly regulated by the intracellular stability of β-catenin (Peifer and Polakis, 2000). Once the β-catenin enters the nucleus, in the presence of *APC* inactivation, it binds to MCF family, and then acts as a transcription factor for Wnt target genes such as *TCF1, MYC* and *CyclinD* leading to increasing in cell proliferation (Figure 1-5). (Polakis, 2007) (He et al., 1998)

1.3.3.2 **Tumour Protein gene (*TP53*):**

The *TP53* gene encodes for tumour protein p53, a tumour suppressor gene and transcription factor that plays a major role in activating cell-cycle arrest, anti-angiogenic signals and the apoptotic pathways (Vousden and Prives, 2009). It is positioned on chromosome 17p13.1, and it is mutated in half of CRC cases (Kheirelseid et al., 2013). In addition to mutations of one allele, loss of heterozygosity (LOH) is thought to be one way of inactivating the other allele in *TP53* gene in 70% of cases (Kikuchi-Yanoshita et al., 1992). *TP53* mutations help the tumour cells in growth continuity and surviving the stress that tumour cells suffer during tumour growth and invasion (Fearon, 2011). A missense mutation in *TP53* may contribute positively to tumour cells proliferation, decreased their apoptosis, increased tumour angiogenesis and reduced tumour hypoxia (Fearon, 2011) (Vousden and Prives, 2009).

1.3.4 **Activation of oncogene Pathways**

1.3.4.1 ***KRAS***

KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) is a member of the mitogen-activated protein kinase (MAPK) pathway (Figure 1-5). Normally KRAS protein performs as a molecular on-off switch, the intrinsic GTPase activity of the protein is compromised by promoted mutated *KRAS* at residues 12, 13 and 61. Activated KRAS leads to changes in proliferation and apoptosis by signals to downstream effectors such as Erk/MAPkinase and PI3K/Akt pathways (Vakiani and Solit, 2011). *KRAS* plays a role in the transition from early to intermediate phase of adenoma-adenocarcinoma sequence and accounts for 35-45 % of CRC cases (Young. et al., 2011) (Markowitz and Bertagnolli, 2009b). *KRAS* mutations are considered an early event in the development of the CRC (Wang et al., 2004) and also associated with large and aggressive adenomatous polyps and CRCs (Monticone et al., 2008).

This oncogene is a target of somatic mutations in many cancers including CRC, the majority of *KRAS* mutations affect codon 12, codon 13 and less commonly mutation in codon 61 (Malumbres and Barbacid, 2003). *KRAS* mutations are more commonly seen in aberrant crypt foci and hyperplastic polyps that are less likely to develop carcinoma (Pretlow and Pretlow, 2005) (Chan et al., 2003). *KRAS* mutations frequency in

adenomatous polyps depend on the degree of dysplasia and the size of the polyp, the larger the polyp the more *KRAS* mutations ranging from 10% to 40% (Vogelstein et al., 1988).

In advanced CRC, *KRAS* has a major role in tumour progress as studies have shown a disruption in the tumour growth by knocking down the *KRAS in vivo* (Shirasawa et al., 1993). Furthermore, RAS protein acts downstream of many different receptors kinase growth factors such as EGFR family (Benvenuti et al., 2007) (Amado et al., 2008). *KRAS* mutations are adverse predictors of patient response to cetuximab, anticipating resistance to anti-EGFR treatment and a poorer outcome in a patient who have received EGFR inhibitors (Winder and Lenz, 2010) (Karapetis et al., 2008). Numerous studies on metastatic colorectal tumours have confirmed that successful anti-EGFR therapy is confined to wild-type *KRAS* and other MAPK activators *NRAS* (Amado et al., 2008).

1.3.4.2 ***BRAF***

BRAF (v-RAF murine sarcoma viral oncogene homolog B1) encodes a serine/threonine protein kinase that mediates the MAPK signalling cascade through the RAF–MEK–ERK1/2 pathway (Barras, 2015) (Weisenberger et al., 2006) (Figure 1-5). Mutations in this gene lead to constitutive activation of the kinase and up-regulation of the MAPK pathway causing a sustained cell proliferation (Davies et al., 2002). *BRAF* mutations are present in about 10% of CRC patients (Barras, 2015). *BRAF* is found activated by mutation in 15% of all known human cancer types including colon neoplasm (Davies et al., 2002). The majority of mutated *BRAF* are V600E mutation, constituting up to 80% of all *BRAF* mutations. This mutation causes amino acid change that has constitutive kinase activity (Davies et al., 2002).

Some colonic polyps such as traditional serrated adenomas and sessile serrated adenomas (SSAs) are considered as premalignant and linked to *BRAF* mutation, which is believed to be an early event in adenoma-carcinoma sequence (Markowitz and Bertagnolli, 2009a) (Noffsinger, 2009). *BRAF*-mutant tumours are mainly right sided, higher grade, and associated with microsatellite instability (MSI), it is more frequent in women, and older age and associated with a poorer prognosis (Gonsalves et al., 2014) (Lochhead et al., 2013) (Missiaglia et al., 2014). *BRAF* mutation is also linked to anti-EGFR resistance in combination to *KRAS* and *PIK3CA* (Kwon et al., 2011) and the

presence of this mutations indicates a poor prognosis in patients with metastatic disease (Spindler et al., 2012).

1.3.4.3 ***PIK3CA***

PIK3CA encodes the catalytic subunit of phosphatidylinositol 3-kinases (PI3Ks) signalling pathways; it has significant role in CRC tumorigenesis by upregulating the cell proliferation and lowering the apoptosis (Manning and Cantley, 2007) (Figure 1-5). Three hotspot mutations are the most common *PIK3CA* genetic alteration; p.*E542K* and p.*E545K* at exon 9 and p.*H1047R* at exon 20 (Kang et al., 2005). These mutations promote the lipid kinase activity of *PIK3CA* and rise phosphorylation of AKT ,which transformate normal cells into cancer cells in vitro and in vivo studies (Board et al., 2010) (Gymnopoulos et al., 2007). In addition, they promote the adenoma-adenocarcinoma sequence (Samuels et al., 2004).

PIK3CA mutations have been identified in 10% to 30% of colorectal cancers (Velho et al., 2005) and have been associated with microsatellite instability (MSI) (Abubaker et al., 2008). It has been linked to a worse patient outcome (He et al., 2009).The PI3K and MAPK pathways interact via oncogenic *KRAS*. *PIK3CA* mutations can often coexist with *KRAS* mutations (Simi et al., 2008) (Parsons et al., 2005), which boosts AKT pathway signalling and cellular transformation further than *PIK3CA* mutations alone (Oda et al., 2008).

PIK3CA with *BRAF, KRAS* are associated with resistance to anti-epidermal growth factor receptor monotherapy (De Roock et al., 2010b) (Spindler et al., 2011). There is evidence that *PIK3CA* mutations can occur early in non-malignant polyps in combination with *KRAS* and *BRAF* mutations (Velho et al., 2008). Adenomas with *KRAS, PIK3CA* or *BRAF* mutations are at higher risk of developing a CRC (Velho et al., 2005). Detecting these mutations in early stages would anticipate those people at high risk of CRC, hence, implementing mutations testing panel would be an enthusiastic screening tool in such cases.

1.4 **Biomarkers in Colorectal cancer and early detection**

Improvements in screening techniques, surgical approaches, adjuvant chemotherapy, radiotherapy, and surveillance techniques have enhanced the survival rate in CRC

patients. CRC is a heterogeneous disease at molecular level, this heterogeneity causes variability in tumour development, response to therapeutic agents and prognosis (Newton et al., 2012). This heterogeneity has been recognised, and more understood by the advances in Genomics (Soreide et al., 2009). It is reasonable to hypotheses that molecular methods will lead to the advancement of better screening technologies and to improve and personalise cancer therapy especially in localised primary tumours (Newton et al., 2012).

A biomarker is defined as a measurable substance, which can be assayed as an indicator of pathological or genetic state of a disease. It can be utilised to detect diseases in early stages, predict the response to treatment and prognosis. Also, the use of biomarkers can potentially improve the life expectancy and quality of life when applied in clinical settings by contributing to targeted therapy (Newton et al., 2012) (Dorner et al., 2015).

Colorectal carcinoma is heterogeneous cancer, and it is not feasible that any one biomarker has high sensitivity and specificity on its own. Table 1-3 shows the current biomarkers that are in use for CRC. The majority of these biomarkers lack sensitivity and specificity and have not been fully validated (Newton et al., 2012).

	Marker	Proposed or Used for	Advantages/Disadvantages
Serum markers	CEA	• Determining prognosis • Surveillance following curative Resection • Monitoring therapy in advanced disease	• Low sensitivity (40%) • Should be used in combination with radiology.
Stool Markers	FOBT	Screening for early CRC	Low sensitivity for both adenomas (10%) and CRCs (40–85%) and low specificity
	DNA-based	Screening for early CRC	• • Fails to detect most cancers and adenomatous lesions. • • False-positive results may be obtained. • • Positive results need to be confirmed by colonoscopy (Burch et al., 2007) (Ouyang et al., 2005)
Tests for genetic susceptibility to CRC	APC	For identifying subjects at high risk of developing FAP	Involved in the initiation of CRC, thus may be useful in detecting early lesions
	MSI/MMRE IHC	Pre-screen for HNPCC	• use as a surrogate marker for hereditary non-polyposis colorectal cancer (HNPCC) • determining prognosis in patients with sporadic CRC and Predicting response to adjuvant chemotherapy in patients with sporadic CRC.
	MLH1/MSH2/MSH6	For identifying subjects at high risk of developing HNPCC	Responsible for at least 80% of the reported mutations in Lynch syndrome that involve defective mismatch repair
Pharmacological markers	DPD	Monitoring 5-FU toxicity	Limited to particular patients group (van Kuilenburg et al., 2000)
Predictive markers	KRAS	response to EGFR therapy in metastatic CRC	Poor prognosis associated with *KRAS* mutations

Table 1-3:Currently used tumour marker for CRC and some of whom are recommeded by the European Group on (EGTM) guidelines for clinical use (Newton et al., 2012) (Duffy et al., 2007).

1.4.1 Circulating free DNA (cfDNA) in colorectal cancer

1.4.1.1 Introduction

Circulating cell free DNA (cfDNA) is a developing biomarker in cancer theragnosis (Schwarzenbach et al., 2011).The utilisation of cfDNA demonstrates theoretical advantages compared to conventional genetic analysis of tumour tissue sampling. CfDNA analysis is a non-invasive procedure that could provide sufficient information about the genetic makeup of tumours, even in early stages without invasive intervention (Bettegowda et al., 2014).

As cancer evolves over time, it is not feasible to get repeated biopsies and patients could lose valuable time. Therefore, cfDNA can present the tool enabling regular monitoring to follow-up a patient's response to treatment and shows any early signs of genetic aberrations (Gorges et al., 2012) (Diehl et al., 2008). cfDNA can provide information on genetic and epigenetic alterations of cancer and can be used to optimise the most beneficial course of treatment and anticipate drug resistance and also assess minimal residual disease (Siravegna and Bardelli, 2016) (Bettegowda et al., 2014) (Mouliere et al., 2014).

1.4.1.2 Discovery and the origin of circulating nucleic acids

Circulating free DNA was described 60 years ago in human in 1948 by Mandel and Metais (Mandel, 1948), in 1971 Leon et al. was the first to describe the importance of cfDNA in cancer (Leon et al., 1977). Subsequent research revealed a high level of cfDNA among patients with autoimmune diseases and cancer as compared with healthy individuals (Elshimali et al., 2013). In cancer, approximately 3.3% of the primary tumour DNA enters the circulation (Lim et al., 2013) (Diehl et al., 2005). The average of cfDNA concentration in plasma of cancer patients is 180ng/ml, while it is limited to an average of 30 ng/ml in healthy individuals (Anker and Stroun, 2000).

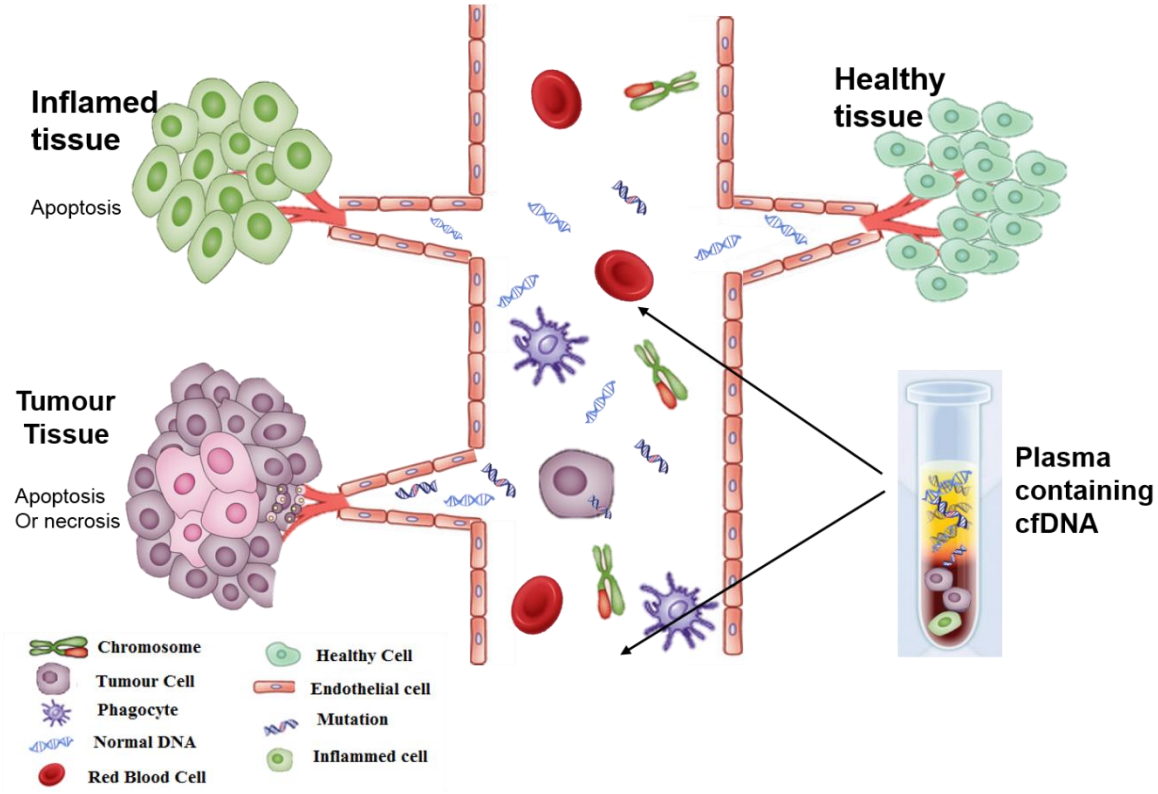

Figure 1-6: Illustration of the main sources of cfDNA that is released from healthy, inflamed and tumour tissues. The process happens due to either apoptosis or necrosis. Genetic alterations can be utilised as surrogate indicators for the genetic makeup of the primary tumour, adapted from (Crowley et al., 2013).

Human genomic DNA can enter the circulation via at least two potential mechanisms, namely passive or active (Figure 1-6) . The passive process suggests that necrotic and apoptotic cells release nuclear and mitochondrial DNA into the blood circulation from dead cells during cellular turnover (Qin et al., 2016) (Schwarzenbach et al., 2011). During normal physiological processes, apoptotic and necrotic cells are cleared by infiltrating phagocytic macrophages. Therefore, cfDNA levels in healthy people are low (Pisetsky and Fairhurst, 2007). Nevertheless, this mechanism does not act efficiently on tumour mass, inflammation or in extensive exercise as a result of cellular debris accumulation including DNA and later discharged into the circulation (Figure 1-6) (Crowley et al., 2013) (Viorritto et al., 2007) (Pisetsky and Fairhurst, 2007). The produced cfDNA fragments are estimated between 180 and 200 base pairs (van Kuilenburg et al.) (Qin et al., 2016) (Mouliere et al., 2014) indicating that apoptosis is the main origin of cell free DNA in the bloodstream (Diehl et al., 2008).

cfDNA in solid tumours can be released as a result of necrosis, autophagocytosis, and under therapeutic pressure (Delgado et al., 2013) (Roninson et al., 2001). Unlike the apoptosis, necrosis produced bigger DNA fragments because of uncompleted and random degradation of the genomic DNA into fragments of 180–1000 bp in size (Qin et al., 2016) (Elshimali et al., 2013). However, new cfDNA can be released by live cells as part of a homeostatically regulated system (Qin et al., 2016) (Stroun et al., 2000), stimulated lymphocytes could also release a significant amount of cfDNA without cell death (Rogers, 1976).

Other mechanisms have been hypothesised to illustrate why tumour cells would actively discharge cfDNA into the bloodstream, including the potentiality that tumour cells could release oncogenic cfDNA to induce the transformation of susceptible cells and develop metastases at distant sites (Trejo-Becerril et al., 2012) (García-Olmo et al., 2010). Cancer patients have much higher of cfDNA levels than healthy individuals; cfDNA levels are broadly variable (Diehl et al., 2005), this variability is linked to tumour burden, Stage, cellular turnover, vascularity, and response to treatment (Diehl et al., 2008) (Kohler et al., 2011).

1.4.1.3 **cfDNA applications for CRC detection**

In CRC, early detection has an enormous impact on the mortality and morbidity of the disease (Schwarzenbach et al., 2008). Interestingly, cfDNA originated from the tumour cells carries the mutational status of the primary tumour, as shown in 1994 by Sorenson et al., when mutations in *KRAS* found in tissue matched to the mutations in plasma samples for the same patients (Sorenson et al., 1994). Hence, the hope of liquid biopsy become possible as cfDNA is non-invasive and convenient while other tools used in CRC screening lack sensitivity and showed poor patient compliance.

Several studies investigated the potentiality of using the level of cfDNA to monitor CRC patients closely and to identify the high-risk recurrence in affected individuals. A study by Frattini et al., illustrated that the levels of cfDNA were significantly high in patients with CRC, while their cfDNA levels declined during follow-up when CRC patients were tumour-free and increase again in cancer recurrence (Frattini et al., 2005).

In another biomarker study that aimed to establish the normal range of cfDNA in a cohort of normal controls in comparison to patients with chemotherapy-refractory metastatic CRC (mCRC), the study found that the levels of cfDNA in mCRC are

markedly higher than the levels in normal individuals (Spindler et al., 2015). The study also demonstrated that overall survival is shorter with increasing levels of cfDNA. Also, they investigated the prognostic value of *KRAS* mutations in the plasma and their relation to the drug resistance (Spindler et al., 2015).

Since the integrity of the cfDNA may indicate tumour cell death, the length of cfDNA fragments might be, therefore, a potential biomarker for cancer detection. In a study by Umetani et al., the DNA integrity was calculated in sera of CRC patients (Umetani et al., 2006B), using qPCR. It was found that serum DNA integrity was considerably increased even with un-metastasised CRC. Hence, it could be a potential tool for early cancer detection. However, longer DNA fragments can release in other conditions such as acute inflammations infarctions or pregnancy. Such conditions may exclude from the screening for the neoplastic lesions (Umetani et al., 2006B). Moreover. It has been suggested that cfDNA can be used with CEA and can be a potential diagnostic tool in the early stages of CRC (Flamini et al., 2006). Flamini et al. investigated how the sensitivity and specificity would increase up to 100% if the ctDNA and CEA combined in the study of early stages of CRC (Flamini et al., 2006)

Heitzer et al., found in a study that a biphasic distribution of cfDNA fragments might occur in about one-third of mCRC patients (Heitzer et al., 2013). The study reported that not all CRC patients with progressive metastasis discharge cfDNA in the bloodstream in measurable amounts (Heitzer et al., 2013). In CRC cases presented with at least a single peak of high cfDNA levels in comparison to healthy individuals. However, it has very low frequency of mutant cfDNA combined with a high background of wild-type DNA, which might explain the raise in total, but not mutant cfDNA. On the other hand, a biphasic distribution may reflect various biological processes as they are linked to the mutant cfDNA levels with an increased number of circulating tumours cells (CTCs) (Heitzer et al., 2013) (Siravegna and Bardelli, 2016). This more likely indicates an extensive cell death that is followed by the release of cfDNA into the bloodstream.

1.4.1.4 **Genotyping cancer alleles in tumour cfDNA of CRC patients**

Interestingly, tumour derived DNA or circulating tumour DNA (ctDNA) is tumour specific, and can potentially reduce or abolish false-positivity results linked to commonly utilised tumour biomarkers. Distinguishing tumour-derived DNA fragments

from normal cfDNA support the fact that tumour cfDNA harbour somatic mutations, which are single base pair substitutions. They are only found in the genomes of tumour cells and pre-cancerous cells, but not in the normal cell of the same person (Mouliere et al., 2013). Detection of these mutations in the cfDNA is the most significant application as a biomarker. Many studies and techniques investigated the hotspot alterations in *BRAF* and *RAS* genes in CRC patients (Siravegna and Bardelli, 2014) (Mouliere et al., 2013).

cfDNA is a potential biomarker of minimal disease residual after surgery and can identify which patients will obtain recurrence. Efficiently, the circulating tumour DNA could be measured after the operation and before commencing the neoadjuvant treatment and helps in therapeutic decision-making (Diaz Jr et al., 2012). In a study by Diehl et al., linked the presence of high levels of cfDNA in 18 metastatic CRC after surgical resection of cancer to the high rate of recurrence while others patients with undetectable cfDNA remain disease free (Diehl et al., 2008).

Recently, a study by Tie and colleagues applied tumour cfDNA analysis to assess tumour burden and predict the therapeutic response to chemotherapy in early stages CRC cases (Tie et al., 2015). Somatic mutations detected in tissue samples collected at the time of the diagnosis were traced in matched plasma to measure the tumour burden non-invasively. This study featured the reflection of mutational load in plasma on the cfDNA release into the circulation and may be utilised to recognise the best therapeutic response (Tie et al., 2015).

1.5 Hypothesis Aims and objectives:

1.5.1 Hypothesis:

The hypothesis to be tested in this thesis is that circulating tumour DNA is a potential clinical biomarker for patients with localised colorectal cancer and may provide an important information on prognosis.

1.5.2 Aims

To investigate the use of cfDNA as a circulating biomarker in CRC.

1.5.3 Objectives:

1. To extracted DNA from primary tumour tissue and matched plasma samples.
2. To quantify the tumour (DNA) tDNA and cfDNA using ALU-qPCR method.
3. To develop a mutation detection assay for oncogenic driver mutations in *KRAS, PIK3CA,* and *BRAF* in CRC cases using AS-LNA/PNA-clamping qPCR in tDNA.
4. To investigate whether mutations detected in CRC tumour tissue can also be detected in the same patient's cfDNA.
5. Can this approach be used to monitor therapy and predict outcome in patients with more advanced tumours?
6. To investigate the importance of the total cfDNA levels in relation to the stage of the disease, lymph nodes metastasis and patient survival

Screening oncogenic mutations in primary tumour tissue and matched cfDNA in plasma can promise reliable biomarkers for early detection of the disease, especially in high-risk group, monitor therapy and predict the outcomes in patients with more advanced tumours. The emphasis will be on optimising detection technique sensitivity in the minority mutant DNA within degraded DNA extracted from clinical tissues and matched cfDNA.

Chapter 2

Materials and Method

2.1 Materials

2.1.1 Patient recruitment and samples

In this study, patients recruitment was carried out by a clinical fellow (Aslam, 2016), who developed the ethics and collected patient samples (Ethics number 05/Q2502/28). Patients with CRC were invited to participate in the study from the Department of Colorectal Surgery at Leicester General Hospital, University Hospitals of Leicester NHS Trust.

Patients attending follow-up outpatient clinic appointments after colonoscopy examination also participated in the study at University Hospital of Leicester NHS Trust. Recruitment started in October 2008 and finished in December 2015. We received fixed tissues with matched plasma from all 84 patients.

Patients with CRCs gave their blood samples that were collected from them 1 to 2 hours before colonoscopy procedure in the endoscopy unit, or with 24 hours before surgical resection of the tumour. Blood samples were also collected from participants in surgical outpatient clinic on the day of their follow-up appointment.

2.1.2 Blood samples processing

Blood samples were processed to recover plasma and buffy coat, as part of the study only blood samples form 74 patients were collected. 15 ml EDTA blood was taken and processed. Blood was centrifuged according to the protocol described by Page et al (Page et al., 2013) to isolate plasma and buffy coat. Plasma aliquots were stored in Eppendorf tubes at -80°C in HTA licenced facility. Each Eppendorf was labelled with a study number; participant's unique identification number and date of collection. The plasma samples were identified, and archived according to the Good Clinical Practice (GCP) Guideline and relocated to HTA freezer room with help from Mrs Linda Potter.

2.1.3 **Assay controls and Cell lines**

Six human cell lines (Table 2-1) were obtained from the American Type Culture Collection (ATCC) and Culture Collections Public Health England – UK (CCPH). These were used as positive controls for the designed assays of gene mutations detection (Table 2-1).

Cell line	**Gene mutation**	**Cell-Line type**	**Manufacture**	**Catalogue No**
A375-P	*BRAF* V600E	Malignant melanoma	ATCC	ATCC® CRL-3224™
MCF7	*PIK3CA* p.E545K	Human Breast adenocarcinoma	ATCC	ATCC® HTB-22™
HCT116	*KRAS* p.G13D and *PIK3CA* p.H1047R,	Human colon carcinoma	ATCC	ATCC® CCL-247™
GP2d	*KRAS* p.G12D	Human Caucasian colon adenocarcinoma	CCPH	95090714
SW626	*KRAS* p.G12V	Human Caucasian ovarian metastasis of a primary colon adenocarcinoma	CCPH	91091203
SW948	*PIK3CA* p.E542K	Human Caucasian colon adenocarcinoma	CCPH	91030714

Table 2.1 Cell lines used as positive controls for the gene mutations.

2.2 Tissue processing

2.2.1 Haematoxylin and Eosin (H&E) staining of FFPE tissue

H&E was carried out by deparaffinising the tissue sections in two tanks of Xylene, 10 mins each and rehydrated gradually 100% ethanol, 90% ethanol, and 70% ethanol, respectively for 10 mins each. Later the slides were washed with water for 3 mins and stained with Mayer's haematoxylin (Fisher) for 5 mins. Slides were washed with acid alcohol (1% HCl, 70% ethanol) for 10 seconds to remove excess stain. After a brief wash with water, slides were stained with 1% Eosin for one min and rinsed briefly with water, then slides were put under running tap water to remove the excess stain. Sections were dried by passing them through tanks of IMS 95%, 99%, and 99%. Slides rank was passed through xylene twice for 5 mins. Finally, stained sections were mounted with coverslips using DPX and examined under a microscope.

2.2.2 Manual Microdissection

For each case, three to four formalin fixed paraffin embedded (FFPE) sections were provided from tumour and normal colonic and rectal tissue. The FFPE sections were cut into approximately 1 to 1.5 cm^2 (Figure 2-1), and related H&E slides were stained by the laboratory technician Mrs. Linda Potter. H&E slides were examined by Dr. Kevin West (histopathologist), and different blocks were checked to obtain blocks rich in tumour tissue. Each section was microdissected to increase the percentage of tumour and normal cells separately (Figure 2-1). Manual microdissection was performed by marking the edges of epithelial tumour or normal tissue on H&E slides then similarly the procedure was carried out on the FFPE sections. One to three tissue sections were chosen, the number of sections taken depended on the amount the tissue in the section and proportion of tumour.

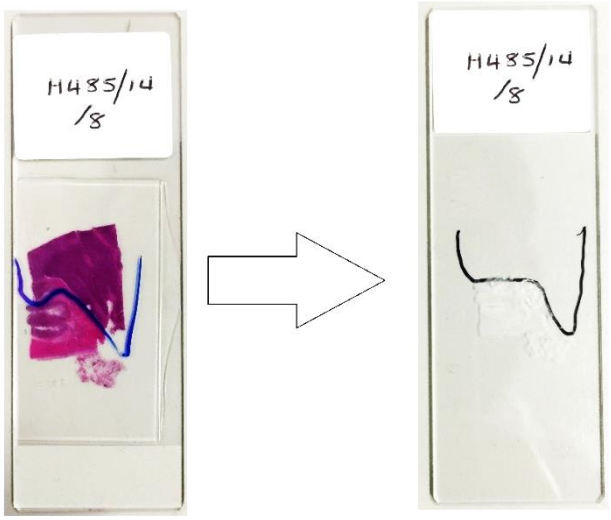

Figure 2-1 Manual microdissection of tumour sections (A) shows H&E slide for tissue section with marked tumour tissue. (B) Tumour section shows the remaining of the section after microdissection of the tumour tissue

2.2.3 **Genomic DNA extraction from fixed tissue**

The wax was removed from tissue sections in Xylene and rehydrated in IMS (99% and 95%). DNA extraction was carried out using QIAamp DNA Blood Mini Kit (Silica Membrane-Based Column Extraction; Qiagen, Hilden, Germany) according to Good Clinical Practice (GCP) Guideline and the manufacturer's recommendations. Sections were treated with ten µl Proteinase K (10mg/ml) for four days at 56°C. 200 µl of Buffer AL was added and incubated at 70°C for ten mins. At room temperature (RT), 200 µl of absolute ethanol (Fisher Chemical, Loughborough, UK) was added.

The mixture was transferred to a QIAamp column and centrifuged for one min at 4300xg. The columns were put in new collection tubes. 0.5 mL of Buffer AW1 was added and centrifuged for one min at 4300xg. The procedure was repeated with 500 µl Buffer AW2 and the columns were centrifuged for one min at 13100xg. To remove the ethanol, columns were put in new collection tubes and then subjected to a dry spin for one min at 13100xg. Elution was performed by adding 80 µl Buffer AE, and incubated for five mins at RT followed by microcentrifuge for one min at 4300xg.

2.2.4 **Circulating free DNA extraction**

The Circulating Nucleic Acid kit (Qiagen) was used to extract cfDNA from the plasma. This was shown previously to give consistently higher yields and more efficient recovery of cfDNA than the other kits tested (Page et al., 2013) (Sorber et al., 2016).The frozen plasma sample was thawed at RT and centrifuged at 1000xg for five mins at 4℃ to remove residual precipitated cellular components. Three mL of plasma was mixed with 300µl of 20 mg/mL proteinase K (Qiagen, Hilden, Germany) the plasma supernatant with 2.4ml of Buffer ACL was incubated at 60℃ for 30 mins. After digestion, 5.4m1 of Buffer ACB was added and lysate-Buffer mix on ice for 5mintes. The samples were loaded into QIAamp Mini columns and connected to Vacuum Manifold. The mix was washed with 600µl of Buffer ACW1, 750µl Buffer ACW2 and lastly with 750µl ethanol in order. Columns were retained to the collection tubes and span at 13100xg for 3 mins. Columns were incubated at 56°C for ten mins to dry membrane completely. Lastly, 150µl of AVE were added, incubated for 3 mins and span at 13100xg for one min at RT to elute nucleic acids. The eluted cfDNA was stored according to GCP guidelines.

2.2.5 **Circulating free DNA lyophilisation**

It was necessary to lyophilise cfDNA to increase the concentration of cfDNA samples for downstream applications. The original volume of 150 µl of cfDNA was prepared in 1.5 ml Low Bind tubes. The lids of these tubes were pierced to make holes using a sterile needle. The samples then were frozen to a lyophiliser shelf temperature of −40°C in liquid nitrogen for 10-15 seconds and carried on dry ice to the lyophiliser.

Vials were placed on the shelf of a lyophiliser (PennTech SP Scientific-USA) which was prepared in advance with a chamber pressure of 50 mTorr to 100 mTorr and temperature of -52°C for 6hr to 10hr to be completely dry. The samples were spun and re-suspended in 20 to 25µl of 1xTE (low EDTA) and then transferred to another Low Bind tubes and stored in at -20°C.

2.3 Nucleic Acid Quantification

2.3.1 Quantification of DNA in FFPE tissue

DNA concentration were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). At the beginning and between samples the pedestal and lid were cleaned with optical instrument cleaner. The Software was initialized with ultra-pure water and blank sample with the buffer used to suspend the nucleic acid. One μl volume of DNA was loaded to the pedestal. At the start in and between samples the pedestal and lid were cleaned with optical instrument cleaner. Samples were measured, and their purity was determined by observing the absorbance readings at 260 and 280 nm, with DNA having an optimal A260/280 ratio of ~1.8.

2.3.2 cfDNA quantification using ALU69 repeat

DNA concentrations were measured using ALU-qPCR based technique and a standard curve. An ALU69 repeat assay was used to determine DNA levels in circulating free DNA samples. The samples in duplicates and no template controls were used to show any potential contamination.

Standard curves were developed by creating a serial dilutions from 10ng DNA to 0.019ng, nine dilutions were diluted 1:2 to and in duplicate and then converted cfDNA concentrations to yield in ng/mL (Page et al., 2011). No Template Controls (NTC) were used to control for potential contamination. Absolute quantification (AQ) of cfDNA was then performed by measuring this relative to the standard curve of human genomic DNA. In addition, efficiency of the subsequent standard curves of cfDNA quantification were within the 90-110% range that required for maximal efficiency and accuracy.

The Protocol was adapted and updated (Last version 2014) from the current protocol that used the housekeeping gene GAPDH as a target (Shaw et al., 2011). Primers and probe sequences are provided in Table 2-2.

Oligonucleotide		Sequence
ALU_F	Forward	GACCATCCCGGCTAAAACG
ALU 69 VIC MGB	Probe	CCCGTCTCTACTAAA
ALU_69_ r	Reverse	CCACTACGCCCGGCTAATTT
ALU_124_r	Reverse	TCCTGCCTCAGCCTCCCAAG
ALU_201_r	Reverse	GCTCTGTCGCCCAGGCTGGAGT

Table 2-1 Primer and probe sequences of the ALUs that were tested and used to quantify the DNA Supplier SIGMA –GENOSYS LTD(Sigma-ALDRICH HOUSE ,HOMEFIELD Road , SUFFOLK – CB9 8QP)

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2.4 **Real-time polymerase chain reaction qPCR**

Real-time quantitative qPCR assays were processed on the StepOnePlus™ v2.3 Real-Time PCR System, microAMP 96-well plates, adhesive films, custom designed and FAM and VIC-labelled TaqMan probes, TaqMan® Fast PCR Universal Master mixes and genotyping were from *Applied Biosystems* (Foster City, CA). Volume of a 10µl reaction was used in each well, composing of 5µl TaqMan® PCR Master Mix (Applied Biosystems™), 0.4µl of primer and 0.2µl probe at 100pM per well, and volume of 3.6µl of 10ng DNA sample.

Human genomic DNA (HG DNA) (Roche Diagnostics Ltd., https://www.roche-appliedscience.com) served as a wild- type DNA control for each experiment and ultra-pure water was also used as 'no template' controls. In each assay, samples were run in duplicate and sometimes in triplicate. StepOne Software v2.3 (Applied Biosystems™, http://www .appliedbiosystems.com) and Microsoft® Excel were used to analysis the data.

Real time data and threshold cycle (Ct) values were collected during the last 50 cycles of the amplification using StepOne Software v2.3 (Applied Biosystems). Also, delta (Δ) Ct values were determined by the difference in the Ct values of mutant and wild-type alleles, ΔCt was calculated according to the formula; ΔCt = (Ct [mutant primer] - Ct [WT primer]).

2.5 Design of Primers and Probes

DNA sequence for the gene of interest was obtained by using the NCIBI's 'Map Viewer' tool. Output sequences were subject to a homology search with other regions in the human genome by pasting the DNA sequence on http://www.ncbi.nlm.nih.gov/ and select 'nucleotide blast'. If there are any SNPs in the sequence, they would be detected at the same time, which should be avoided as they misrepresent the result of the qPCR. Oligonucleotide primers and TaqMan® MGB probes (Applied Biosystems™, Life Technologies Corporation, http://www.appliedbiosystems.com) were initially designed using Primer3 v. 0.4.0 (Whitehead Institute, MIT http://primer3.wi.mit.edu/). The amplicon size was restricted to <180bp with primer Tm 57-70ºC and GC% of 36-60. The software was also able to pick up appropriate probes between the forward and reverse primers.

After obtaining the primer and probe sequences, UCSC *in-silico* PCR (https:// genome.ucsc.edu) was used to check the feasible amplicons produced from every primer pair and were screened for distinctiveness in the human genome. In further step OligoAnalyser online software by IDT (https://www.idtdna.com/calc/analyzer) was used to check the primers for any Hairpin formation and their self-complementarity. Sequences were then imported into Primer Express® v3.0.1 (Applied Biosystems™) for further optimisation. Target sequences were also investigated for genetic anomalies such as amplifications, deletions, and LOH using the Catalogue of Somatic Mutations in Cancer (COSMIC). Primers and probes were supplied by SIGMA –GENOSYS LTD (Sigma-ALDRICH HOUSE, HOMEFIELD Road, SUFFOLK – CB9 8QP) and Applied Biosystems UK (7 Kingsland Grange, Cheshire –WA1 4SR)

Oligonucleotide sequences detailed in Tables 2-3 to 2-5.

Oligonucleotides	Primer /Probe	Sequence (5' → 3')	Tm °C	GC %	length
BRAF **V6000E**	F	TCATGAAGACCTCACAGTAAAAATAGGT	58.7	36	28
	R	ATCCAGACAACTGTTCAAACTGATG	58.2	40	25
	wt- P	CTAGCTACAGTGAAATC	68	41	17
	mut- P	TAGCTACAGAGAAATC	68	38	16
	wt-F-LNA	TAGGTGATTTTGGTCTAGCTACAG+T	-	40	25
	mut-F-LNA	TAGGTGATTTTGGTCTAGCTACAG+A	-	40	25
	R	ATCCAGACAACTGTTCAAACTGATG	58.2	40	25

Table 2-2. Primers, probes, primers with LNAs for BRAF V600E mutation assay. (P) probe; (F) forward primer; (R) reverse primer; wt (P) wild-type probe; mut-P mutant probe; (LNA) locked nucleic acid, supplied by *Applied Biosystems* UK (7 Kingsland Grange, Cheshire –WA1 4SR)

Oligonucleotides	Primer /Probe		Sequence (5' → 3')	Tm °C	GC %	length
PIK3CA Primers	E542K / E545K	F	GGAAAATGACAAAGAACAGCTCAA	58.5	38	24
	E542K / E545K	P	TATGGAGTCACAGGTAAGT	68	42	19
	E542K / E545K	R+LNA	ATCTCCATTTTAGCACTTACCTG+T	50.1	37	24
	E542K / E545K	R	ATGCTGAGATCAGCCAAATTCA	58.4	41	22
	A3140G:H1047R	F	CAAGAGGCTTTGGAGTATTTCATG	58	42	24
	A3140G:H1047R	P	ATGGATTGGATCTTCCACACA	68	43	22
	A3140G:H1047R	R	5'ACAGTGCAGTGTGGAATCCAGA'3	59.2	50	22
PIK3CA Probes	G1633A:E545K	wt-P	ATCACTGAGCAGGAGAA	69	47	17
	G1633A:E545K	Mut-P	ATCACTAAGCAGGAGAA	66	41	17
	G1624A:E542K	wt-P	CTCTCTCTGAAATCAC	68	44	16
	G1624A:E542K	Mut-P	CTCTCTCTAAAATCAC	66	38	16
	A3140G:H1047R	wt-P	ATGCACATCATGGTGG	67	50	16
	A3140G:H1047R	Mut-P	ATGCACGTCATGGTG	66	53	15

Table 2-3 Primers, primers with LNAs, wild –type and mutant probe for *PIK3CA* mutation assay, supplied SIGMA –GENOSYS LTD (Sigma-ALDRICH HOUSE, HOMEFIELD Road, SUFFOLK – CB9 8QP).

Oligonucleotides	Primer /Probe	Sequence (5' → 3')	Tm °C	GC %	length
***KRAS* FP1**	F	TATAAGGCCTGCTGAAAATGAC	55 °C	41	22
***KRAS* G12D (35G>A)**	Mut R+LNA	GCACTCTTGCCTACGCCAT-LNA	57.5 °C	58	19
***KRAS* G12V (35G>T)**	Mut R+LNA	CACTCTTGCCTACGCCAA-LNA	54.5 °C	56	18
***KRAS* G13D (38G>A)**	Mut R+LNA	CAAGGCACTCTTGCCTACGT-LNA	56.7 °C	55	20
KRAS WT	WT R+LNA	ACTCTTGCCTACGCCAC -LNA	57.9 °C	59	17
***KRAS* FP78:**	F	AGGCCTGCTGAAAATGACTGA	58.5° C	48	21
***KRAS* RP78:**	R	TGTATCGTCAAGGCACTCTTGC	59°C	50	22
Probe (FAM-MGB)	Probe	AACTACCACAAGTTTATATTCA	70 °C	59	22

Table 2-4 Primers, probes, and primers with LNAs for *KRAS* mutations assay, supplied by *Applied Biosystems* UK (7 Kingsland Grange, Cheshire –WA1 4SR)

2.6 Peptide Nucleic Acids

2.6.1 PNA: definition and chemical properties:

The Peptide Nucleic Acid (PNA) oligomer is a synthetically created nucleic acid analog in which ribosephosphate backbone is substituted by peptide bond-linked of N-(2-aminoethyl)-glycine (NA-G) units to which nucleobases are joined via a methyl carbonyl linker. (Kwon et al., 2011) (Araki et al., 2010) (Beau-Faller et al., 2009) (Paulasova and Pellestor, 2004). PNA is an artificially synthesised oligomer, which was invented by Peter E. Nielsen (Univ. Copenhagen), in 1991. The PNA special chemical makeup produces strong hybridization to the nucleic acid sequence to form a double strand product (Nielsen et al., 1991).

Contrary to DNA and RNA, the PNA oligomer backbone is not charged. Therefore, PNA hybridises to the DNA sequence without electrostatic repulsion, providing a strong and stable PNA–DNA duplexes than the natural homo- or hetero-duplexes. As a result of this bonding, high thermal melting temperature (Tm) is produced than what was noticed in DNA–DNA or DNA–RNA duplexes (Figure 2-2) (Wittung et al., 1994) (Paulasova and Pellestor, 2004) (Jeong et al., 2011).

Polyamide backbone also makes PNA hybridisation almost independent of the salt concentration. Hence, melting temperature (Tm) of PNA–DNA combination is hardly altered by low ionic level. This feature promotes the hybridisation with the PNA (Beau-Faller et al., 2009). Moreover, Tm of a completely matched PNA-DNA duplex is greater than that of the DNA-DNA of the same sequence, and a single mismatch will disrupt the PNA-DNA hybrids, leading to a Tm shift of 10-18˚C.

PNA prevents the amplification of targeted DNA sequence confined by a pair of primers PNA does not work as primers and does not affected by *Taq* polymerase enzymatic activities. Therefore, PNA is resistant to the enzyme degradation, and its life span is extended both in vivo and in-vitro. (Oh et al., 2010) (Paulasova and Pellestor, 2004)

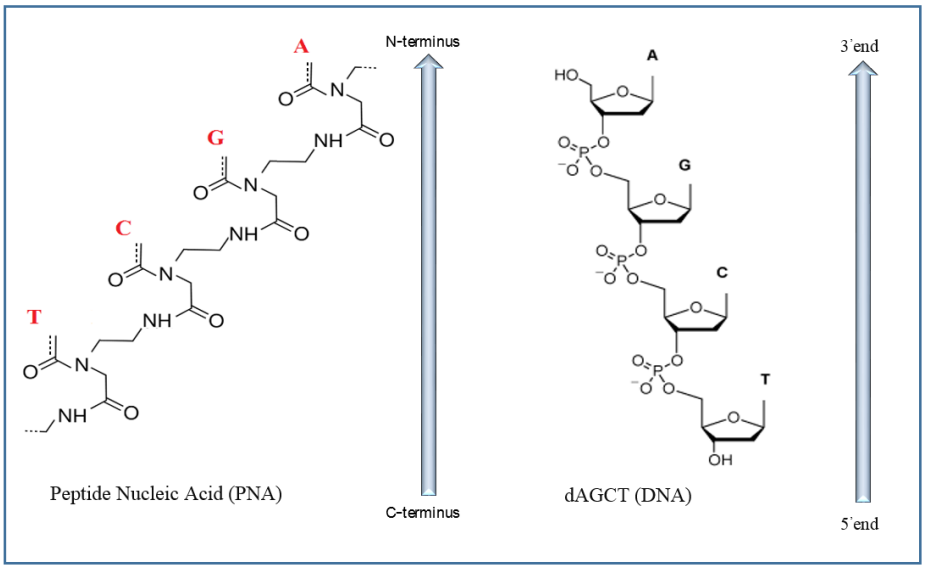


Figure 2-2: Comparison between the structure of PNA and DNA. The backbone of PNA shows 2-NA-G links in the position of phosphodiester backbone of DNA, and the nucleotides bases are linked to this backbone at the amino-nitrogens by methylene carbonyl bonds. (Gilje et al., 2008) (Paulasova and Pellestor, 2004).

2.6.2 **The importance of the PNA**

Cancer biopsies contain mixtures of stromal cells and cancer cells, and even pure tumour samples are genetically heterogeneous leading to low–level mutation detection, which is a challenging issue in the genetics of cancer. Moreover, the detection of mutant DNA in body fluids in early stages, including blood and urine needs a sophisticated approach in terms of mutation detection (Oh et al., 2010) (Parsons and Heflich, 1997).Therefore, several strategies have been established to detect tumour-specific mutations sensitively in presences of an excess of wild-type DNA (Gilje et al., 2008).

Many techniques aimed to optimise the sensitivity of mutation detection, but most of them are not suitable for clinical applications due to multiple procedural manipulations that are both cost-ineffective and time-consuming. Moreover, the risk of contamination during multiple transfers is high. Hence, it is important to establish simpler and useful approaches for clinical settings of low-level mutations detection (Beau-Faller et al., 2009) (Oh et al., 2010) (Kwon et al., 2011) (Jeong et

al., 2011). Therefore, PNA-mediated PCR clamping assay has been presented as highly sensitive and cheap technique for mutation detection (Kwon et al., 2011).

2.6.3 **PNA-mediated PCR clamping**

PNA-mediated PCR clamping technique is one of the most recent methods which have proven success in low mutation detection (Kang et al., 2015) (Jeong et al., 2013) (Miyano et al., 2012).The PNA-mediated PCR clamping technique has recently been improved to enrich the mutant alleles. It has become wildly used in combination with different PCR applications (Oh et al., 2010) (Araki et al., 2010) (Gilje et al., 2008). In qPCR, excess wild-type DNA exhausts necessary reagents and tends to veil mutations sequence signals during the detection process (Gilje et al., 2008). Thus, PNA has been used to overcome this difficulty to suppress wild-type DNA amplification and enrich the mutant alleles, so it can be detected by a procedure that provides an acceptable resolution to reveal mutant signals. (Jacobson and Mills, 1994) (Toyooka et al., 2003).

The PNA peptide nucleic acid (PNA) probes, which is complementary to wildtype DNA, clamp to the matched sequence and suppresses amplification of wild-type target, as a result it enhances the amplification of mutant sequences by competitively inhibiting DNA primer binding to wild-type DNA (Figure 2-3).

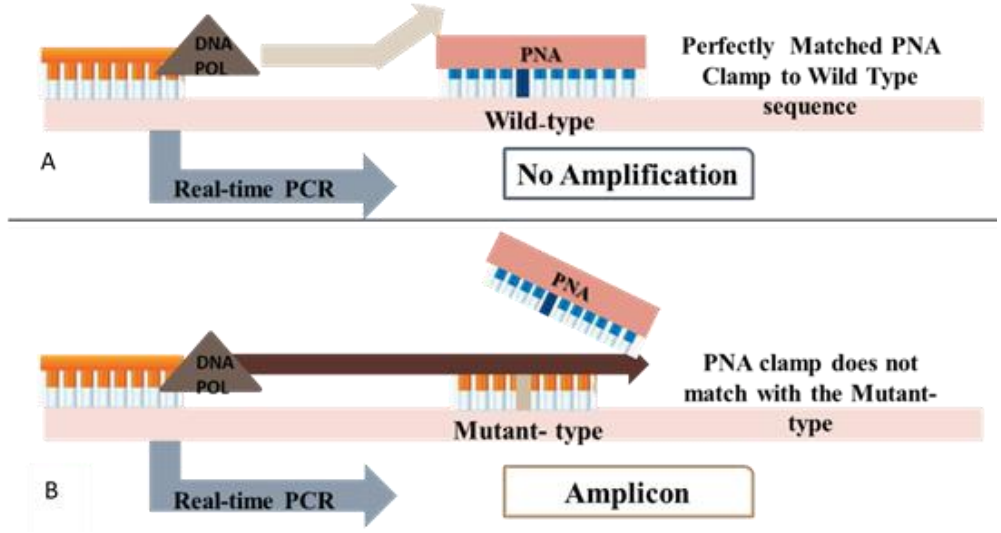


Figure 2-3: The mechanism of PNA action, (A): The PNA and the primer bind competitively to part of the same sequence. With wild-type targeted nucleic sequence the PNA binds to DNA and blocks primer annealing and elongation (B): in the case of the mutant allele, there is a mismatch between PNA and DNA causing a much weaker binding, allowing the primer to bind DNA, and elongation can take place.

2.6.4 **PNA design and synthesis**

All PNA oligomers were designed using the PNA Bio tool (107 N. Reino Rd. 242 Thousand Oaks, CA 91320 USA http://pnabio.com/support/PNA_Tool). This tool facilitates and aims to give information about the optimal design of the PNA oligomers, and it gives the recommended redesign possibility if the PNA sequence (Table 2-6) does not match the following conditions according to the manufacturer guideline:

- Anti-parallel orientation is preferred to design the PNA, although it can both orientations.
- Length is ideally between 12-18 bases. Short PNA are more specific and the effect of a mismatch is greater. Long PNA tends to accumulate, and it is hard to characterise and purify.
- Purines: PNA rich in purines tend to aggregate, the worst are G-rich oligomers. As a rule never have more than seven purines in any stretch of ten nucleotides.
- Self-Complementary sequences were avoided such as inverted repeats, hairpin forming, and palindromic sequences because a PNA/PNA duplex is more robust than PNA/DNA duplex.
- The position of mutant mismatch base was in the middle of the PNA rather than the peripheries.

PNA oligomer	Sequence (5' → 3')	Tm °C	Purines %	length
***PIK3CA* E542K**	CTCTCTCT**G**AAATCACTG	66.34 °C	33.3%	18
***PIK3CA* E545K**	TCACT**G**AGCAGG	64.86 °C	58.3%	12
***PIK3CA* H1047R**	ATGCAC**A**TCATGGTG	64.41°C	53.3%	15
***BRAF*: V6000E**	TAGCTACAG**T**GAAATC	61.8°C	56.3%	16
KRAS	TACGCCA**CC**AGCTCC	75°C	33.3%	15

Table 2.6 PNA oligomers for the most common hot-spot mutations in *PIK3CA*, *BRAF*, and *KRAS*. The underlined base is the location of the mutant mismatch base which is almost in the middle of the PNA.

The PNAs were synthesised by Eurogentec S.A. Liège Science Park-Rue Bois Saint-Jean 5-4102 SERAING BELGIUM, http://www.eurogentec.com). They were supplied in dry form and dissolve in pre-heated (60°C) sterile water up to 100µM.

PNA-mediated PCR clamping assay was further developed in combination with locked nucleic acid, the technique that will be explained in details later in this chapter. PNA-mediated PCR clamping sensitivity test's results -will be shown in the results chapter.

2.7 Allele Specific Locked Nucleic Acid (LNA)

2.7.1 What is the LNA?

Locked Nucleic acid (LNA) is bicyclic modified RNA nucleotide where the ribose sugar is structurally bounded by a methylene bond between the 4'–carbon and the 2'-oxygen atoms (Figure 2-4). This bond locks the ribose in a 3'-endo structural conformation (Vliegen et al., 2015). The melting temperature (Tm) of the duplex rises by 2–8 °C for each integrated LNA monomer (Dominguez and Kolodney, 2005). Therefore, an oligonucleotide with LNAs exhibit remarkable thermic stability as anneals to a targeted RNA or DNA strand. These properties allow shorter oligonucleotides while maintaining the desired Tm and enhance the mismatch discrimination (Chen et al., 2014) and it increases resistance to certain exo- and endonucleases (Oldenburg et al., 2008) (Kauppinen et al., 2006) (Morandi et al., 2012).

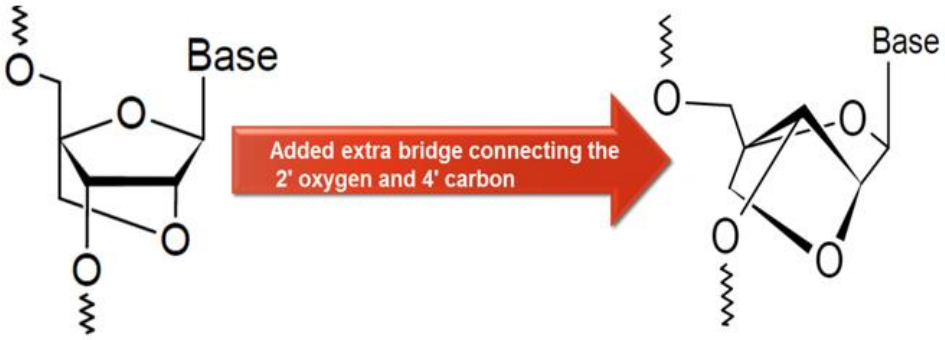


Figure 2-4 Locked nucleic acid structure. Showing the extra modified bond connecting the 4' carbon and 2' oxygen of the ribose moiety in a 3'-endo structural conformation.

2.7.2 Locked nucleic acid applications:

LNA has been used to improve the sensitivity and specificity of expression in DNA microarrays, FISH probes, quantitative PCR probes, enhancing Sanger sequencing sensitivity and other molecular biology techniques based on oligonucleotides (Ishige et al., 2016) (You et al., 2006) (Vester and Wengel, 2004). Moreover, The enhanced *Tm* led to LNA-modified nucleotides, which enhanced the *Tm* in several applications, such as in situ hybridization (Kubota et

al., 2006), whole genome amplification (Sun et al., 2008), methylation sensitive PCR (Morandi et al., 2012), germline SNP genotyping (Latorra et al., 2003), as blocker probe in order to suppress wild-type alleles and to improve the PCR sensitivity. Blocker LNA oligonucleotides showed considerable level of sensitivity to detect mutations such as *KRAS* and *BRAF* mutations (Arcila et al., 2011) (Morandi et al., 2012).

2.7.3 **Allele Specific LNA (AS-LNA) QPCR and mutation detection**

Since most clinical specimens contain a mixture of normal and tumour cells, mutation detection techniques must be able to detect mutations in tissue or/and plasma samples that often carry an excessive wild-type DNA combined with mutant DNA. Allele-specific LNA qPCR (AS-PCR) can detect at least one mutant copy of genomic DNA in 10^2 wild-type species (Jarry et al., 2004). This technique uses a forward primer with a 3' terminal that is unique to the mutant rather than the wild-type alleles. The selectivity of this method is limited by unwanted amplification of wild-type DNA, creating a false mutant template that is generated by advance PCR cycles. In this project, this disadvantage has been fixed by incorporated PNA-mediated PCR clamping method as described in the previous section 2-8.

In this project, Allele Specific LNA (AS-LNA) qPCR is based on allele specific discrimination qPCR. Hence, two forward primers for mutation- and non-mutation-specific primers. The forward or the reverse mutation-specific primer was modified with LNA nucleotides at the 3′-end of the oligonucleotide sequence as described by Morandi *et al*. (Morandi et al., 2012). The primers with LNA are listed in Table 2-3, 2-4 and 2-5. They were supplied by Eurogentec S.A. Liège Science Park-Rue Bois Saint-Jean 5-4102 SERAING BELGIUM, http://www.eurogentec.com).

2.8 Touchdown-qPCR

2.8.1 What is the touchdown thermo-cycling programme?

It is a modified technique in which initial annealing temperature should be several degrees higher than the estimated *Tm* of the primers, and is gradually decreased over subsequent cycles until the calculated annealing Tm temperature is reached. Lowering the temperature gradually permits more annealing during the course of cycling. The amplification of the desired amplicon then continues at the favoured annealing temperature (Don et al., 1991) (Roux, 1994) (Hecker and Roux, 1996).

2.8.2 How does TD-PCR work better?

In PCR, optimal annealing temperature is an essential requirement. This is normally based on the melting temperature (*Tm*) of the primer template pair. At temperatures just lower than this point, only specific base pairing happens between primers and the template. More temperatures below, the primers become less specific and obscure the polymerase chain reaction results. Moreover, the *Tm* of the primers can be affected by individual buffer elements, primers and templates concentrations.

Therefore, calculation of *Tm* value is just estimation and sometimes difficult to find the correct temperature for the targeted templates and designed primers (Korbie and Mattick, 2008).Very low annealing temperatures can cause primer-dimers formation and non-specific products. This will obscure the PCR results as non-specific sequences will bind to primers in early stages of the amplification and block any specific sequences due to the exponential nature of the PCR amplification , whilst, too high temperatures causes weak primers annealing and decrease the yield (Don et al., 1991)) (Hecker and Roux, 1996).

The initial steps of the Touchdown-qPCR (TD-PCR cycle) have high annealing temperatures than the estimated *Tm*. The annealing temperature decreases in the following subsequent set of cycles. Then the primers will anneal at high temperature to form desired amplicons that is less likely to non-specific sequence to tolerate. Hence, the first amplicon to be produced will be at the region of highest

primer specificity, which most likely the area of the interest (Don et al., 1991)) (Hecker and Roux, 1996).

These sequences will be further amplified in the subsequent cycles at lower temperatures, and will outcompete with non-specific products or primer dimers that primers may bind to at lower temperatures. Therefore, TD-PCR boosts specificity at higher temperatures and raises the efficiency toward the end of the reaction by lowering the annealing temperature and that increases the outcome of the PCR (Don et al., 1991) (Hecker and Roux, 1996) (Wu et al., 2005).

2.8.3 **Touchdown-qPCR development and conditions**

Wu et al in 2005 were the first to recruit the touchdown thermos-cycling programming in allele specific PCR (AS-PCR), which led to progressive decreasing in annealing temperature and stressing the difference between the match and mismatch allele specific primers to raise the specificity and the sensitivity of AS-PCR (Wu et al., 2005).

In this project the touchdown thermos-cycling programme was utilised to optimise the efficiency of the qPCR during the primary stages of the *PIC3CA* assay design. The conditions of the touchdown-qPCR included denaturation at 95 ^{0}C for 10 min, followed by a total of 50 cycles at 95 ^{0}C for 15 sec. The annealing temperature of the first 12 cycles was set 10 ^{0}C higher than the estimated melting temperature, calculated with the nearest method starting by the cycle 2 and during 10 cycles, the temperature decreased by 1 ^{0}C each cycle. The annealing temperature was maintained for the subsequent 40 cycles at 60 ^{0}C (Figure 2-5).

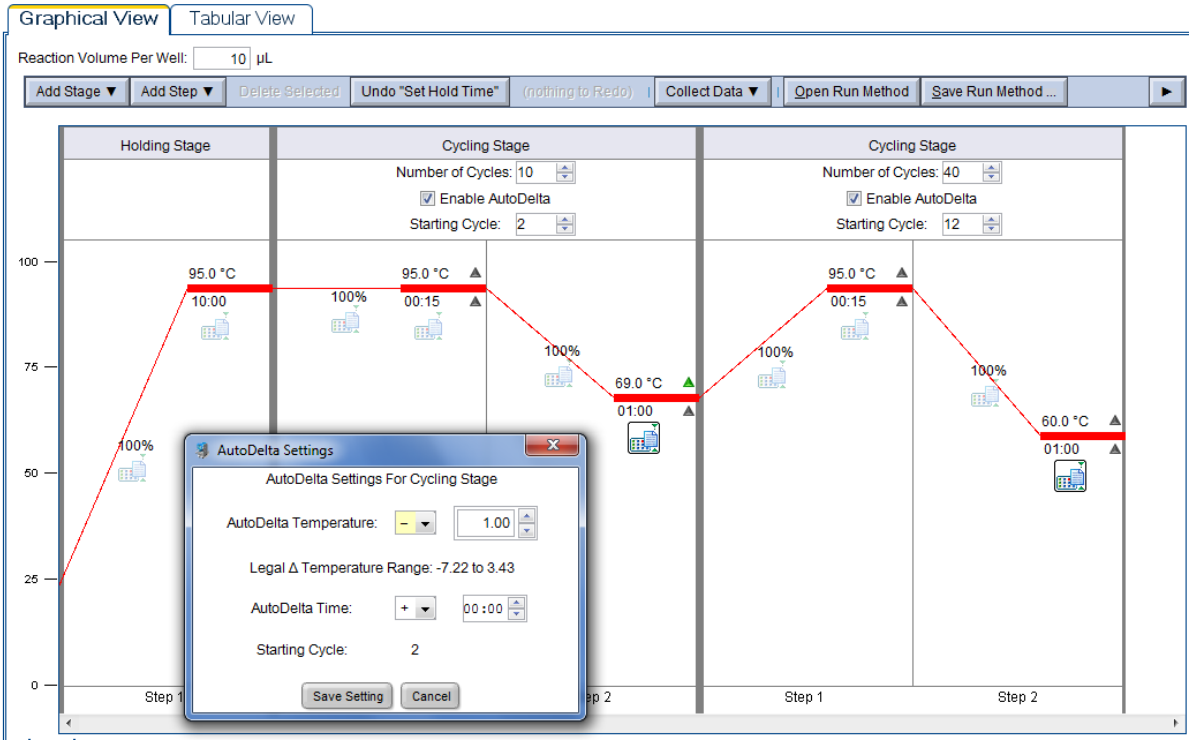


Figure 2-5 TD-qPCR, the temperature was started with is 10 0C higher than that calculated annealing temperature and dropping by 1 0C every cycle. The remaining cycles was carried out at the annealing temperature of 60 0C.

2.9 STATISTICAL ANALYSIS

Descriptive statistics and Mann-Whitney U test, Kruskal Wallis test, Jonckheere-Terpstra test, Fisher's Exact test and chi-square, One-way analysis of variance were used to assess the clinic-pathological variables associated with baseline ctDNA levels and mutations. The Wilcoxon-matched pairs signed-rank test was used to compare pre and post-treatment cfDNA levels. Receiver operating characteristic (ROC) curve was used to determine the most appropriate cfDNA cut-off for differentiating patients with negative and positive lymph nodes and to measure the sensitivity and specificity of the assays. Statistical analysis was carried out using SPSS version 24 and GraphPad Prism version 7 (GraphPad Software, Inc., CA), where P values of <0.05 were considered significant. Kaplan-Meier and Cox regression analysis, was used to measure the overall patient survival.

Chapter 3

Assay development

3.1 Introduction

It has been shown that plasma carries small quantities of circulating cfDNA. Levels of cfDNA increase remarkably in some patients with cancer compared with healthy individuals especially in advanced disease (Lecomte et al., 2010). Therefore, cfDNA provides a promising biomarker for various cancers (Hauser et al., 2010). In healthy individual circulating cfDNA is predominantly derived from apoptosis of white blood cells (fragment size range 185 or 200*bp*) (Kohler et al., 2011). In contrast, tumour cells release DNA fragments of varying sizes by necrosis, apoptosis, and autophagy in the circulation (Umetani et al., 2006B). Recent studies have investigated using ALU-qPCR to detect total cfDNA in serum or plasma, to introduce interesting new prospects for diagnosis and follow-up of patients with cancer (Mead et al., 2011) (Umetani et al., 2006B) (da Silva Filho et al., 2013).

In cancer genetics, somatic mutation detection is important but this is also challenging in cfDNA due to the excess wild-type DNA and low frequency mutations. To improve mutation detection in cfDNA, previous assays have enriched mutant alleles or suppressing wild-type alleles amplification, followed by a detection process that produces a sufficient resolution to reveal mutant signals (Toyooka et al., 2003, Qiu et al., 2008) (Miyano et al., 2012). In the present study, PNA-mediated PCR clamping combined with Allele-Specific LNA (AS-LNA) oligonucleotides were used in a single step to detect different types of *BRAF*, *KRAS* and *PIK3CA* mutations in a ratio of less than 1:1000 wild-type alleles in both FFPE tissue samples and cfDNA.

3.2 Aims and objectives

The aim of this chapter is to develop a sensitive mutation detection and DNA quantification assays for analysis of primary tumours and matched cfDNA in CRC patients.

Objectives

1. To compare three ALU repeat -qPCR assays of increasing amplicon size to determine, which is optimal in terms of assay efficiency.
2. To compare the selected optimal ALU-qPCR assay with the housekeeping gene GAPDH assay for cfDNA quantification.
3. To utilise the ALU assay in determining the quantities of cfDNA in plasma samples of CRC patients.
4. To develop mutations detection assays for key oncogenic drivers and validate the assays using cell lines that harbour the targeted mutations and apply the technique on a cohort of CRC using DNA extracted from primary tumours and matched cfDNA.

3.3 Results:

3.3.1 cfDNA quantification using ALU repeats

In this study, three different length assays were compared to target ALU repeats (ALU 69, ALU 124, and ALU 201). The three ALUs vary in their length and share the same forward primer and probe (Table 2-2) (Figure 3-1). A standard curve of a serial dilution of 1:2 was performed on human genomic DNA reference to compare between the three ALU assays. The efficiency of ALU69 was 99.3%, the Regression coefficient (R^2) value >0.99, which made it the best choice for DNA quantification assay (Figure 3-3). The other two ALUs had lower efficacy, <90, which is out of the recommended efficiency range within 90-110% that required for maximal efficiency and accuracy (Herbst-Kralovetz et al., 2008) (Figure 3-2).

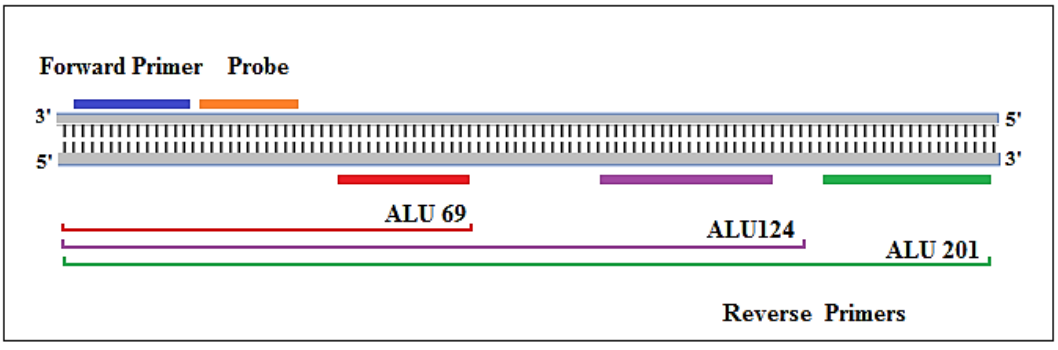


Figure 3-1 Illustration of the three ALU assays. The ALUs assays have the same forward primer and probe and different reverse primers, so the amplicons vary in length according to the reverse primer used.

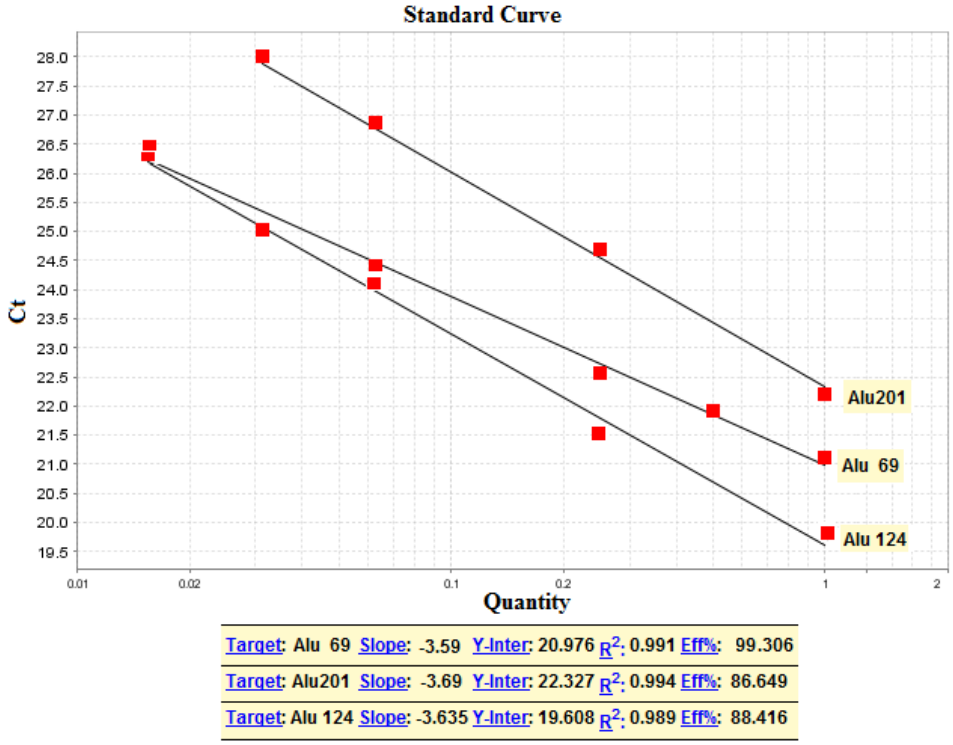


Figure 3-2 Comparison between three ALU assay, the illustration shows ALU 69 as the most efficient assay for DNA quantification.

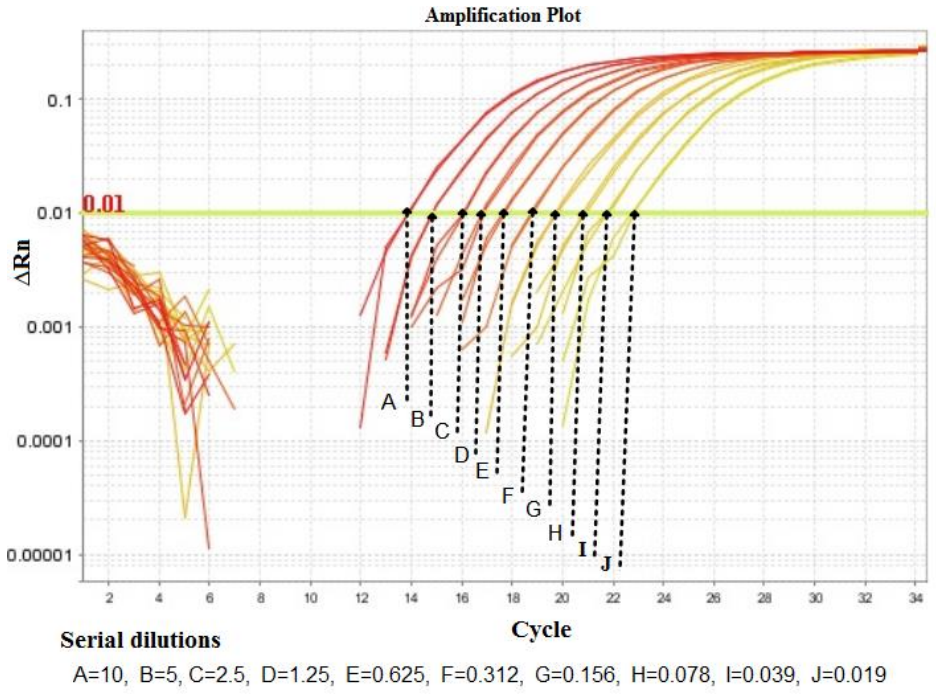


Figure 3-3 Validation of the ALU69 assay efficiency. Serial dilutions of human genomic DNA was used to validate the assay. The dilution started from 10ng as to 0.019ng. The CT values range from 14 to 22 indicating excellent efficiency with very low-frequency DNA.

An assay was designed to compare between ALU69 and GAPDH on serial dilutions of Human genomic DNA reference (HG-DNA) (Figure 3-4). The pilot experiment showed the efficacy of both techniques, with efficiencies of 94% and 92% for the ALU69 and GAPDH respectively. The cycle threshold (Ct) of the amplicons were from 12 to 17 with the ALU69 assay and 22 to 27 with GAPDH assay. Both assays had an R^2 value of >0.99, indicating good efficiency and repeatability. The 10 Ct difference between the 2 assays can be explained by the fact that the ALU assay targets a common repeat, which is a shorter amplicon to maximise amplification from heavily fragmented cfDNA, whereas the GAPDH targets a single copy locus in an intron.

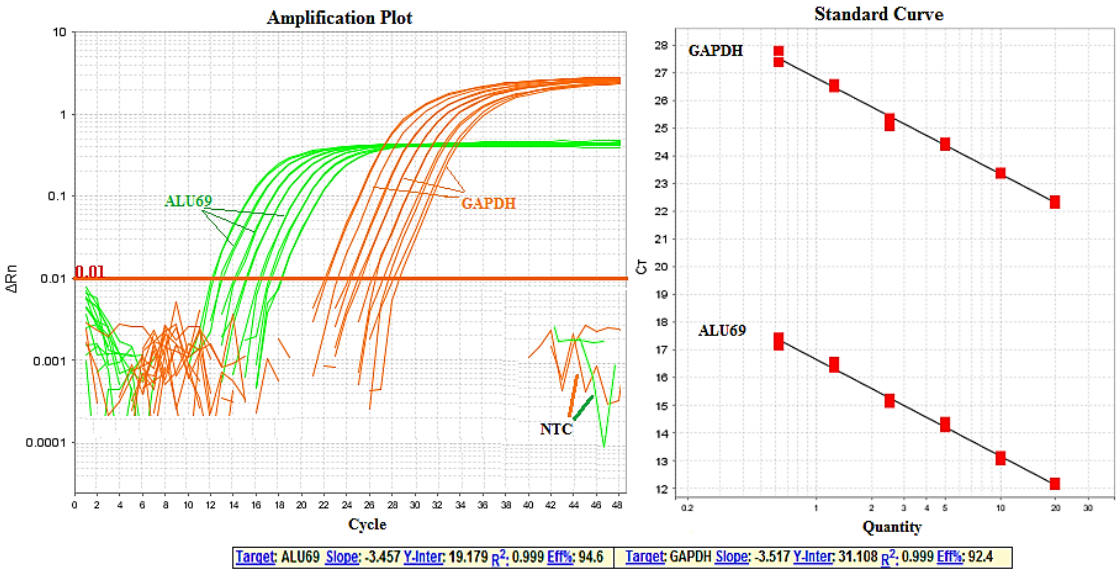


Figure 3-4 Comparison between ALU69 and GAPDH in relation to DNA quantification on the same DNA concentration of 5ng. The ALU69 was more efficient than GAPDH and can quantify smaller DNA fragments.

3.3.2 **Mutation Detection Assay Design**

Development of mutation detection assays was based on a combination of the three techniques described in Chapter 2; PNA-mediated qPCR clamping, Allele-

Specific LNA (AS-LNA) qPCR and Touchdown qPCR, which are described in details below.

3.3.2.1 *BRAF* and *KRAS* mutation detection assay

Mutation detection assays were developed for *BRAF* (V600E) and *KRAS* mutations (*G12D, G12V* and *G13D*). This method was based on allele-specific qPCR using LNA primers for the mutant alleles. In the *BRAF* assay, specific LNA was integrated with the forward primer of the mutant allele (*V600E*), in addition to wild-type forward LNA primer (Figure 3-5). While, in the *KRAS* assays, three reverse LNA primers were designed for *G12D, G12V* and *G13D* mutation sites, besides a wild-type reverse LNA primer (Figure 3-5). The sequences and characteristics of the selected primers and probe are presented in Table 2-3 and 2-5. A single PNA oligonucleotide was also designed for each assay to clamp the wild-type sequence and prevent it from being amplified. The PNA was designed according to the manufacturer recommendations as described in section 2.8.4, the sequences and characteristics of *BRAF* and *KRAS* PNAs are illustrated in Table 2-6.

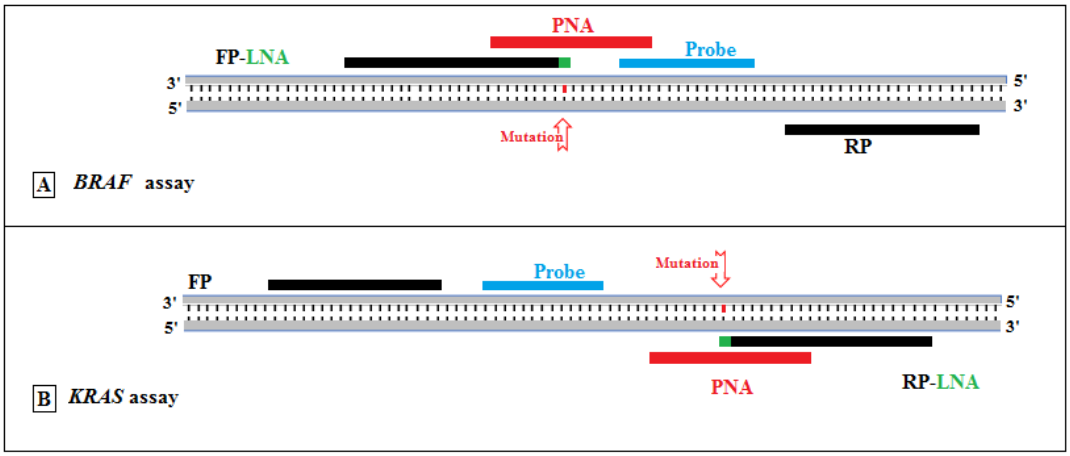


Figure 3-5 Illustration of designed *BRAF* and *KRAS* assays, A: *BRAF* assay and B: *KRAS* assay show the positions of the PNAs and LNA and the conventional probe. FP: Forward primer, RP: Reverse primer, LNA: Locked nucleic acid, and PNA: Peptide nucleic acid.

3.3.2.2 *PIK3CA* mutation detection assay

A different strategy was used to develop *PIK3CA* mutation detection assays. The most three common *PIK3CA* hotspot mutations (*p.E542K*, *p.E545K* and *p.H1047R*) were investigated. Specific probes were designed for the mutant and wild-type alleles (Figure 3-6). Moreover, in order to increase the specificity of the *PIK3CA* assay, a reverse primer was used for the hotspots *p.E542K* and *p.E545K* with ligated LNA (Locked Nucleic Acid) at 3', this was included to prevent the amplification of a pseudogene on chromosome 22, which has >95% homology to exon 9 of *PIK3CA* (Figure 3-7). The method was established by Ang et al., who found that LNA-qPCR was more sensitive than standard Sanger sequencing in discriminatory amplification of the known pseudogene (Ang et al., 2013). The sequences and characteristics of the selected primers and probe are presented in Table 2-3.

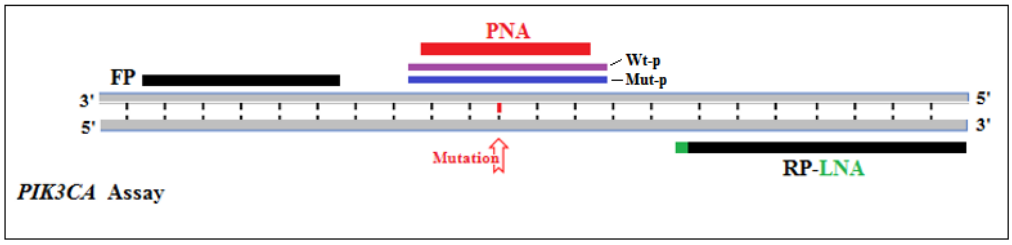


Figure 3-6 Illustration of designed *PIK3CA* assay. The diagram shows the positions of the PNAs and LNA and the conventional probe. FP: Forward primer, RP: Reverse primer, LNA: Locked nucleic acid, and PNA: Peptide nucleic acid.

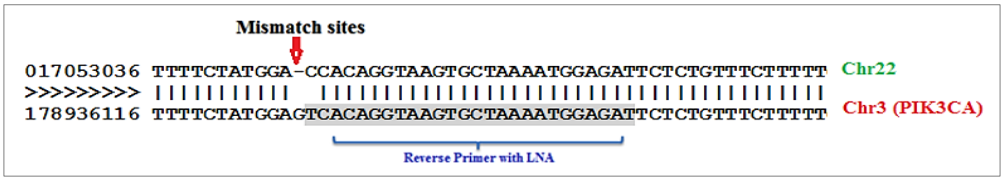


Figure 3-7 Homology sequence of the both chromosome 22 and chromosome 3 (*PIK3CA*) the LNA was designed on the base of mismatch between the locations to allow amplification of the targeted sequence only.

Three PNA oligonucleotide were designed for each hotspot mutations (*p.E542K*, *p.E545K* and *p.H1047R*) to clamp and block the wild-type sequence amplification. The PNA was designed according to the manufacturer's recommendations as

described in section 2.8.4, the sequences and characteristics of PNAs are illustrated in Table 2-6 (Figure3-9).

At the beginning of this study, several methods were tested to develop *PIK3CA* mutations detection assays. MCF7, a heterozygous cell line for the *p.E545K* mutation, was tested for allele specific-LNA and specific probe-based assay (Figure 3-8). A similar experiment also carried out on SW948, homozygous cell line for *p.E542K* mutation to examine the two assays. The experiments confirmed that Specific probe-based assays were better than allele specific-LNA assay in terms of amplification efficiency. The reason could be the rich area of C and T bases around the *p.E542K*, *p.E545K* mutations, which make it difficult to put specific primers on those mutant bases. In contrast, AS-LNA prime assay was successful in the case of p.*H1047R* hotspot mutation.

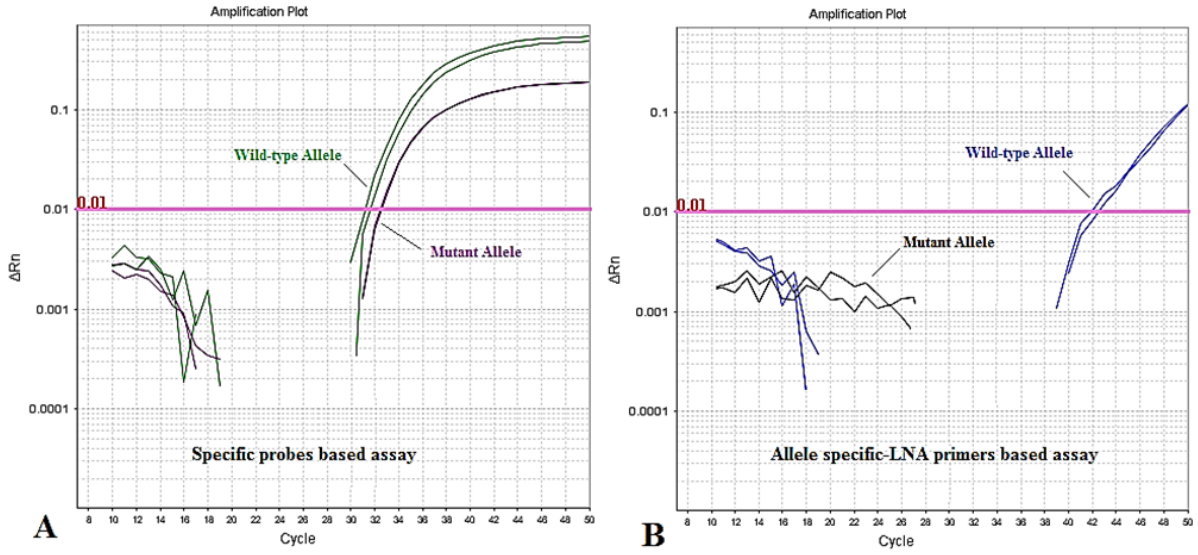


Figure 3-8 Comparison of *PIK3CA* mutation detection assays. MCF7 cell line was used as a template to test the two assays A: specific probe-based assay showed the amplification of both Wild-type and mutant alleles whereas AS-LNA primer-based assay failed to do so.

3.3.3 **Mutation detection assay validation**

As validation, all assays were tested on relevant cell lines harbouring the specific mutations (Table 2-1) and Human genomic DNA (HG-DNA) was used as a mutation reference (negative) control to measure the sensitivity and the reproducibility of the assays. In order to mimic the clinical settings, cell line DNA samples were serially diluted with HG-DNA at 1:10, 1:100 and 1:1000. The

starting concentration was 10 ng/3.6µl for both HG-DNA and the cell lines DNA. (Figure 3-10 and 3-11). Also, sterile water was used as a negative template control in each assay. The Touchdown qPCR was set as an essential profile to match the most appropriate annealing temperature in each assay, described in details in Chapter 2-10. An example of the mechanism of PNA action is illustrated as HCT-116 cell line (heterozygous mutant for p.*H1047R*) was tested by mutation detection assay with and without PNA (Figure 3-9). PNA clamped the wild-type alleles and completely suppressed its amplification.

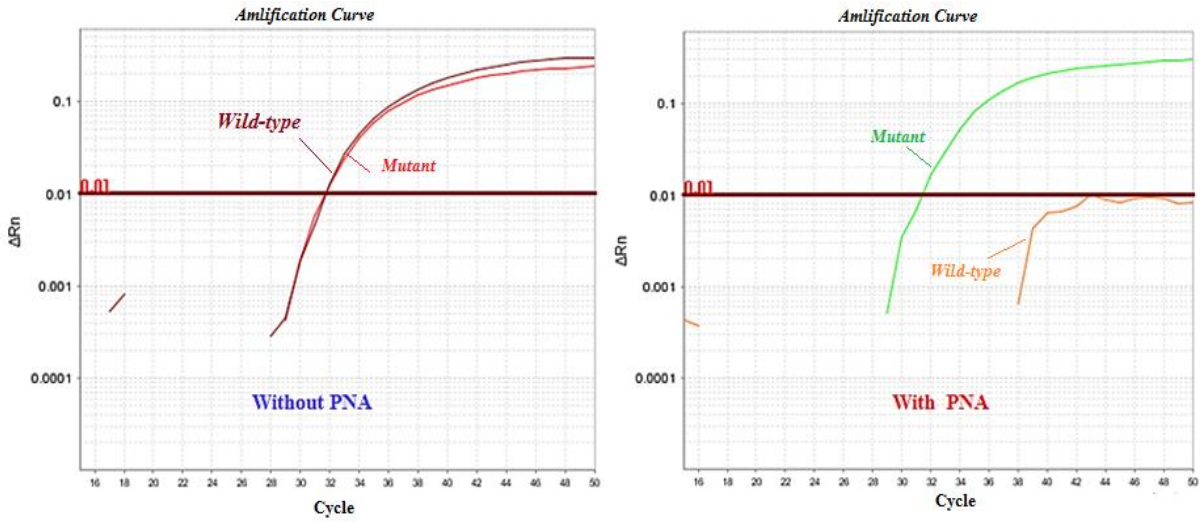


Figure 3-9 Mechanism of PNA action, *PIK3CA* mutation detection assay left panel without PNA, right panel with PNA. The PNA suppresses the wild-type allele amplification allowing specific mutant allele amplification.

3.3.3.1 **Sensitivity and Reproducibility of the AS-LNA /PNA qPCR assays**

The specificity and selectivity of AS-LNA /PNA qPCR assays were determined by calculating the difference between Ct values, ΔCt = (Ct [mutant primer] - Ct [WT primer]). The Ct values were varies depending on several factors including DNA quality and the efficiency of the primers and probes. Analytical sensitivity of AS-LNA/PNA qPCR was examined with a mixture of wild-type HG-DNA: mutated cell line serial dilutions at 1:10. 1:100 and 1:1000 confirming that the primers were specific to targeted mutations. Also, patient DNA samples were amplified by the designed assays (Figure 3-10).

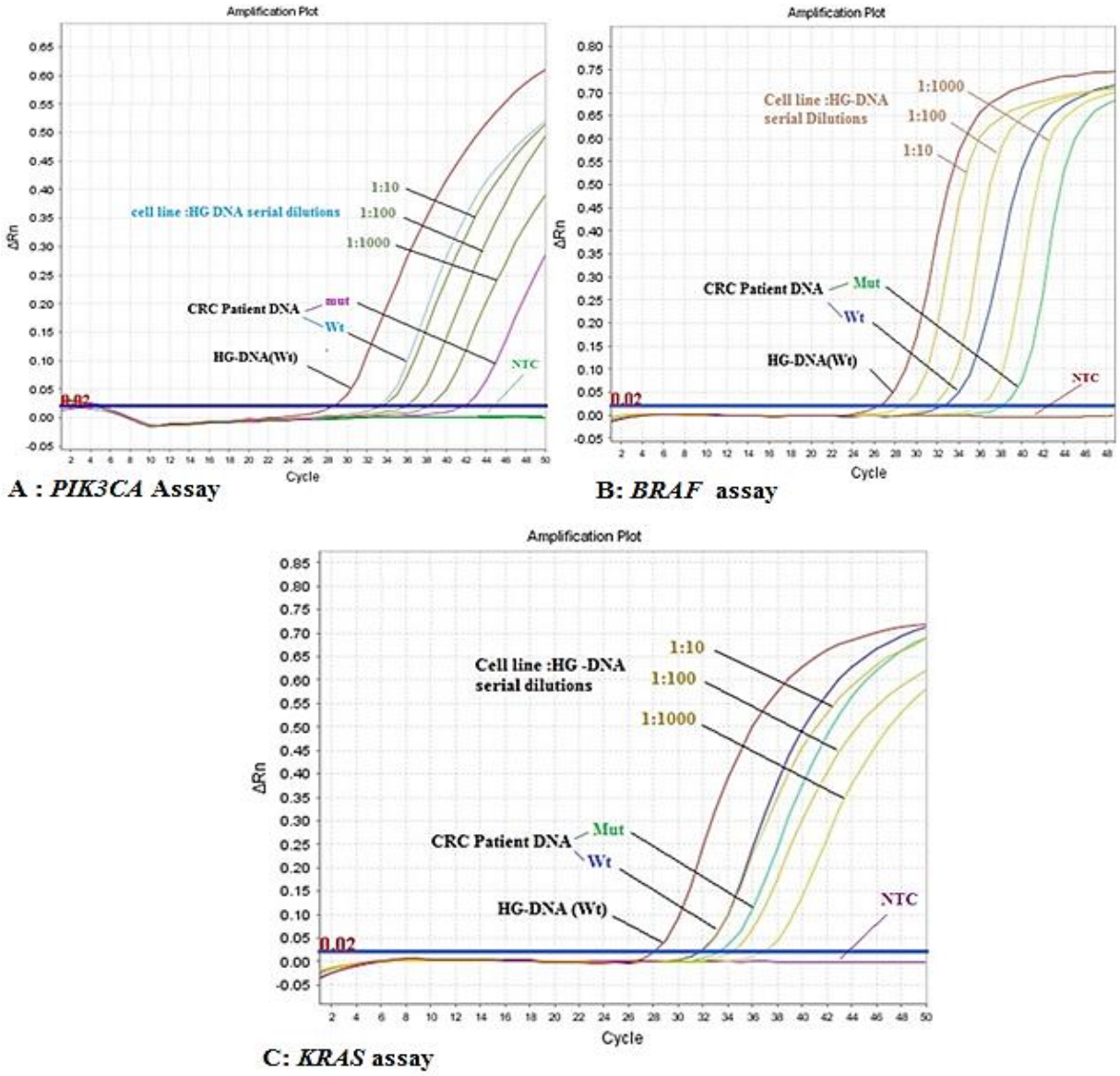


Figure 3-10 Validation of mutation detection assay. A: *PIK3CA* assay B: *BRAF* assay C: *KRAS* assay showing Positive and Negative controls, wild-type (Newton et al.) Ct of the HG-DNA compared to the Wt Ct signal from CRC patient normal tissue. Serial dilutions of HG-DNA and positive cell line to the relevant gene mutation at (1:10, 1:100 and 1:1000) compared to the mutant allele (Mut Ct) signal from CRC.

There was a significant difference between *BRAF* and *KRAS* control assays and the various levels of mutant:non-mutant alleles mixtures (one way ANOVA, $P< 0.001$). Also, *PIK3CA* assay has a similar level of sensitivity ($P= 0.007$) confirming that mutation detection assays are sensitive and can detect mutant alleles as low as 0.001% in 10ng DNA (Figure 3-11).

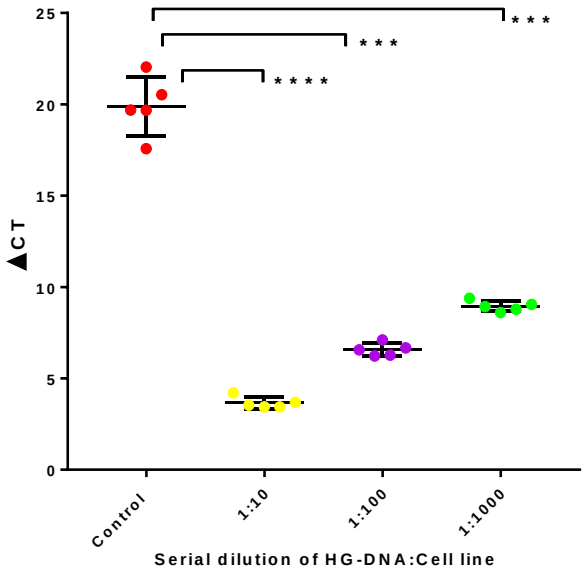


Figure 3-11 Sensitivity of AS-LNA/PNA clamping qPCR assays. Serial dilutions HG-DNA and positive cell line to relevant gene mutation at (1:10, 1:100 and 1:1000), ΔCt = (Ct [mutant primer] - Ct [WT primer]), * annotates the strength of the significance $p < 0.001$.

Reproducibility, measuring how repeatable the assays were, was investigated by comparing results from experiments conducted on different occasions. *BRAF* and *KRAS* assays were repeated on five occasions. A repeated measures ANOVA with a Greenhouse-Geisser correction determined that mean ΔCts do not differ statistically between time points (F (1.9, 5.9) P = 0. 5) and (F (1.211, 3.634) P = 0.6 for *BRAF* and *KRAS* assays respectively. The *PIK3CA* assay was reproducible without any significate difference between three independent experiments F (1.052, 3.155) P = 0.7 (Figure 3-12).

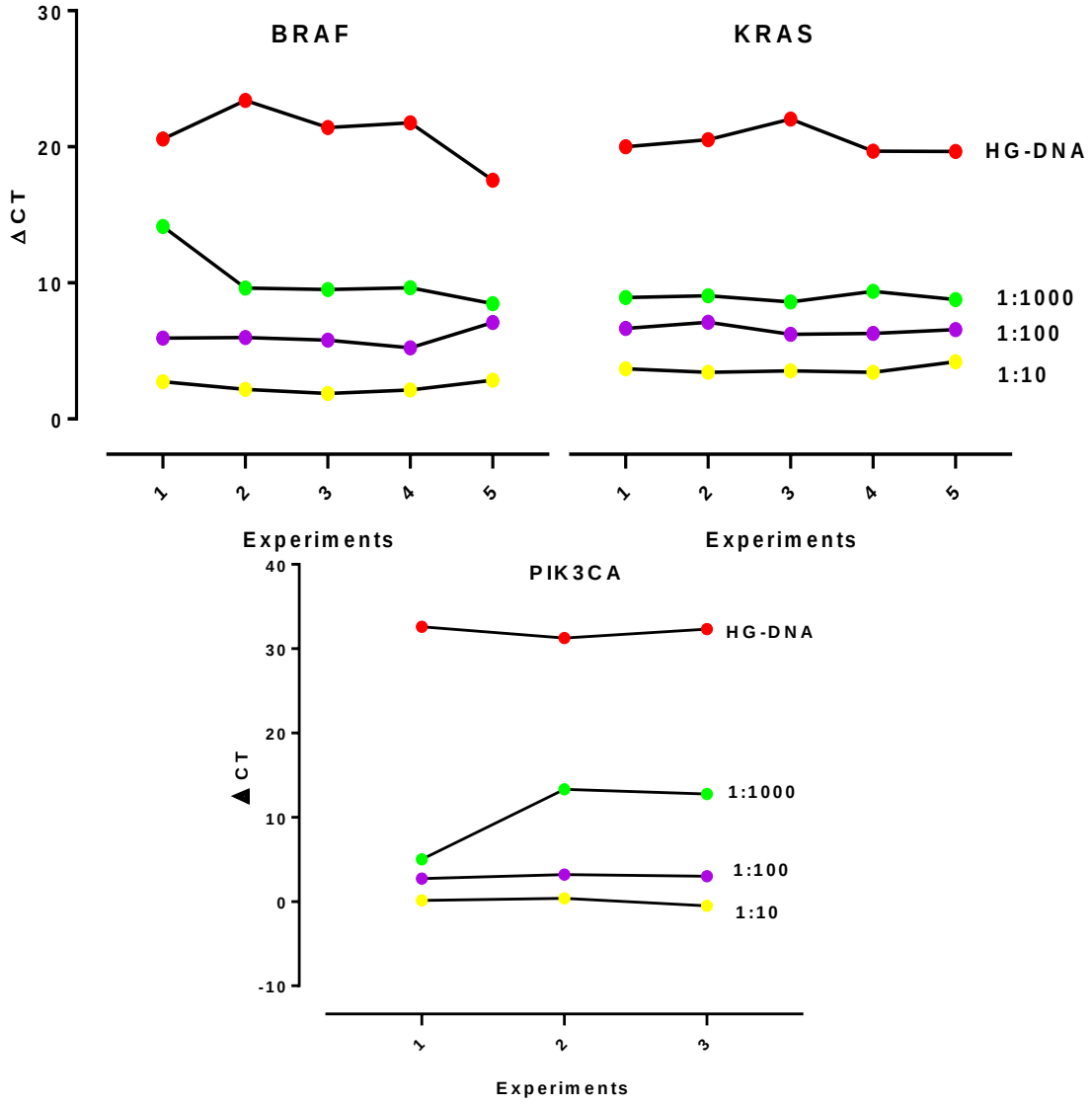


Figure 3-12 The reproducibility of the *BRAF*, *KRAS* and *PIK3CA*, There is no significant difference between the assays over various experiments, Serial dilutions HG-DNA and positive cell line to relevant gene mutation at (1:10, 1:100 and 1:1000).

3.4 Discussion

3.4.1 Validation cfDNA quantification using ALU repeats

In recent years, several studies have been examined the feasibility of using the non-coding DNA particularly repetitive sequences such ALU repeat. ALU have been used to measure the integrity or levels of cfDNA in cancers such as breast and CRC (Umetani et al., 2006A) (Umetani et al., 2006B). The shortest ALU used for fragmented DNA was ALU115 (Umetani et al., 2006A). In this study, ALU69 is to be the shortest ALU repeat to be used in cfDNA quantification. Therefore, ALU (69bp) used to optimise the assay detection of cfDNA especially in localised primary cancers of the disease and was better than longer amplicons such as ALU124 and ALU 201.

In this thesis, ALU-qPCR was developed as an accurate, highly sensitive technique to measure very low concentrations of cfDNA. Preliminary results presented in this Chapter are in agreement with the findings found by Umetani *et al.*, who investigated the potentiality of using the ALU repeat as a biomarker for CRC detection and evaluation (Umetani et al., 2006B) (Mead et al., 2011) (da Silva Filho et al., 2013).

Although, GAPDH, a single copy reference, is better in estimating DNA concentration, the ALU69 assay detects multiple copies, is small in size and more sensitive for detecting low concentration DNA. This data showed that ALU69 repeats is very sensitive as one copy of GAPDH was equivalent to ≈ 4000 copy of ALU 69.

3.4.2 Validation mutation detection assay

Three different techniques were integrated to the Quantitative PCR based on suppressing the wild-type allele amplification and increasing of the mutation detection sensitivity as well as optimising the assay efficiency. The assays are a particular form of qPCR-based on allele-specific differentiation. It is worth stating that this technology has not been combined or evaluated by any other studies as it is a novel approach of mutation detection techniques. Hence, it was

challenging for developing and enhancing the assay strategy. Moreover, Touchdown-qPCR method has been done for the first time on real-time PCR to allow annealing of PCR oligonucleotides at the most appropriate temperature to improve the sensitivity and specificity of the assays.

The AS-LNA qPCR, as described by Morandi et al., (2012) is a new AS-qPCR assay using forward or reverse mutation-specific primers modified together with LNA at the 3′-end sequence that recognises and quantifies oncogenic mutations having higher specificity and sensitivity. Allele-specific PCR is suitable for detecting gene mutations when these are produced by either a single base mutation or few base changes from certain hotspots of the gene. However, conventional primers within qPCR can easily miss-anneal the target amplicon, especially when PCR conditions tend to be suboptimal (e.g., DNA degraded by formalin fixation), hence, causing false positive outcomes, which could have unwanted consequences regarding preferred treatment of choice (Morandi et al., 2012).

Other techniques have been attempted to overcome the non-specific amplification of targeted genes. PCR-restriction fragment length polymorphism mapping (PCR-RFLP) is a method that includes PCR amplification followed by restriction digestion with an enzyme, which can selectively digest mutant DNA (Cohen et al., 2003). However, this method is qualitative and cannot be utilised for low ratios of mutant to wild-type alleles. Clamping or wild-type blocking PCR (WTB-PCR) techniques use the discriminatory properties and exo-endonucleases resistance of locked nucleic acid (LNA) oligomers. These oligomers selectively attach to the wild-type DNA alleles to prevent elongation of the amplicon of wild-type template but not the mutant template (Dominguez and Kolodney, 2006). Nevertheless, elongation of the forward primer upstream of the mutation produces a partially extended product, which is more likely to amplify wild-type sequence owing to its raised annealing temperature (Oldenburg et al., 2008)

In recent studies, combined PNA-mediated PCR clamping with a melting curve analysis detected *KRAS* mutations in a ratio of 1:1000 wild-type molecules (Oh et al., 2010). It has been proven that it is sensitive method even in the challenging

clinical samples such as blood (Oh et al., 2010), urine (Roos and Jakubowski, 2010) and pleural fluids (Kang et al., 2015).

Kwon, Lee, *et al*. 2011, compared the detection rates of direct sequencing to PNA. They found in his study that PNA-mediated PCR clamping raised the mutation detection rate by up to 1.2%, 5.4%, and 7.6% for *BRAF*, *PIK3CA*, and *KRAS* mutations respectively. In NSCLC, Beau-Faller, et al. (Beau-Faller et al., 2009) found that direct sequencing (DS) detected *KRAS* mutations in 10% of tumours. Meanwhile, PNA-mediated PCR clamping identified ten more cases with *KRAS* mutation (18%). Kang *et al*. found similar results and higher detection yield for PNA-mediated PCR clamping over the direct sequencing (Kang et al., 2015) which demonstrates that PNA-mediated PCR clamping manifests high accuracy and sensitivity in the detection of *KRAS* mutations. By combining the AS-LNA and PNA clamping method the sensitive of the assay has recorded to 0.001% better than yielded previously (Wilson and Lackner, 2014), that making it equivalent to and more practical than digital droplet PCR.

In conclusion, the utility of AS-LNA/PNA clamping qPCR as sensitive and reliable technology were demonstrated for detection of the *BRAF*, *KRAS* and *PIK3CA* mutations. This study was designed to evaluate new qPCR assays based on allele specific discrimination and to test the colorectal cancer samples with their matched plasma to identify a marker that can be useful to track mutation in the blood circulation.

Chapter 4

Patient Demographics

4.1 Introduction

Eighty-four patients with colorectal cancer were invited to participate in the study from the Department of Colorectal Surgery at Leicester General Hospital, University Hospitals of Leicester NHS Trust. Tumour tissue and matched plasma samples were collected between August 2008 and November 2010. The histological and clinical data were obtained from patients reports by Dr Aslam (REC: 05/Q2502/28- 'Markers of tumour progression in colorectal cancer'). Clinical data were updated and reviewed by two clinical fellows. Dr Lava Krishna collected the post-treatment data and samples. Dr Eyad Issa updated the clinical data which included therapy, response and survival follow-up of the participating cohort.

4.2 Aims and objectives

The aims of this chapter were to analyse patient tumour DNA samples for hotspot mutations in key driver genes and compare the cohort demography with similar studies.

Objectives

1. To review patient demographic data.
2. To extract DNA from 84 tumour samples.
3. To investigate point mutations in *KRAS*, *BRAF* and *PIK3CA* using AS-LNA/PNA clamping qPCR in CRC primary tumours.
4. To relate the results to patient clinicopathological characteristics.

4.3 Results

4.3.1 Demographics

Eighty-four patients with colorectal cancer were included in this study, comprising 54 males (64%) and 30 females (36%) with a median age of 72 years (range 26 to 92 years), Patient features are shown in Table 4-1. The majority of

the cases involved in this study were between 50 and 74 years of age (64%) (Figure 4-1).

Of the 84 cases, 55 (64%) were colonic cancers, the majority of which were surgically resected. The remaining 29 cases (36%) were rectal cancers, two-third (20/29) of which were treated by neoadjuvant therapy (Chemotherapy or/and Radiotherapy) and the rest (9/29) were treated with surgical resection.

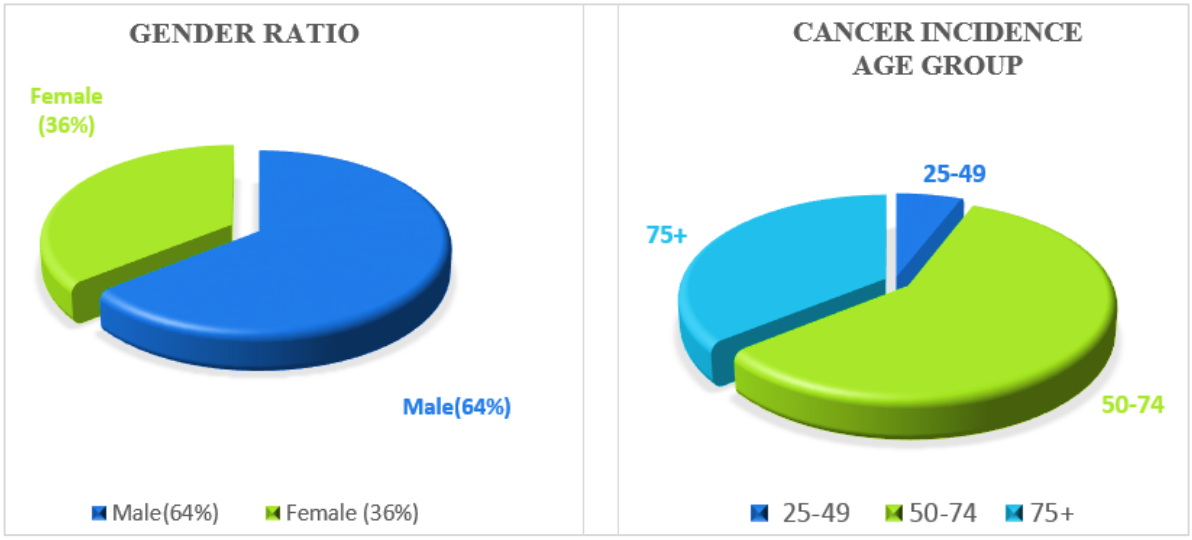


Figure 4-1 Gender and age (in years) of 84 patients with CRC in the study.

Histologically, one tumour was well-differentiated, 72 (87%) were moderately differentiated, and 6 (7%) were poorly differentiated carcinomas. The majority of the cases had a T stage between T2-T3, of these 71 (87%), and 54 (64%) had no lymph node involvement. Almost two-third of the cases were in localised primary cancers including early stages: Dukes' A 18 (22%) and Dukes' B 37 (44%) respectively (Figure 4-2) and (Table 4-1).

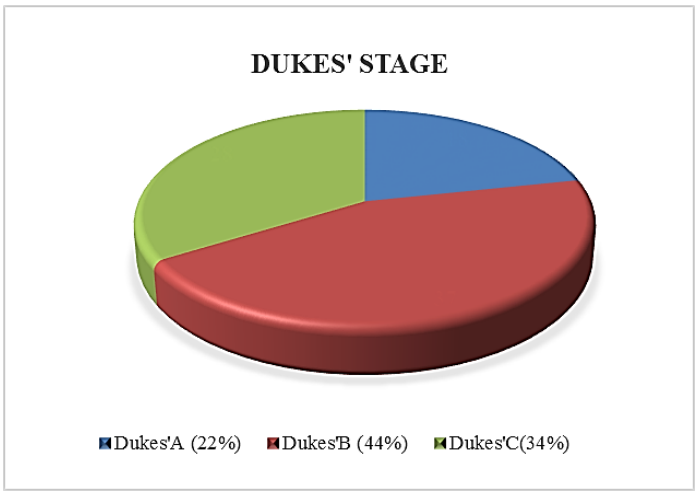


Figure 4-2 Dukes' stage of the cohort of 84 CRC.

Characteristics		
Patients	*n*=84	
Age	Median	72
	Range	(26-92)
Gender	Male	54 (64%)
	Female	30 (36%)
Primary Tumour location	colon	55 (Right 31 +Left 24)
	Rectum	29
Differentiation	Well	2
	Moderate	74
	Poor	6
	Unknown*	2
T stage	T0	1
	T1	3
	T2	19
	T3	52
	T4	8
	Unknown*	1
Lymph node	N0	54
	N1	17
	N2	12
	Unknown*	1
Tumour Stage **	I	18
	IIa	32
	IIb	2
	IIIa	2
	IIIb	19
	IIIc	4
	IVa	6
	Unknown*	1
Dukes' stage	A	18
	B	37
	C	28
	Unknown*	1

Table 4-1 Patients demographics of 84 CRC including the tumours staging (TNM and Dukes'), tumour differentiation, location, and lymph node metastasis. *Unknown: information lacking from histopathology reports.** Tumour Staging according to the (American Joint Commission on Cancer- AJCC) classification (Kin et al., 2013) and (Blanke and Faigel, 2011).

4.3.2 **Mutation analysis in Primary tumours**

DNA was extracted from FFPE tissue sections and quantified by ALU repeat qPCR (described in Chapter 2 and Chapter 3). Based on ALU results, 10 ng of DNA was subjected to mutation analysis by AS-LNA /PNA qPCR assays targeting key point mutations in *BRAF*, *KRAS,* and *PIK3CA* genes The mutation frequencies shown in the table represent the frequency of the mutations according to the online database Cosmic from the Sanger Institute (http://cancer.sanger.ac.uk/cosmic) (Table 4-2) (Figure 4-3). The mutation analysis was performed by PNA clamping the wild-type allele, while, AS-LNA primers were used to amplify the target mutant alleles specifically. Each mutation detection experiment was always compared to a mutant and a wild-type DNA control.

Gene	Frequency of TCGA		Mutation percentages in CRC
BRAF	V600E	80%	13%
KRAS	G12D	35.10%	30%
	G12V	22.3%	
	G13D	19.3%	
PIK3CA	E542K	12%	17%
	E545K	21%	
	H1047R	16%	

Table 4-2 Frequency of hotspot mutations of *PIK3CA*, *BRAF* and *KRAS*. Data illustrated are according to the online database Cosmic from the Sanger Institute (http://cancer.sanger.ac.uk/cosmic).

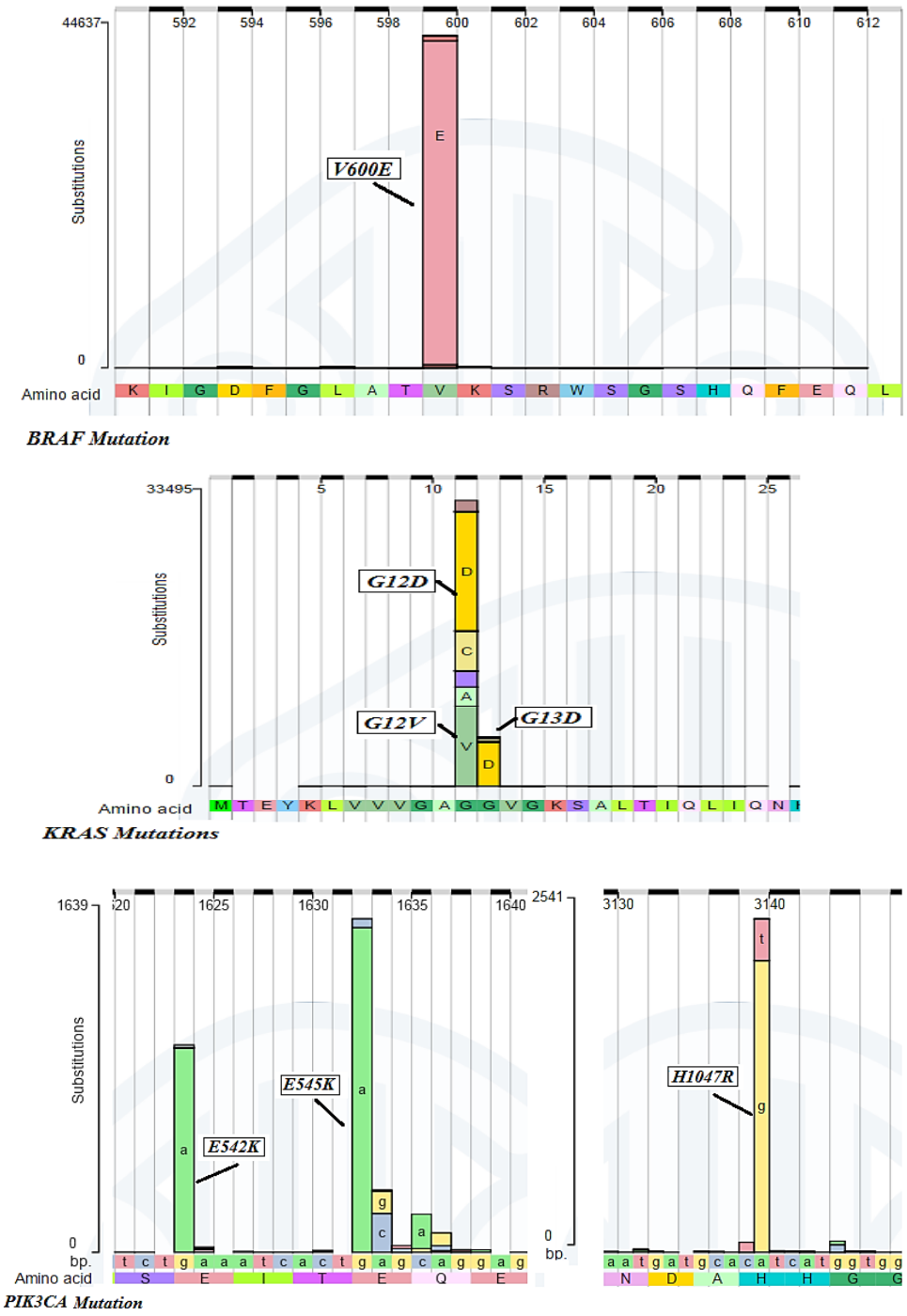


Figure 4-3 Illustration of frequency of hotspot mutations in *PIK3CA*, *BRAF* and *KRAS* genes according to online database Cosmic from the Sanger Institute (http://cancer.sanger.ac.uk/cosmic)

The overall proportion and association between *BRAF*, *PIK3CA*, *KRAS* mutations were analysed. Altogether, mutations were detected in 41/84 (49%) of the CRC cohort: 12 (14%) for *BRAF*, 19 (22%) for *PIK3CA* and 17 (20%) for *KRAS*. Among the 41 mutant CRCs, 10 tumours exclusively had a *BRAF* mutation, 12 had *KRAS*, and 12 had *PIK3CA*. Concomitant mutations occurred in two patients for *BRAF* and *PIK3CA*, and five patients for *KRAS* and *PIK3CA* respectively (Table 4-2) (Figure 4-4). Mutations in *BRAF* and *KRAS* were mutually exclusive (Table 4-3) (Figure 4-4).

GENE		**CRC**	**Exclusive Mutation**	**Colon**		**Rectum**
				Right	Left	
BRAF	41/84 (49%)	12 (14%)	10	8	-	4
PIK3CA		19 (22%)	12	9	6	4
KRAS		17 (20%)	12	4	5	8

Table 4-3 The frequency of the oncogenic mutations of *BRAF*, *PIK3CA*, *KRAS* in the FFPE of the CRC cohort.

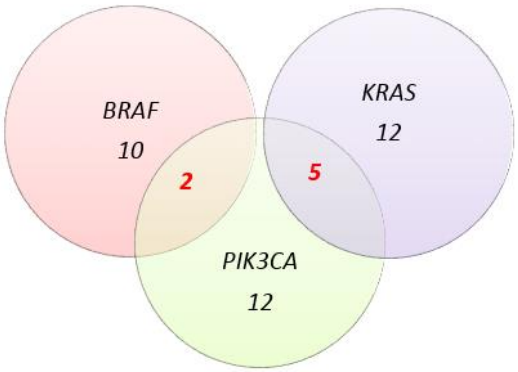


Figure 4-4 Overall proportions and associations between *KRAS*, *BRAF*, and *PIK3CA* mutations identified in CRC cohort. Red numbers indicated the concomitant mutations between the three genes mutations.

	Patient ID	Mutations	Additional mutations	Tumour Location	Duke's staging	Age
1	H467/14	PIK3CA H1047R	PIK3CA E545K	Left Colon	C	70
2	H486/14	PIK3CA H1047R		Left Colon	B	71
3	H433/14	PIK3CA H1047R		Left Colon	C	74
4	H460/14	PIK3CA H1047R		Right Colon	B	91
5	H928 /13	PIK3CA H1047R	PIK3CA E545K	Right Colon	C	87
6	H473/14	PIK3CA H1047R	PIK3CA E545K	Right Colon	C	73
7	H911/13	PIK3CA H1047R		Right Colon	B	53
8	H930 /13	PIK3CA H1047R		Rectum	C	54
9	H470/14	PIK3CA H1047R		Rectum	C	77
10	H454/14	PIK3CA H1047R		Rectum	A	78
11	H459/14	PIK3CA E545K		Left Colon	C	74
12	H487/14	PIK3CA E545K		Rectum	B	78
13	H497/14	KRAS G13D	PIK3CA E542K	Right Colon	A	71
14	H448/14	KRAS G13D		Rectum	B	79
15	H490/14	KRAS G13D		Rectum	B	73
16	H450/14	KRAS G13D		Rectum	A	65
17	H916 /13	KRAS G12V		Left Colon	B	84
18	H439/14	KRAS G12V	PIK3CA E542K	Left Colon	A	73
19	H446/14	KRAS G12V		Left Colon	B	79
20	H484/14	KRAS G12V		Rectum	B	71
21	H488/14	KRAS G12V		Rectum	A	76
22	H492/14	KRAS G12V		Rectum	B	86
23	H927 /13	KRAS G12D		Left Colon	B	71
24	H469/14	KRAS G12D	PIK3CA H1047R	Left Colon	C	65
25	H435/14	KRAS G12D	PIK3CA H1047R	Right Colon	C	78
26	H462/14	KRAS G12D		Right Colon	B	85
27	H456/14	KRAS G12D	PIK3CA E545K	Right Colon	B	87
28	H1030 /13	KRAS G12D		Rectum	C	86
29	H479/14	KRAS G12D		Rectum	A	87
30	H918 /13	BRAF V600E		Right Colon	B	83
31	H519/13	BRAF V600E		Right Colon	C	90
32	H447/14	BRAF V600E		Right Colon	B	76
33	H912 /13	BRAF V600E	PIK3CA E542K	Right Colon	B	82
34	H493/14	BRAF V600E		Right Colon	B	97
35	H451/14	BRAF V600E		Right Colon	C	31
36	H475/14	BRAF V600E		Right Colon	A	83
37	H436/14	BRAF V600E	PIK3CA H1047R	Right Colon	A	79
38	H932 /13	BRAF V600E		Rectum	B	56
39	H463/14	BRAF V600E		Rectum	B	68
40	H434/14	BRAF V600E		Rectum	B	67
41	H458/14	BRAF V600E		Rectum	B	90

Table 4-4 The mutational status of primary tumours and clinicopathological characteristic of 41 CRC patients.

Mutations were detected in 25/55 (45.5%) of colonic cancer cases and 16/29 (55%) of rectal cancer cases (Figure 4-5). Two thirds of *BRAF* mutation (66%) were found in colonic cases, all of which were in the right colon, only 13.7% of rectal cases had mutant *BRAF*.

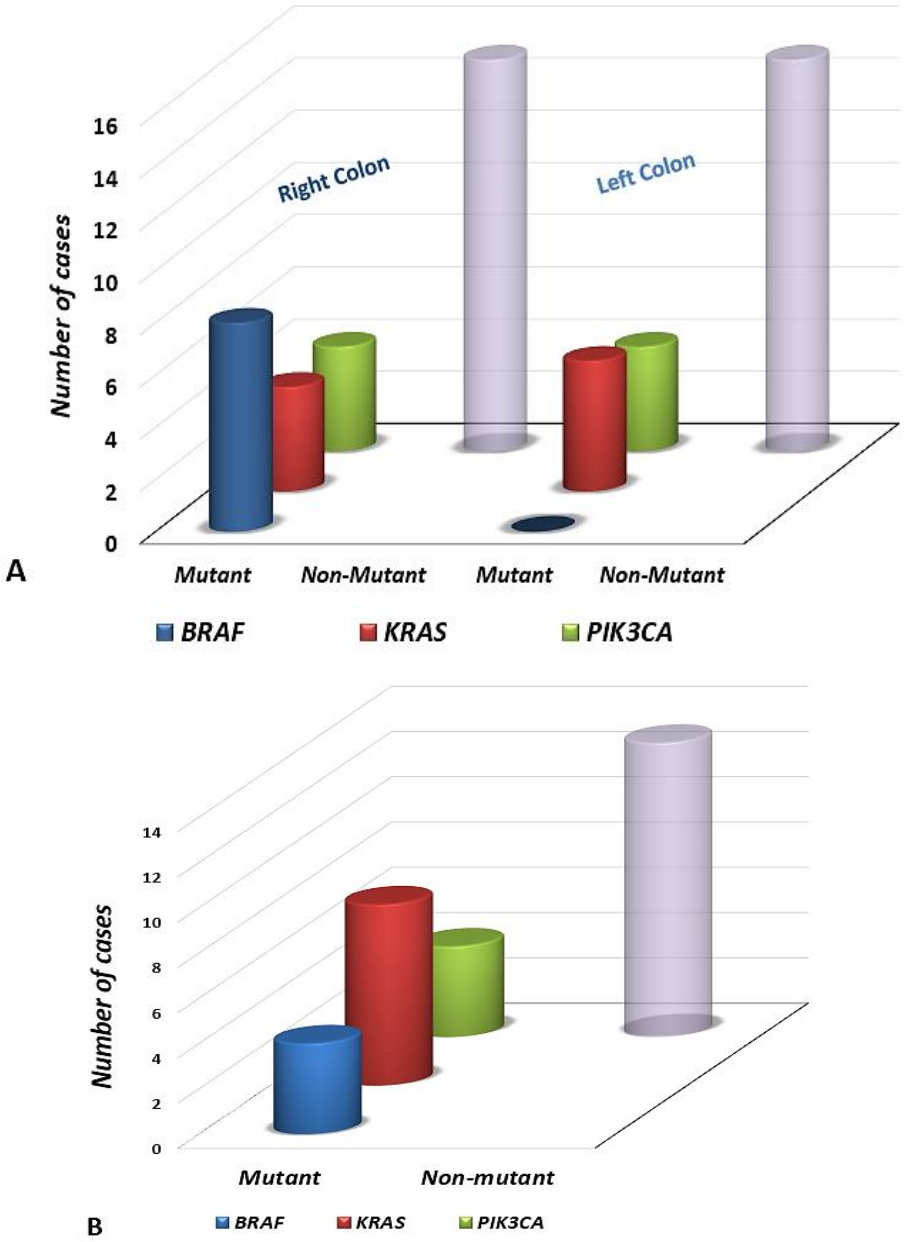


Figure 4-5 Distribution of *BRAF*, *KRAS* and *PIK3CA* mutations in patients with CRC according to tumour location. (A: Colon *n*= 55, B: Rectum *n*= *29*).

KRAS mutations were detected in 8/55 (14.5%) colonic and 9/29 (31%) rectal cases (Figure 4-5). *p.G12D* and *p.G12V* were the most common hotspot mutations in *KRAS* by 41% and 35% respectively (Figure 4-6). *PIK3CA* mutations were more common in colon 15/55 (27%) than in rectal tumours 4/29 (17.8%). The hotspot *p. H1047R* was the most detectable mutation (52%), and 16% of the cases had concomitant mutation of *p.H1047R* and *p.E545K* (Figure 4-6). V600E point mutation was the only one was tested in this study for *BRAF*.

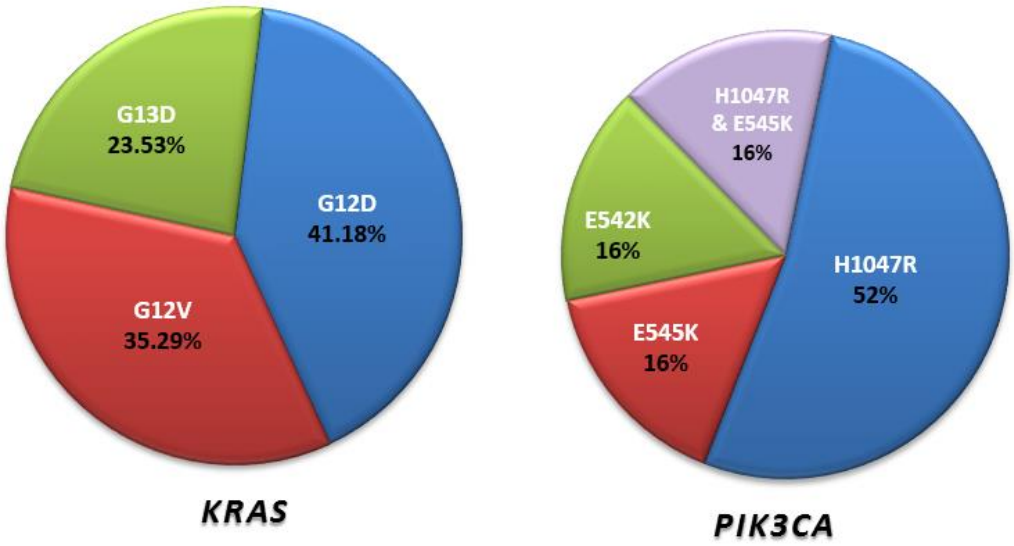


Figure 4-6 The proportions of mutated hotspot codons in *KRAS* hot spot mutations (G12D, G12V and G13D) and *PIK3CA* hot spot mutations (H1047R E545K and E542K).

Regardless of the tumour location, there was a significant relation between the tumour stage and gene mutations in CRC tissue (Kruskal-Wallis, *p=0.05*). *BRAF* and *KRAS* were more common in early stages (Dukes' A and B) than in Dukes' C. Dukes'C cases had the highest proportion of *PIK3CA* mutation (Figure 4-7), some of Duke's C tumours are aggressive and heterogenetic in terms of gene mutations as it harbours more than one hot spot of *PIK3CA* (H1047R and E545K).

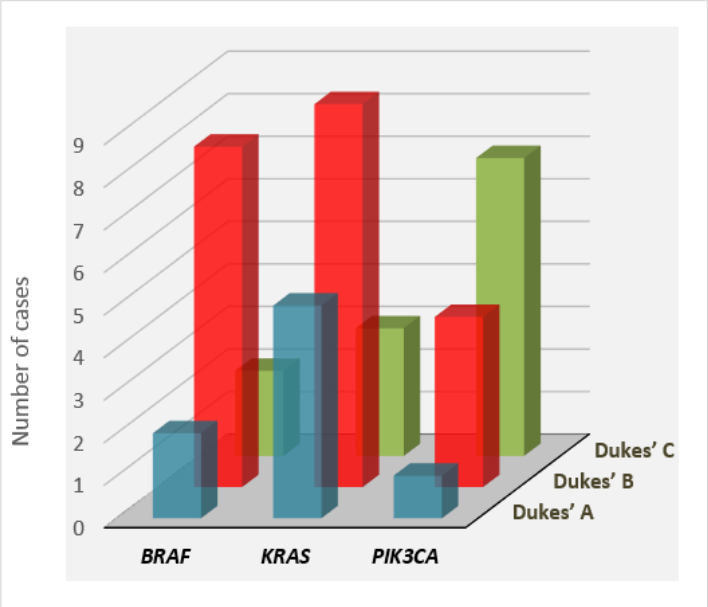


Figure 4-7 Distribution of *BRAF*, *KRAS* and *PIK3CA* mutations with Dukes' stages.

70 % of the mutant tumours were from male and 30 % from female patients. There was a significant relation between the three genes (*KRAS*, *BRAF*, and *PIK3CA*) mutations in tissue and the gender of the patient (Chi-Square Test, $p = 0.008$) (Figure 4-8). *KRAS* mutations correlated with a higher frequency in men compared with women (26% 14/54 vs. 10%, 3/30). No further correlations of mutations to clinicopathological data were observed.

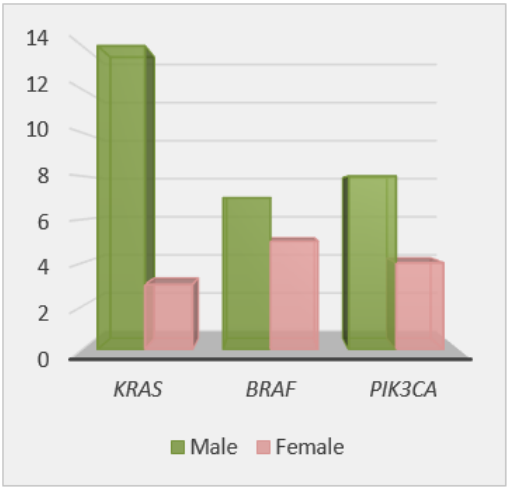


Figure 4-8 Gene (*KRAS*, *BRAF*, and *PIK3CA*) mutations distribution according to the gender, (Chi-Square Test, $p = 0.008$).

4.4 Discussion

In this study, the age range of patients closely matched the incidence by age groups stated in national data provided by Cancer Research UK (CRUK, 2015). The age from 50 to 74, remains the most common age group acquiring CRC. Also, this is in line with the age-associated risk linked to population aged over 50 in 90% of cases (CRUK, 2015). There was a higher proportion of men affected by cancer than women in this study at a ratio of M: F 1.8:1. This was greater than that reported by Cancer Research UK Statistics that report a male to female ratio is 1.3:1.

Microdissection was the first step toward analysing FFPE sections before extracting DNA to overcome sensitivity issues and increase the proportion of the tumour DNA (Arcila et al., 2011). 10ng of quantified DNA by ALU-qPCR was subjected to the novel AS-LNA/PNA assay for mutation analysis, which was described in the previous Chapter.

Few studies have investigated matched tumour samples with plasma and most of them had a limited sample size from 14 to 85 cases. The current study is close in the sample size to the study by Lecomte et al., (Lecomte et al., 2002). The sample size limitation could be related to common cancer stages at the time of diagnosis and study as these studies involved cases with early stages of cancer (Table 4-5) Meanwhile, studies that recruited bigger numbers of patients were in stage more advanced than Dukes' stage C (Roth et al., 2009) or studies conducted in countries with a large population such as China (Pu et al., 2013) (Table 4-5). Furthermore, 10 cases of had no plasma left from the previous projected that were collected to, which made the sample size become even smaller than original number of the patient cohort. This challenge has faced many studies studied the cfDNA in early stages of the CRC. Therefore, bigger cohort and properly prospective study can give a clear picture for the potentiality of cfDNA as surrogate of the primary tumours. Although, this study does not have healthy controls (participants without CRC), positive and negative DNA controls were provided from positive cell line and normal human genomic DNA, respectively, for the mutation analysis.

Author/Country	Age, median	Sex, M/F	No of cases	Stages
(Anker et al., 1997)England	63 (41–78)	N/A	14	Dukes' stage A-D
(Lecomte et al., 2002) France	69 (39–93)	29/29	85	stage I to IV
(Diehl et al., 2005) Germany	68(33-93)	18/15	33	Dukes' stage A-D
(Frattini et al., 2008) Italy	65 (38–87)	31/39	18	stage II to IV
(Lindforss et al., 2005) Sweden	72 (49–90)	7/15	22	Dukes' stage A-D
(Miyano et al., 2012) Japan	68 (33–84)	30/12	42	stage I to IV
Our study	72(26 -92)	56/31	84	Dukes' stage A-C

Table 4-5 Illustration of the similar studies, which investigated gene mutations in CRC primary tumours and matched cfDNA. Those studies were carried out in early stages mainly in plasma and used qPCR-based techniques in comparison to our study.

The mutation analysis has detected *PIK3CA* (mainly the hotspot *p.H1047R*) as being the most common in this study with a frequency of 22% across the patient cohort. This mutation more commonly occurred in colonic cases (27%) than rectal cases (13.8%), which remain in the range reported by previous studies from 10% to 30% (Velho et al., 2005) (Samuels et al., 2004). The presence of two difference *PIK3CA* mutations in the same patients is an example of tumour heterogeneity and convergent evolution with each mutation belonging to a separate clone.

KRAS point mutations were at a frequency of 20%, which is lower than previously reported in a number of studies, which indicated an incidence between 30 to 45%. *p.G12D* and *p.G12V* remain the most detectable *KRAS* point mutations as seen in this data (Kwon et al., 2011) (Fearon, 2011). *KRAS* mutations were detected in 14.5% colonic and 31% rectal cases in agreement with Smith et al. who stated that *KRAS* mutations were more frequent in rectal than in colon tumours, suggesting potential differences in the molecular pathways of tumourigenesis in these tissues (Smith et al., 2002). However, Frattini et al. identified that *KRAS* mutations were more prevalent in colon tumours than in those of the rectum (Frattini et al., 2008).

BRAF V600E mutation was identified at 14% in this cohort. This is higher than previously published incidences of only 5-10% (Fearon, 2011). V600E point mutation is the most common, constituting up to 80% of all *BRAF* mutations (Barras, 2015), which was the only point mutation tested in this study. 66% of the colon (exclusively the right colon) and 13.8% of rectal tumours were *BRAF* mutant, this is in agreement with studies that found highly enrichment in right-sided proximal tumours, and associated with a poorer prognosis (Missiaglia et al., 2014) (Meguid et al., 2008).

BRAF and *KRAS* mutations were found to be mutually exclusive, matching the data reported by other studies (Frattini et al., 2004) (Rechsteiner et al., 2013). However, Kwon *et al.* found a 1.1% of concomitant mutation between *KRAS* and *BRAF*. Five CRC cases had *PIK3CA* mutations coexisting with *KRAS* mutations. Similarly, some studies suggested that these concurrent mutations increase the AKT pathway signalling and cellular transformation further than *PIK3CA* mutations do alone (Oda et al., 2008) (Simi et al., 2008) (Parsons et al., 2005).

The current study included localised primary CRCs , Dukes' A, B and C. These stages are curable and non-metastatic tumours, whereas, several types of research emphasised on metastatic lesions while studying primary CRC tumours and matched cfDNA (Mostert et al., 2013) (Tie et al., 2015). *KRAS*, *PIK3CA* or *BRAF* mutations have been detected in early stages such as Dukes' stage A and B. This finding indicates the importance of mutations in these genes in early CRC tumorigenesis, which was also found by other studies (Bose and Ahuja, 2010). Nevertheless, these data showed that *PIK3CA* is more common in advanced stages.

The mutations of the main gene drivers were more commonly found in male patients than females. Nevertheless, it should be kept in mind that the number of men in this study was almost double the number of female patients. This relationship comes in disagreement with the findings by Roth et al. who claimed that *BRAF* mutation was significantly linked to female sex, however, his study had much larger cohort (Roth et al., 2009).

Chapter 5

Circulating free DNA analysis (cfDNA)

5.1 Introduction

Circulating free DNA (cfDNA) in colorectal cancer and other cancers is being investigated as a biomarker for early diagnosis, predicting prognosis and monitoring response to therapeutic medicine (Catarino et al., 2008) (Kohler et al., 2011, Mead et al., 2011) (da Silva Filho et al., 2013).

Increased levels of total cfDNA in the circulation of patients with cancer arises as a result of apoptosis, necrosis and secretion from tumours. This cfDNA can also carry the genetic makeup of the primary tumour, the circulating tumour DNA fraction, or ctDNA (García-Olmo et al., 2010). Hence, cfDNA/ctDNA has become an active area of translational cancer research (Mead et al., 2011).

5.2 Aims:

The aim of this chapter is to investigate the relationship between total cfDNA levels and the clinicopathological features of the early stage CRC cohort. A secondary aim was to compare total cfDNA levels before and after treatment with patient survival.

Objectives

1. To extract and quantify cfDNA from cases matched to CRC tissue samples.
2. To relate results to patients clinicopathological characteristics.
3. To investigate total cfDNA levels before and after treatment and patient survival.

5.3 Results

5.3.1 Patient samples and controls

74/84 patients' plasma samples subjected to cfDNA analysis, starting by DNA quantification and later mutation analysis, these preoperative samples were stored for at least 7 years before using them in this project. 10 cases were excluded because of unavailability of the plasma and sample size went down from 84 to 74.

Normal human genomic DNA was used to make the standard curves and to quantify the cfDNA.

The ALU69-qPCR assay was used to quantify total cfDNA levels in 74/84 patients with CRC. 10 cases had no plasma samples available for the analysis. In each case, cfDNA was extracted from 1mL of plasma and 3.6 µl of eluted cfDNA was quantified in duplicate relative to a standard curve using the AQ method as described previously (Page et al., 2011). The efficiency of the standard curves were within the desired 90-110%. Based on comparison with the standard curves, the cfDNA yield of 74 patients ranged from 0.02 to 89.8 ng /mL (Table 5-1) (Figure 5-1).

Patient ID	Age	Gender	Survival	Tumour Location	cfDNA Yield ng/mL	Duke's staging	T	N
H453/14	75	F	Dead	Right Colon	6.97	A	T1	N0
H483/14	74	F	Alive	Right Colon	29.16	B	T1	N0
H497/14	66	F	Alive	Right Colon	0.02	A	T2	N0
H436/14	74	F	Alive	Right Colon	6.49	A	T2	N0
H478/14 H477/14	71	M	Dead	Right Colon	2.37	B	T3	N0
H493/14	92	F	Dead	Right Colon	2.92	B	T3	N0
H456/14	82	M	Alive	Right Colon	4.94	B	T3	N0
H912 /13	77	M	Dead	Right Colon	7.82	B	T3	N0
H464/14	75	F	Alive	Right Colon	9.38	B	T3	N0
H913/13	77	F	Dead	Right Colon	12.6	B	T3	N0
H460/14	86	M	Dead	Right Colon	15.58	B	T3	N0
H462/14	80	M	Alive	Right Colon	16.04	B	T3	N0
H465/14	76	M	Alive	Right Colon	19.99	B	T3	N0
H915 /13	72	M	Alive	Right Colon	20.47	B	T3	N0
H918 /13	78	F	Alive	Right Colon	45.29	B	T3	N0
H447/14	70	M	Dead	Right Colon	67.91	B	T3	N0
H917 /13	59	M	Alive	Right Colon	68.63	B	T3	N0
H928 /13	82	F	Dead	Right Colon	28.25	C	T4	N0
H451/14	26	M	Alive	Right Colon	5.03	C	T2	N1
H461/14	64	M	Alive	Right Colon	14.85	C	T3	N1
H473/14	69	M	Dead	Right Colon	23.54	C	T3	N1
H914 /13	73	M	Alive	Right Colon	30.57	C	T3	N1
H440/14	73	F	Alive	Right Colon	89.8	C	T3	N1
H519/13	85	M	Dead	Right Colon	49.05	C	T4	N1
H468/14	69	F	Alive	Right Colon	12.35	C	T3	N2
H933 /13	90	F	Dead	Right Colon	10.23	C	T4	N2
H435/14	73	M	Alive	Right Colon	34.02	C	T4	N2
H910/13	60	M	Alive	Right Colon	44.38	C	T4	N2
H495/14	86	M	Alive	Left Colon	0.1	A	T2	N0
H439/14	68	M	Alive	Left Colon	16.78	A	T2	N0
H442/14	57	M	Alive	Left Colon	45.47	A	T2	N0
H499/14	62	F	Alive	Left Colon	2.63	B	T3	N0
H486/14	66	M	Alive	Left Colon	2.77	B	T3	N0
H514/13	75	F	Alive	Left Colon	4.08	B	T3	N0

H919 /13	64	M	Alive	Left Colon	5.5	B	T3	N0
H916 /13	80	M	Alive	Left Colon	11.17	B	T3	N0
H927 /13	67	M	Alive	Left Colon	15.02	B	T3	N0
H443/14	81	M	Alive	Left Colon	35.64	B	T3	N0
H446/14	73	M	Alive	Left Colon	5.82	B	T4	N0
H467/14	67	M	Alive	Left Colon	19.3	C	T3	N1
H433/14	69	M	Dead	Left Colon	20.9	C	T3	N1
H480/14	65	M	Dead	Left Colon	0.02	C	T3	N2
H459/14	69	M	Alive	Left Colon	5.31	C	T3	N2
H455/14	69	M	Alive	Left Colon	6.28	C	T3	N2
H469/14	60	M	Alive	Left Colon	6.74	C	T3	N2
H518/13	85	F	Dead	Left Colon	14.34	C	T3	N2
H929 /13	71	M	Dead	Left Colon	25.49	C	T3	N2
H438/14	70	F	Alive	Left Colon	63.32	C	T3	N2
H481/14	64	F	Dead	Left Colon	7.09	C	T4	N2
H432/14	82	F	Alive	Rectum	19.59	N/A	N/A	N/A
H932 /13	51	M	Alive	Rectum	34.71	B	T0	N0
H472/14	48	F	Alive	Rectum	1.22	A	T2	N0
H476/14	83	F	Alive	Rectum	3.25	A	T2	N0
H454/14	73	F	Alive	Rectum	3.7	A	T2	N0
H488/14	71	M	Dead	Rectum	5.2	A	T2	N0
H931 /13	59	F	Alive	Rectum	5.63	A	T2	N0
H449/14	72	M	Alive	Rectum	46.25	A	T2	N0
H494/14	66	M	Alive	Rectum	2.74	B	T3	N0
H490/14	69	F	Dead	Rectum	3.3	B	T3	N0
H471/14	75	M	Alive	Rectum	4.55	B	T3	N0
H484/14	67	M	Dead	Rectum	6.42	B	T3	N0
H487/14	73	M	Alive	Rectum	6.73	B	T3	N0
H463/14	63	M	Dead	Rectum	14.73	B	T3	N0
H448/14	74	M	Alive	Rectum	16.43	B	T3	N0
H434/14	62	F	Dead	Rectum	17.12	B	T3	N0
H431/14	41	F	Dead	Rectum	19.27	C	T1	N1
H515/13	87	F	Dead	Rectum	2.31	A	T2	N1
H470/14	73	M	Alive	Rectum	7	C	T2	N1
H450/14	60	M	Dead	Rectum	21.6	A	T2	N1
H489/14	63	M	Alive	Rectum	1.01	C	T3	N1
H445/14	82	M	Alive	Rectum	10.77	C	T3	N1
H930 /13	50	F	Alive	Rectum	13.96	C	T3	N1
H1030 /13	81	M	Dead	Rectum	9.81	C	T4	N1
H492/14	81	F	Dead	Rectum	22.13	B	T3	N0

Table 5-1 The total cfDNA Levels of CRC patients and clinicopathological features including age, gender, patient survival, tumour location, Dukes' stages and TNM stages.

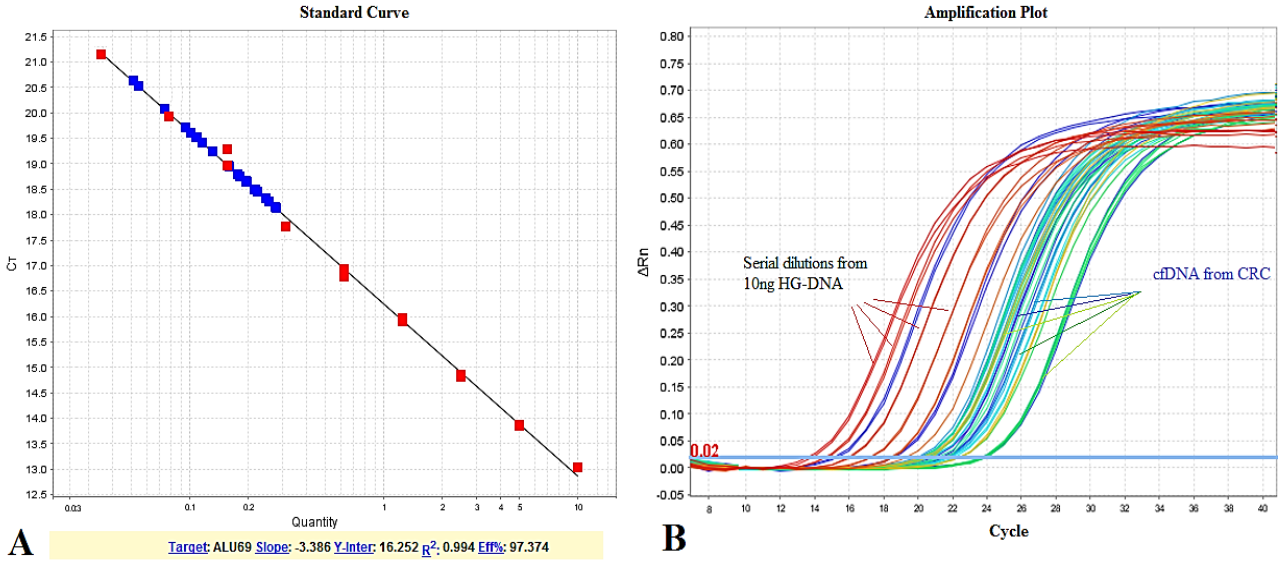


Figure 5-1 Quantification of cfDNA from CRC patients. A: shows the standard curve of serial dilation of Human Genomic DNA using ALU69 as a target, the result is an efficiency of 97.3% and R2 of 0.99. B: Serial dilutions of the Human genomic DNA was used to validate the assay. The dilution started from 10ng down to 0.019ng. The CT values range from 14 to 24.

5.3.2 **Clinicopathological parameters and cfDNA levels**

The concentration of cfDNA was independent of age, gender, tumour location (colon *vs* rectum) T stage and lymph node status. However, there was a significant relation between the total cfDNA level and tumour stage (*p*= 0.03 Jonckheere-Terpstra) (Figure 5-2).

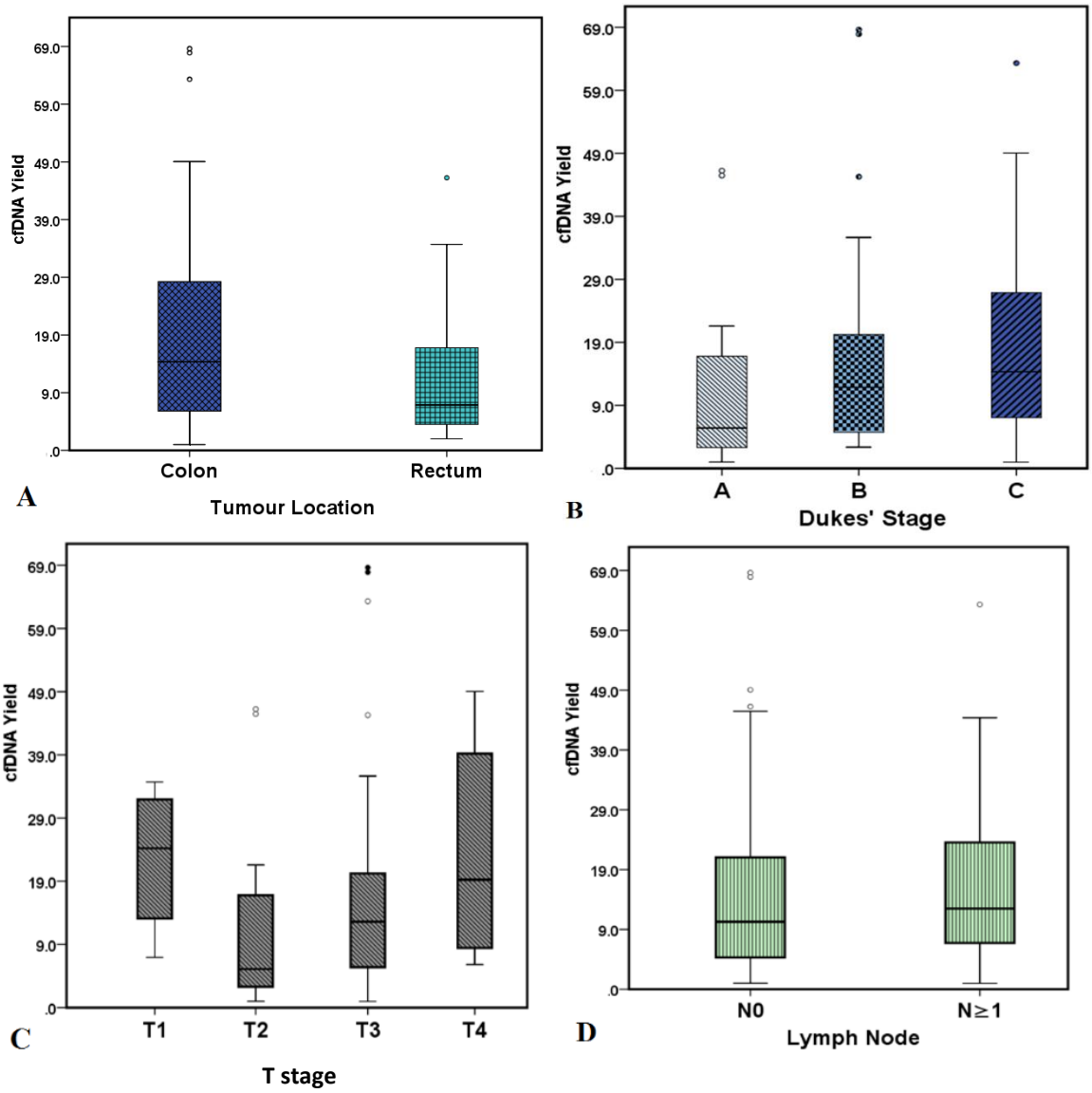


Figure 5-2 Figure show box and whisker plot for actual total cfDNA yield. cfDNA yield values in ng/µl. The relation between total cfDNA levels and clinic-pathological features of the entire CRC cohort. A: Tumour location (NS) B: Dukes' stage *(P*= 0.03 Jonckheere-Terpstra).C: T stage (NS). D: Lymph node status (NS*).*

5.3.3 **cfDNA analysis in colonic cancers**

In the 55 colonic cancers, 6 had no plasma for cfDNA analysis. In the remaining 49 cases the total cfDNA level was independent of age, gender, tumour location, T stage and lymph node status. Although, no statically significant relationship was

observed between cfDNA levels and tumour stage, there was a steady increase in cfDNA levels with more advancement in disease stage (Figure 5-3).

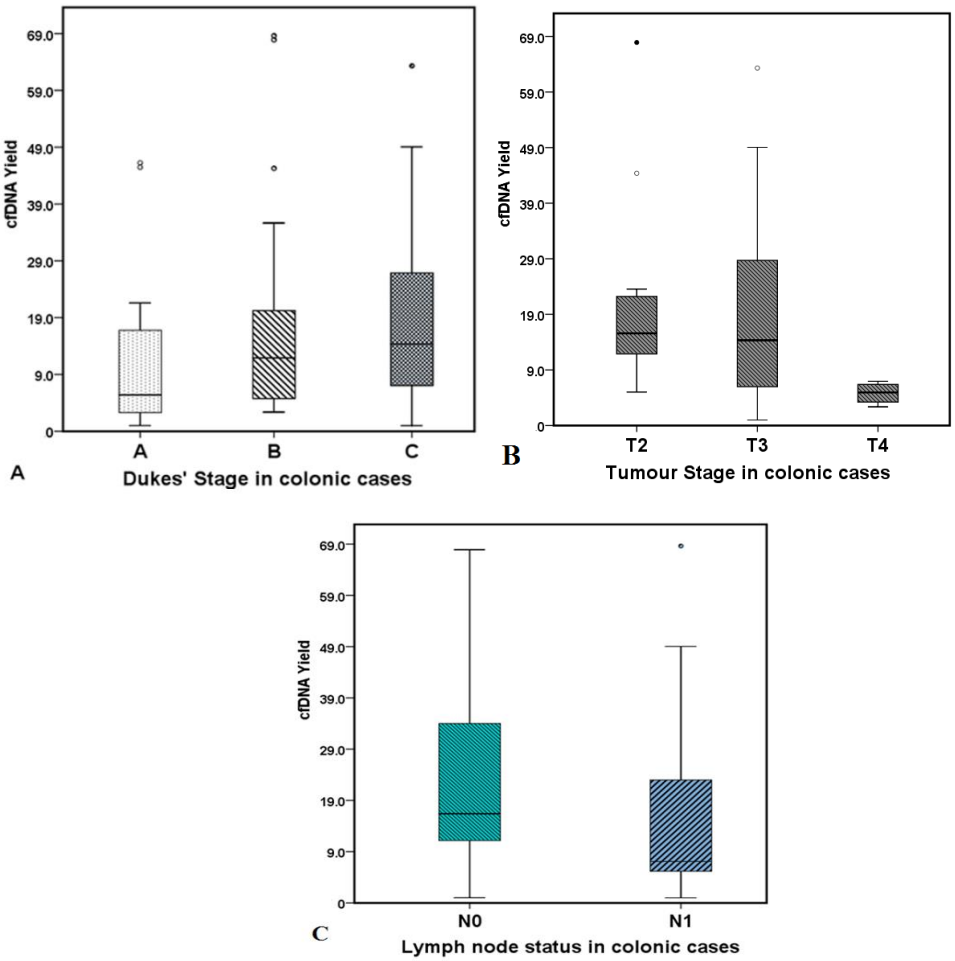


Figure 5-3 Figure show box and whisker plot for actual total cfDNA yield. cfDNA yield values in ng/µl. The relation between the cfDNA levels and the clinicopathological features of the colonic tumours. A: Dukes' stage *(NS*). B: T stage (*NS*)). C: Lymph node status (*NS).*

5.3.4 **cfDNA analysis in rectal cancers**

In 29 rectal cancers, 4 had no plasma for cfDNA analysis. Of the remaining 25 there was no association between cfDNA levels and the various clinicopathological parameters. Some rectal cases 17/25 were treated with neoadjuvant treatment before surgery, for such cases, the time of blood sample was after the treatment. However, there was no significant difference between

cases treated with neoadjuvant treatment and cases who treated with surgery in relation to the cfDNA levels (Figure 5-4).

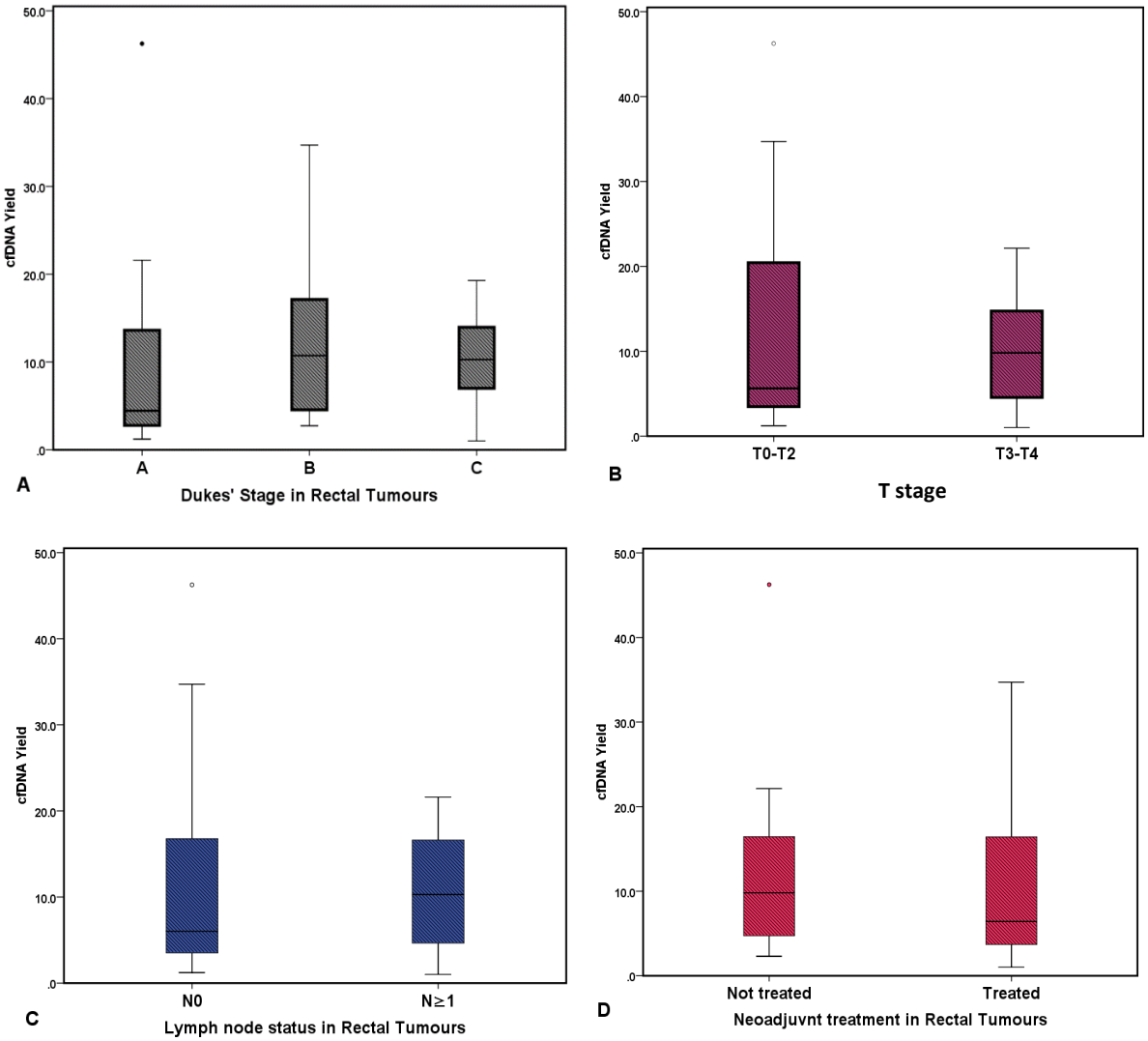


Figure 5-4 Figure show box and whisker plot for actual total cfDNA yield. cfDNA yield values in ng/µl. The relation between the cfDNA levels and the clinicopathological features of the rectal tumours. A: Dukes' stage *(NS)*. B: T stage *(NS)*. C: Lymph node status *(NS)*. D: Type of treatment *(NS)*.

5.3.5 **cfDNA analysis and treatment**

Treatment of CRC in this cohort included surgical resection of colonic tumours and some of the rectal tumours. The majority of rectal tumours (69%) underwent neoadjuvant therapy before surgery. Total levels of cfDNA were measured for all colonic cases and some rectal cases before treatment. Fourteen cases returned for follow up and also had their blood samples taken after their surgery: 7 rectal cases treated with neoadjuvant therapy and 7 colonic cases treated with surgery. The analysis of cfDNA in those cases before and after treatment showed that there were significantly higher cfDNA levels post -treatment in both colonic and rectal cases [Wilcoxon matched-pairs signed rank test (p=0.015) and (p=0.01) respectively], (Figure 5-5).

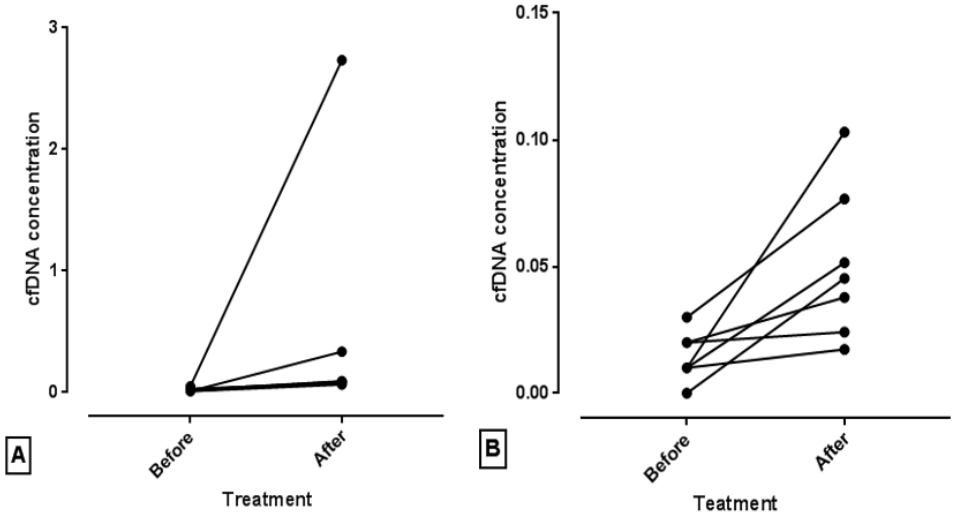


Figure 5-5 Total cfDNA levels before and after treatment, A: Colon cases, B: Rectum cases.

5.3.6 **cfDNA levels and patient survival**

Overall survival analysis was performed using cox regression and Kaplan–Meier methodology. There was no evidence of a greater risk of death associated with the detected levels of the cfDNA, *p> 0.05*. Also, high cfDNA concentrations >20ng/mL (> upper 25% quartile) and low cfDNA concentrations ≤20ng/mL (<75% quartile) had no effect on the survival of the CRC patients in this study (Kaplan–Meier curves *P>0.05),* (Figure 5-6). The overall survival analysis in

relation to the total cfDNA levels was also conducted separately on the colonic cases and rectal cases and no significant relation was observed.

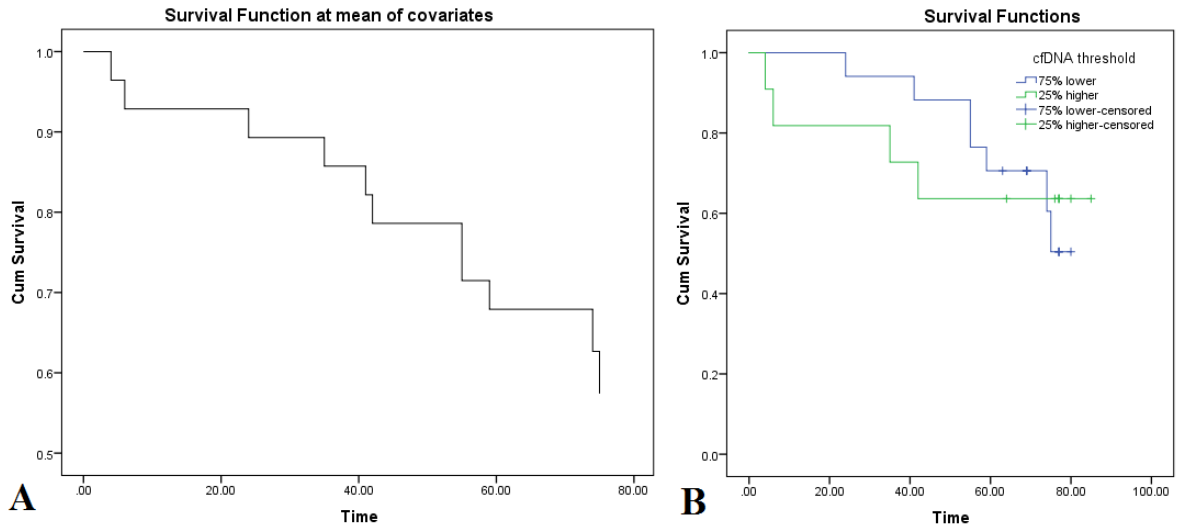


Figure 5-6 Association of cfDNA concentration with overall survival A: Cox regression analysis for the cfDNA B: Kaplan–Meier curves from low levels of cfDNA and high cfDNA ≥ 20ng/mL (<75% quartile) levels *P>0.05.*

5.4 Discussion

This chapter describes the total levels of cfDNA in localised primary CRCs with different tumours involving the colon and the rectum. To the best of our knowledge, this is the first study that utilised the ALU69 repeat assay to quantify total cfDNA levels in CRC. The yield of cfDNA had various relations to the clinicopathological of this CRC cohort. cfDNA concentrations were below or equal to 89 ng/mL. This result is comparable to those previously published (da Silva Filho et al., 2013) (Flamini et al., 2006).

There were a heterogeneity in total cfDNA yield and this could be due to multiple factors including the quality of the cfDNA samples as they were stored for longe time before the analysis (Sozzi et al., 2005) (Xue et al., 2009). Also, different tumour stages could affect the cfDNA yield as more advanced tumours are capable of producing significant levels of cfDNA. therapeutic agents has been proven that they have a major impact on the total levels of the cfDNA (Schwarzenbach et al., 2008), therefore, variety in the cfDNA levels can be seen in the study.

Use of ALU repeats in cfDNA quantifications, especially in early stages with the CRC has shown a good discriminatory test with high detection sensitivity up to 92% and inexpensive to be used in early stages of the disease (Umetani et al., 2006B). da Silva Filho and his colleges in 2013 illustrated the potentials of the using ALU repeats as distinctive levels between normal controls , and people with CRC, as well as operated and non-operated patients (da Silva Filho et al., 2013).

Most cases (66%) in this study were Dukes' A and Dukes' B. Almost all tumours at these stages can be cured with conventional surgery alone, without the need for adjuvant treatments. Interestingly, these data showed that early cancers give rise to considerable levels of cfDNA that can be detected with sufficiently sensitive assays. Moreover, small tumours were able to yield significant cfDNA levels as was seen in colonic cancer cases, which give more importance to cfDNA in early diagnosis and monitoring of the disease (Diehl et al., 2005). Importantly, at these early stages there is no lymph node or distal metastasis and patients have a good 5-year survival rate at 75-80%, while other studies have involved more advanced

stages (Frattini et al., 2005) (Diehl et al., 2008) (Spindler et al., 2012) (Mostert et al., 2013) (Tie et al., 2015).

5.4.1 Correlations between the cfDNA and T stage

Most CRC-related deaths can be preventable through early detection and removal of early-stage cancer or precancerous lesions (Kim et al., 2007), those lesions are usually small in early stages and sometimes not detectable before going deeper in more critical stages.

In the present study, the relationship between the T stage and the cfDNA level signifies the potential use of cfDNA in early cancer detection. Previously, studies investigated the stage rather than the size in relation to the cfDNA integrity or levels in CRC. Small tumours containing ~50 million malignant cells could produce a detectable cfDNA in the circulation (Yamada et al., 1998), a tumour of this size is far below that needed for definitive imaging at present (Diaz Jr et al., 2012) (Bettegowda et al., 2014). This advantage of cfDNA could promise early management of small non-detectable tumours by conventional imaging.

5.4.2 Correlations between the cfDNA and lymph node metastasis

The diagnosis of lymph node- negative CRC should indicate a good prognosis. Furthermore, the most important indicator of overall survival and therapeutic response to adjuvant chemotherapy is the presence of metastatic tumour cells in local lymph nodes. Although histopathologic analysis of lymph nodes is principal to all CRC staging criteria, its prognostic and predictive value is limited. Indeed, about 25% of patients with histopathology-negative lymph nodes die from metastatic cancer. CRC with stage I and II may just undergo surgical resection with no adjuvant therapy, usually without LN metastasis (Hyslop and Waldman, 2013) (Benson et al., 2014) (Aldecoa et al., 2016). Hence, early detection of LN involvement could change the treatment choices and give a better prognosis. Total cfDNA levels have no relation to the status of the LN in this cohort, neither in colonic cases nor with rectal ones.

In the rectal tumours, there was no relation between the cfDNA and any of the clinicopathological features, which could be explained by the small number of the

cases, and the majority of the rectal cases were treated with neoadjuvant therapy before the blood samples were taken. This finding contradicts the data illustrated by Agostini et al., 2011 who measured the cfDNA integrity of rectal cancer cases pre and post treatment using ALU repeats, his study indicated how cfDNA could distinguish responsive from unresponsive cases and showed that cfDNA levels were low in response to the neoadjuvant therapy (Agostini et al., 2011). These findings confirm the effects of the neoadjuvant treatment on the levels of cfDNA and could explain the weak correlation of cfDNA levels with the clinical parameters in rectal tumours.

5.4.3 **Distinctive features of colon and the rectum**

There is still debate about whether colon and rectum should be recognised as a single organ or two separate entities. There is already a correlation between cancer incidence rates for both parts perceived in various ethnic populations (Boyle et al., 1985). Epidemiologically, there are studies stated an increase in the incidence of right-sided CRC and a decrease in recto-sigmoid tumours suggesting different aetiology for both types of tumours (Cucino et al., 2002) (Rabeneck et al., 2003). Biologically, there are >1,000 genes distinguishably expressed in adult right-sided versus recto-sigmoid epithelium (Glebov et al., 2003). Minoo et al., 2010 illustrated that right-sided CRC is significantly larger in T stage , exhibit significantly higher tumour grade than the rest of colon and rectum (Minoo et al., 2010). *MSI-H* status is more common in proximal colon cancers than distal ones (Minoo et al., 2010). *BRAF* and *KRAS* mutations are also more prevalent in colonic tumours than in those of the rectum (Frattini et al., 2008) (Minoo et al., 2010).

In this study, there is no difference between the colonic and rectal cancer cases in relation to the cfDNA levels, which disagrees with the data reported by Frattini et al.,2008 that a higher significant level of cfDNA in colonic cases more than the rectal case, which could be referred to the various vascular supply for both types of cancer (Frattini et al., 2008). Moreover, oncogenic driver mutations and different types of treatment for colonic and rectal cancers might explain why

cfDNA levels are higher in colonic cases more than the rectal cancers, which will be explored in details in the next Chapter.

This data shows that there were higher cfDNA levels post-treatment than pre-treatment, in disagreement with the findings by da Silva Filho et al., 2013 that cfDNA levels were significantly lower in post-operative cases than pre-operative ones (da Silva Filho et al., 2013) (Frattini et al., 2008). The analysis was conducted on a small number of patients who returned for follow up, the amount of plasma used for pre-treatment less than post-treatment analysis, 1mL and 3mL respectively. It was depended on the availability of the plasma for the analysis in the first place. Moreover, pre-treatment plasma was stored for at least seven years before cfDNA was extracted, while post-treatment recovered within two years after blood samples taken. These cases had no relapse and still alive at end of the study. Low pre-treatment cfDNA levels be explained by the long-term effects of the plasma storage on the quality of the cfDNA (Sozzi et al., 2005) (Xue et al., 2009). Nevertheless, storage duration has no impact on the detection of specific sequences or mutations in cfDNA as mutations can be discovered several years after freezing plasma samples (El Messaoudi et al., 2013). Hence, the results of pre and post therapeutic cfDNA levels in this study could depend on technical issues (as mentioned) rather than reflect a true biological phenomenon or as a result of treatment.

In this cohort, total cfDNA levels was not correlated to the survival of the patients. This finding is in disagreement with previous studies in colorectal cancer, which reported that high cfDNA levels correlate with shorter survival (Schwarzenbach et al., 2008) (Thierry et al., 2010). Most of studies analysed the patient survival in relation to the mutant cfDNA rather total cfDNA such as *BRAF* and *KRAS* mutant cfDNA (Kin et al., 2013). However, high cfDNA levels was correlated to poor outcome in metastatic CRC in a study by Spindler et al. (Spindler et al., 2012)

The presence of no relation between the total cfDNA levels and patient survival might linked to the heterogeneity of cfDNA levels, as this cohort included localised, non-metastatic tumours as well as the small sample size cannot show a significant link between the cfDNA levels and the patient survival. This comes in line with Frattini et al., concluded in 2008 (Frattini et al., 2008).

No doubt, that large tumours are more likely to be cfDNA secretory masses, which could accompany with metastatic lesions. mCRC has been studied more than any stage, and there is a high chance of obtaining as cfDNA in better quality and containing the genetic alterations of the primary tumours (Spindler et al., 2012) (Mostert et al., 2013) (Tie et al., 2015). However, early stages remain challenging in regard to cfDNA presence and genetic alteration detection.

Chapter 6

Circulating free DNA mutation analysis

6.1 Introduction

The MAPK pathway is an essential intracellular signalling cascade that intermediates vital functions such as cell growth, cell survival and proliferation (Friday and Adjei, 2008). Proteins including KRAS and BRAF play a fundamental role in regulating this pathway and are prominent members in the biological downstream biomarkers of the EGFR (Simi et al., 2008) (Parsons et al., 2005). Therefore, *KRAS* and *BRAF* mutations are key drivers in CRC tumourigenesis leading to inappropriate functioning of the majority of cellular responses. In addition to *PIK3CA*, which has a significant role in CRC by upregulating cell proliferation and lowering apoptosis (Vivanco and Sawyers, 2002).

Therefore, detecting mutations in these 3 genes in tumours tissues and matched plasma of CRC patients may help and guide treatment and predict therapeutic response. In this study, PNA-mediated PCR clamping, locked primers with Allele Specific LNA and touchdown qPCR was used to establish a robust, sensitive method to detect gene mutations in minority DNA species.

As described in chapter 2 and 3, AS-LNA/PNA-mediated PCR clamping is very sensitive assay in comparison to other detection and sequencing techniques. Both assays can achieve and be sensitive down to a limit of detection of 0.001% of the mutant allele. They were validated independently and later combined in one assay to optimise the detection sensitivity and lower the likelihood of technical errors.

In this chapter, the emphasis will be on the analysis of cases with mutations in the tissue and matched plasma, starting with a comparison between specimens that had the mutations only in their tissue in the whole cohort and then progressing with the separate analysis of the colonic and rectal cases.

6.2 Aims and objectives:

The aim of this chapter was to study the association between mutations in 3 key genes in cfDNA and the clinicopathological features of the localised primary cancers including early stage CRC cohort and relate this to the total cfDNA levels. A second aim was to compare mutational cfDNA status before and after treatment with patient survival.

Objectives

1. To extract cfDNA from cases matched to mutant CRC tissue samples.
2. To investigate *KRAS*, *BRAF* and *PIK3CA* mutations using AS-LNA/PNA clamping qPCR in cfDNA matched to mutant CRC tissue samples.
3. To relate results to patients clinicopathological characteristics.
4. To investigate key gene mutations in mutant cfDNA before and after treatment and patient survival.

6.3 Results:

In this chapter, descriptive analysis for the mutational status of the whole cohort and what gene mutations they harboured in either tissue or matched plasma, then further investigation has been carried out on the ''Tissue mutation positive'' cases (38/84) based on the cfDNA levels analysed in chapter 5.

Afterwards, a comparison between the cases of the whole cohort according to the mutation status of tissue or/and matched plasma in relation to the total cfDNA levels section 6.3.2.

The following section (6.3.3) concerns the relation between the clinicopathological features of the patients' cohort and the mutational status of the plasma mutation positive cases. Section 6.3.4 describes the analysis of the relation between the colonic and rectal cases with regard to total cfDNA as well as and the presence or absence of mutations in the plasma samples. In addition, the relation between the colonic and rectal cases in terms of the number of mutant cases (Plasma mutation positive) is analysed for the genes *KRAS*, *BRAF* and *PIK3CA*

with descriptive analysis of mutation frequency for each gene according to the tumour location either in the colon or in rectum.

The subset analysis for the colonic cases is described in section 6.3.5, starting by a comparison between the ''Tissue mutation positive'' colonic cases in relation to the total cfDNA levels, then the relation between the clinicopathological features of the and the mutational status of the plasma (Plasma mutation positive) in terms of total cfDNA levels. The same analysis is done for the rectal cases in section 6.3.6.

6.3.1 **Patient samples and controls**

74/84 patients' plasma samples subjected to cfDNA mutation analysis, starting by detecting the mutations for the genes *KRAS, BRAF* and *PIK3CA* in the tumour tissue, which accompanied by analysing the normal tissue of the same patient, if the mutation was found in the any case the same hot spot mutation will be analysed in the matched cfDNA. Mutant cell lines and non-mutant normal human genomic DNA were used as controls in these assays.

Plasma preoperative samples were stored for at least 7 years before using them in this project. 10 cases were excluded because of unavailability of the plasma and sample size went down from 84 to 74. Moreover, 10 negative plasma samples were used to screen for all mutational assays to avoid any false positive results.

6.3.2 **Mutation analysis in primary tumours and matched cfDNA**

The mutational analysis for the genes *KRAS*, *BRAF* and *PIK3CA* was performed on cfDNA of cases that had at least one of oncogenic mutations in their primary tumour tissue 38/41 mutant cases (Tissue mutation positive). Unfortunately, no plasma samples were available for the three mutant cases. As a control group, 10 plasma samples from the tissue mutation negative cases were also tested by the mutation detection assays, and none of their cfDNA was tested positive for the targeted three gene mutations. The number of cases studied for the comparison between tissue and peripheral blood presented in (Table 6- 1).

Mutation analysis		*n*	Positive	Negative
FFPE	Colonic	55	25	30
	Rectal	29	16	13
	Total	84	41(49%)	43 (51%)
cfDNA	Colonic	24/25*	13	11
	Rectal	14/16*	2	12
	Total	38	15 (39.4%)	23 (60.5%)

Table 6-1 Illustration of number of the FFPE tissues and cfDNA were involved in the mutational analysis. The analysis included colonic and rectal, also, showing the number of positive and negative cases,* indicate the cases were excluded because of unavailability of the plasma for analysis.

6.3.2.1 ***BRAF* mutation analysis**

Based on AS-LNA/PNA clamping qPCR technique, mutational status of the CRC cohort was detected for the *BRAF* ^{V600E} mutation. Overall *BRAF* mutation in the CRC cohort was 12/84 (14%) in FFPE tissue. Two cases were excluded from the cfDNA mutation analysis due to unavailability of plasma samples. 6 of 7 cases had the same mutation detected in cfDNA:all of them were right-sided colonic cases at concordance rate of 86% (Table 6-2 and 6-6). No *BRAF* ^{V600E} mutation was found in the cfDNA of the rectal cases (Table 6-2). Reference controls of human genomic (HG-DNA) DNA and A375 cell line (a homozygous cell line for *BRAF* ^{V600E} mutation at serial dilutions of 1:10, 1:100 and 1:1000 were used in each experimental assay.

	FFPE	Excluded cases	Mutant cfDNA	FFPE/cfDNA Concordance
Colonic Ca (*n*=55)	8/55	1	6/7	86%
Rectal Ca (*n*=29)	4/29	1	0/3	-

Table 6-2 *BRAF* ^{V600E} mutational analysis in colorectal carcinoma primary tumours and matched plasma (cfDNA) samples.

6.3.2.2 *KRAS* mutations analysis

KRAS point mutations *(p.G12D, p.G12V and p.G13D)* were detected by AS-LNA/PNA clamping qPCR technique in primary tumour tissue samples of 17/84 (20%) patients and in matched plasma for 6 of 17 cases (35.2%) (Table 6-3, 6-4 and 6-6). The concordance rate was higher in the colonic than rectal cases (Table 6-3). Reference controls of HG-DNA at serial dilutions of 1:10, 1:100 and 1:1000 including mixtures of HG-DNA and GP2d, SW626 and HCT116 for the *KRAS* point mutations *(p.G12D, p.G12V and p.G13D)* respectively.

	FFPE	Mutant cfDNA	Mutation rate in FFPE	FFPE/cfDNA Concordance
Colonic Ca (*n*=55)	9/55	4/9	16.3%	44.4%
Rectal Ca (*n*=29)	8/29	2/8	27.5%	25%

Table 6-3 *KRAS* mutational analysis in colorectal carcinoma primary tumours and matched plasma (cfDNA) samples.

***KRAS* point mutations**	**FFPE**	**%**	**Mutant cfDNA**	**FFPE/cfDNA Concordance**
p.G12D	7	41%	3	50%
p.G12V	6	35%	2	33%
p.G13D	4	23.5%	1	16%

Table 6-4 Distribution of detected *KRAS* mutations in primary tumour tissue and matched cfDNA samples.

6.3.2.3 *PK3CA* mutations analysis

Analysis of *PIK3CA* mutations in tissue and plasma samples was performed based on PNA-qPCR clamping and specific probes technique. The three most common *PIK3CA* hotspot mutations (*p.E542K, p.E545K and p.H1047R*) were investigated. 19/84 (22%) FFPE in the CRC cohort had *PIK3CA* mutations. Only 3 of 19 cases had the same mutation in matched cfDNA, and all of them were colonic cases (Table 6-5 and 6-6). *p.H1047R* was the only point mutation detected in the cfDNA of this cohort. Reference controls of HG-DNA at serial dilutions of 1:10, 1:100 and 1:1000 including mixtures of HG-DNA and, SW948, MCF7 and HCT116 for the mutations (*p.E542K, p.E545K and p.H1047R*) respectively (Table 6-5).

	FFPE	**Mutant cfDNA**	**FFPE/cfDNA Concordance**
Colonic Ca (n=55)	15/55	3/15	20%
Rectal Ca (n=29)	4/29	0/4	-

Table 6-5 the *PIK3CA* mutational analysis in colorectal carcinoma primary tumours and matched plasma (cfDNA) samples.

	Patient ID	Mutations	Additional mutations	Tumour Location	Pre-treatment cfDNA mutations	Post-treatment cfDNA mutations
1	H467/14	PIK3CA H1047R	PIK3CA E545K	Left Colon		PIK3CA H1047R
2	H486/14	PIK3CA H1047R		Left Colon		
3	H433/14	PIK3CA H1047R		Left Colon		
4	H460/14	PIK3CA H1047R		Right Colon	PIK3CA H1047R	
5	H928 /13	PIK3CA H1047R	PIK3CA E545K	Right Colon	PIK3CA H1047R	
6	H473/14	PIK3CA H1047R	PIK3CA E545K	Right Colon	PIK3CA H1047R	
7	H911/13	PIK3CA H1047R		Right Colon		
8	H930 /13	PIK3CA H1047R		Rectum		
9	H470/14	PIK3CA H1047R		Rectum		PIK3CA H1047R
10	H454/14	PIK3CA H1047R		Rectum		
11	H459/14	PIK3CA E545K		Left Colon		
12	H487/14	PIK3CA E545K		Rectum		
13	H497/14	KRAS G13D	PIK3CA E542K	Right Colon		
14	H448/14	KRAS G13D		Rectum	KRAS G13D	
15	H490/14	KRAS G13D		Rectum		
16	H450/14	KRAS G13D		Rectum		
17	H916 /13	KRAS G12V		Left Colon		
18	H439/14	KRAS G12V	PIK3CA E542K	Left Colon	KRAS G12V	
19	H446/14	KRAS G12V		Left Colon		
20	H484/14	KRAS G12V		Rectum		
21	H488/14	KRAS G12V		Rectum		
22	H492/14	KRAS G12V		Rectum		
23	H927 /13	KRAS G12D		Left Colon	KRAS G12D	
24	H469/14	KRAS G12D	PIK3CA H1047R	Left Colon		
25	H435/14	KRAS G12D	PIK3CA H1047R	Right Colon	KRAS G12D	PIK3CA H1047R
26	H462/14	KRAS G12D		Right Colon		
27	H456/14	KRAS G12D	PIK3CA E545K	Right Colon	Plasma N/A	
28	H1030 /13	KRAS G12D		Rectum	KRAS G12D	
29	H479/14	KRAS G12D		Rectum		
30	H918 /13	BRAF V600E		Right Colon	BRAF V600E	
31	H519/13	BRAF V600E		Right Colon	BRAF V600E	
32	H447/14	BRAF V600E		Right Colon	BRAF V600E	
33	H912 /13	BRAF V600E	PIK3CA E542K	Right Colon	BRAF V600E	
34	H493/14	BRAF V600E		Right Colon	BRAF V600E	
35	H451/14	BRAF V600E		Right Colon	BRAF V600E	
36	H475/14	BRAF V600E		Right Colon	Plasma N/A	
37	H436/14	BRAF V600E	PIK3CA H1047R	Right Colon		
38	H932 /13	BRAF V600E		Rectum		
39	H463/14	BRAF V600E		Rectum		
40	H434/14	BRAF V600E		Rectum		
41	H458/14	BRAF V600E		Rectum	Plasma N/A	

Table 6-6 The mutational status of cfDNA CRC patients, including the mutation found before and after treatment

6.3.3 **Analysis of cfDNA based on tumour-specific mutations**

Plasma of 74/84 was available for the analysis; the ALU 69-qPCR assay was used to quantify the cfDNA of the CRC patients. Following the mutation analysis of the tumour tissues for the genes *KRAS*, *BRAF* and *PIK3CA* , only matched cfDNA of Tissue mutation positive CRC cases were subjected to the mutation analysis, cfDNA samples were not tested for the mutations in patients were negative for *BRAF*, *KRAS* and *PIK3CA* mutations in tissue.

In the patient with mutations in the tissue, the group was further subdivided as following (A) mutation in tumour and plasma (B) mutations in tumour but not plasma and all tissue negative cases for the group C combined when the plasma was not analysed (Figure 6-1). This analys is based on the total cfDNA levels that were investigated in chapter 5.

There was no significant difference between three groups regarding total cfDNA levels. However, there was a statically significant difference between the first and second group in relation to cfDNA yields (Mann–Whitney U = 93, p=0.02), meaning that, the presence of gene mutations was associated to higher cfDNA levels. There was no significant correlation between other groups (Figure 6-1).

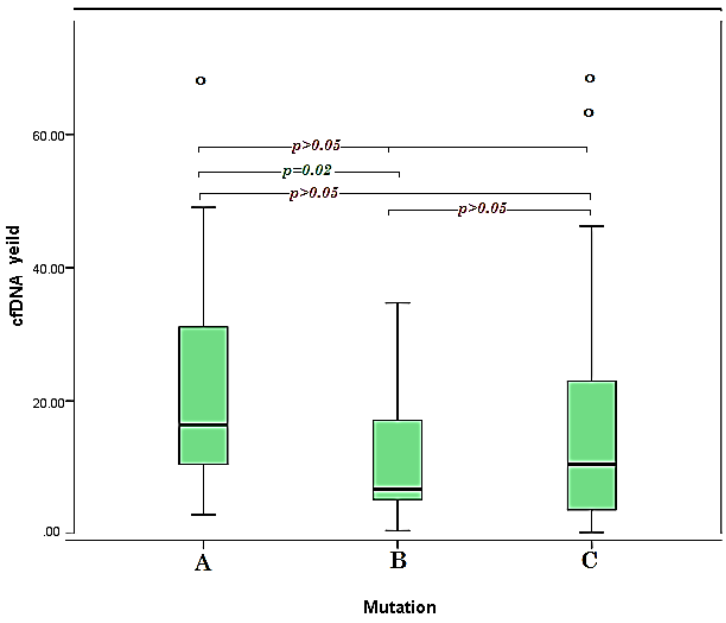


Figure 6-1 Figure show box and whisker plot for actual total cfDNA yield. cfDNA yield values in ng/µl. Comparison between the CRC cases in relation to gene (*BRAF*, *KRAS* and *PIK3CA*) mutations and cfDNA yield. A: cfDNA levels in CRC cases with mutations in tissue and plasma.

B: cfDNA levels in CRC cases with mutations in tissue but not in the plasma. C: cfDNA levels in CRC cases with no mutations in tissue and plasma was tested.

6.3.4 **cfDNA mutation analysis in the CRC cohort**

Oncogenic mutations for the genes *KRAS*, *BRAF* and *PIK3CA* in the cfDNA analysed in the plasma of mutation CRC tissue specimens were examined in relation to the CRC clinicopathological features, including the tumour stage, T stage and lymph node metastasis. Plasma mutation positive cfDNA in the whole cohort had no significant relationship with the tumour stage (Chi- square test $p>0.05$). Although mutations were detectable in cfDNA of cases (approximately 50%) with localised disease, particularly Stage 1 and Stage 2, there was an increasing likelihood of cfDNA mutation detection in advanced stages (Chi-square test for trend $p= 0.01$) (Figure 6-2).

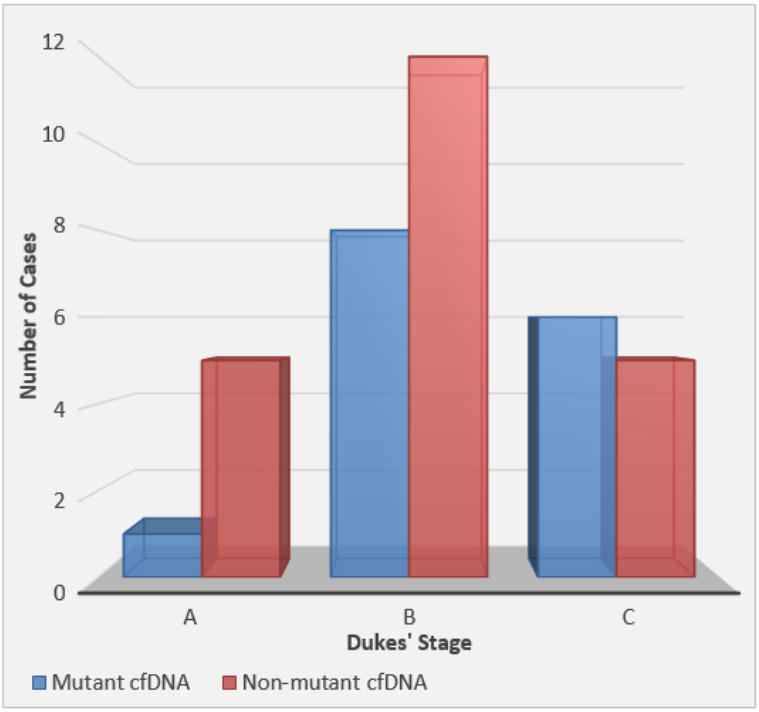


Figure 6-2 Relationship between the tumour stage and the Plasma mutation positive cfDNA (Mutations including *BRAF*, *KRAS* and *PIK3CA*) in the whole cohort.

There was no significant difference between the T stage in relation to gene mutations in cfDNA (Chi- square test $p>0.05$) (Figure 6-3). Nevertheless, it can be seen that some mutations were detectable in small tumours (tumours with T stage T2) and more detectable Plasma mutation positive cfDNA in cases of

tumours with T stage T3, taking into consideration the relatively large number of tumours with this size(Figure 6-3).

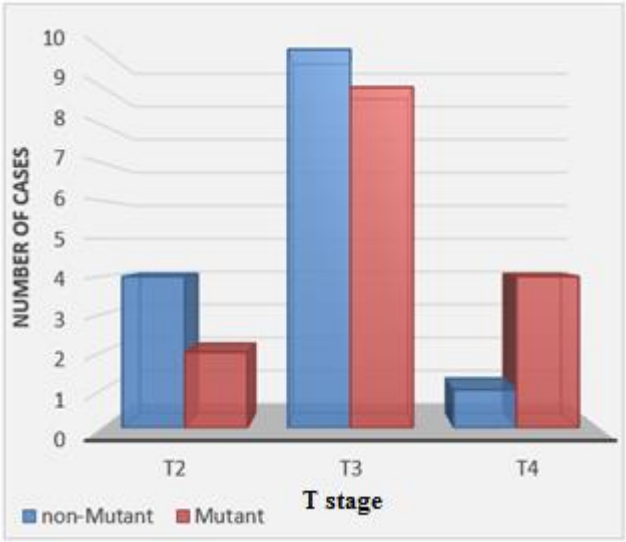


Figure 6-3 Relationship between the T stage and the Plasma mutation positive cfDNA (Mutations including *BRAF*, *KRAS* and *PIK3CA*) in the whole cohort.

There was no significant relationship between lymph node status and Plasma mutation positive cfDNA in the CRC cases (Chi- square test $p > 0.05$) (Figure 6-4). Interestingly, more than 40% of cases with negative lymph node status had a detectable mutation in cfDNA, compared with 55% of cases that had lymph nodes metastasis and harboured a mutation in cfDNA.

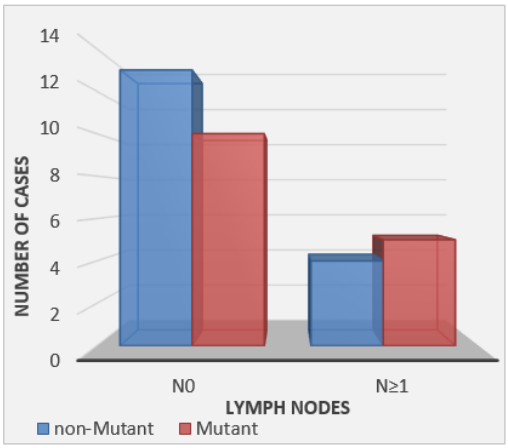


Figure 6-4 Relationship between the lymph node status and the Plasma mutation positive cfDNA (Mutations including *BRAF*, *KRAS* and *PIK3CA*) in the whole cohort, N0 = no lymph nodes involved, N≥ 1 = at least one lymph node has been involved.

6.3.5 **cfDNA mutation analysis in Colon vs. rectum**

There were no statistically significant differences between the colonic cases and rectal cases in relation to total cfDNA levels (*p>0.05*). Also, there was no relation either between all colonic cases *vs.* all rectal cases or Plasma mutation positive colonic cases *vs.* Plasma mutation positive rectal cases (Figure 6-5).

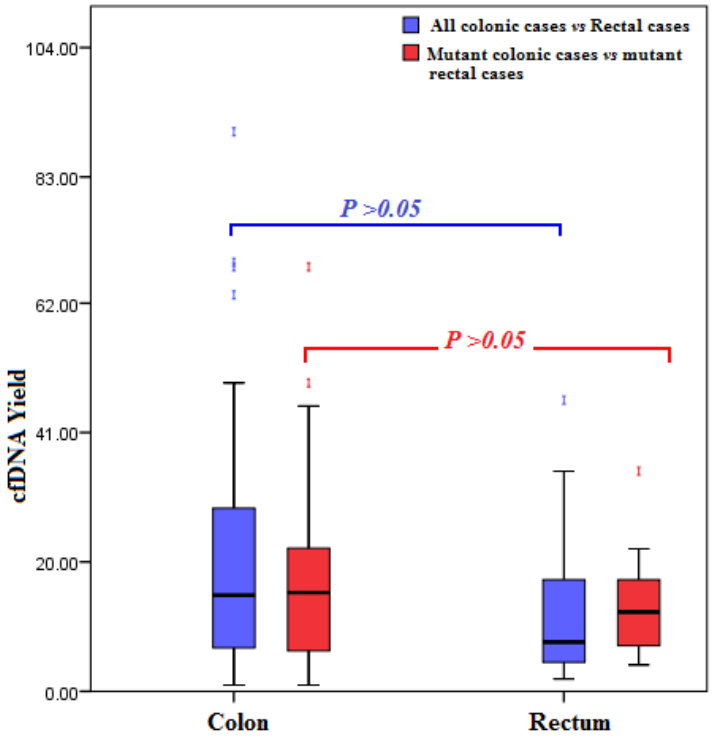


Figure 6-5 Figure show box and whisker plot for actual total cfDNA yield. cfDNA yield values in ng/µl. The relationship between the colonic and rectal cases in relation with cfDNA levels. Blue: both Plasma mutation positive for the genes KRAS, BRAF and PIK3CA and non-mutant colonic cases vs. rectal cases (*p>0.05*). Red: Plasma mutation positive colonic cases vs. rectal cases (*p>0.05*).

6.3.5.1 **Mutation analysis Colon *vs.* Rectum**

There was a significant difference between the colon and rectum in relation to mutations in cfDNA (Chi- square test *p*=0.01) (Figure 6-6), including rectal cases that were treated by neoadjuvant therapy. However, the relationship becomes insignificant if treated rectal cases were excluded.

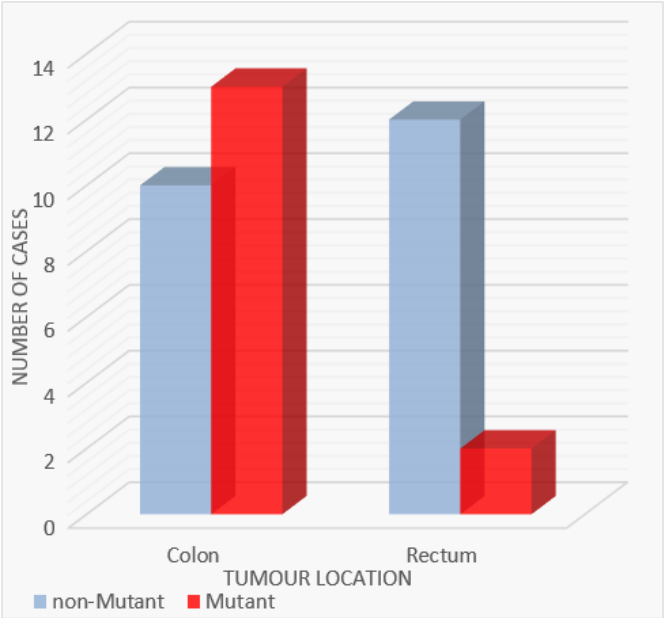


Figure 6-6 Comparison between colonic and rectal cancer cases in relation to mutations in cfDNA.

As a comparison between colonic and rectal cases, the charts below compare mutations in cfDNA and FFPE of three different genes *BRAF, PIK3CA* and *KRAS* in this CRC cohort (Figure 6-7). It is clear that the three genes mutations were more detected in cfDNA and FFPE of the colonic cases than the rectal cases. Of the three genes, *BRAF* mutation was only detectable in cfDNA of colonic cases and not in rectal ones. *PIK3CA* mutations in cfDNA were the least common in the cfDNA of colonic cases. *KRAS* mutations were identified in cfDNA of two rectal cases, and were the only gene mutations detected in the plasma of rectal tumour cases.

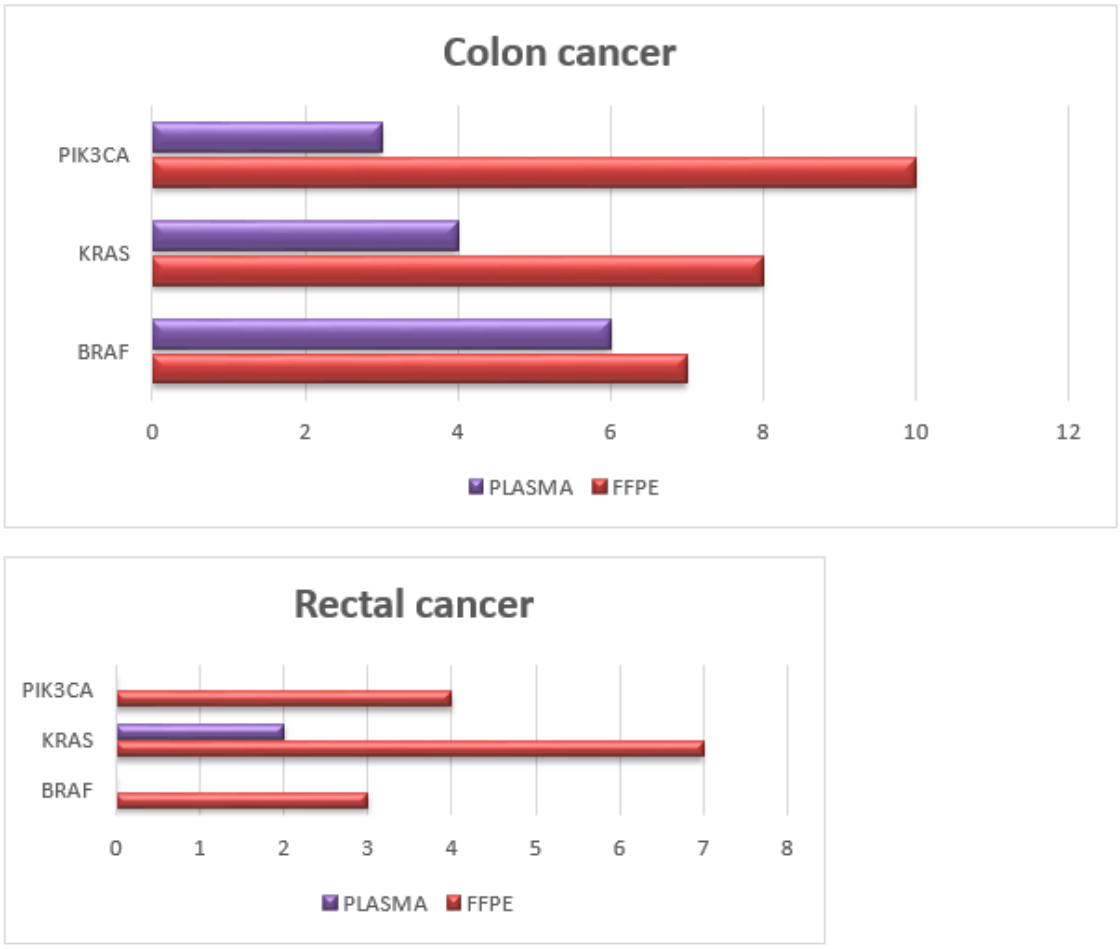


Figure 6-7 Mutation were detected in FFPE and matched plasma (cfDNA) in colon and rectal cancer cases.

Patients who received treatment (including Surgery, Chemotherapy and/or Radiotherapy) had a post-treatment blood sample tested for mutations that were found pre-operatively either in tissue or cfDNA. Mutations were found in three cases (two colonic and one rectal case), and all of them were mutant for *PIK3CA*. Two of these cases were treated with neoadjuvant therapy and one treated with surgery. No *BRAF* or *KRAS* mutations detected in pre-operatively were found in postoperative cfDNA samples (Table 6-6).

6.3.6 **cfDNA analysis in Colonic tumours**

Plasma samples of (22/55) colonic cancer cases were investigated for total cfDNA levels. Only 22 patients for which gene mutations were previously found in

primary tumours were selected. The relationship between the levels of the cfDNA and clinicopathological features including the T stage, tumour stage and lymph node of the colonic cancer cases were investigated.

6.3.6.1 **Plasma mutation positive colonic cases versus non-mutants**

There was a significant difference between the colonic cases with mutations for the genes *KRAS*, *BRAF* and *PIK3CA* in the primary tumour tissue (*n*=22) and non-mutant cases (*n*=30) in relation to cfDNA levels (Mann–Whitney U = 29, *p*= 0.02), (Figure 6-8), this analysis is different from the one conducted in section 6.3.2 as this is a subset comparison between the tissue mutation positive in colonic cases versus tissue mutation negative colonic cases. There was no difference in the distribution of Plasma mutation positive and non-mutant cases when they tested for cfDNA levels in relation to tumour stage and size as well as the lymph node metastasis (*p*>0.05, Fisher's exact test), (Figure 6-9), indicating that clinicopathological features of tumours without mutation were similar to those of tumours with mutations.

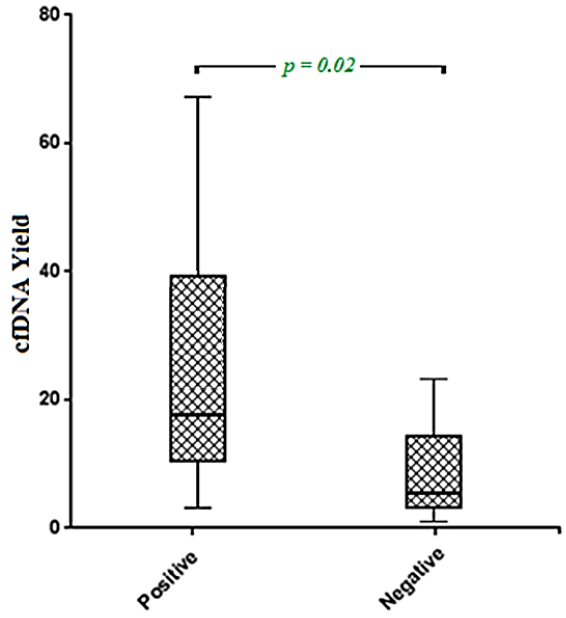


Figure 6-8 Comparison between the colonic cases with a mutation in the primary tumour tissue (*n*=22) and non-mutant cases (*n*=30) in relation to cfDNA levels (Mann–Whitney *U = 29, P=0.02* two-tailed), Positive = Mutation found in the FFPE, Negative =no mutation in the FFPE.

When the analysis was carried out for all colonic cases (*n*=52), including mutants and non-mutants, there was no difference between the total cfDNA levels in relation to tumour stage, T stage and lymph node status (Figure 6-9). However, analysis of mutant cases only showed a significant relationship between total cfDNA levels and tumour stage *(p= 0.03)*, T stage *(p= 0.04)* and lymph node status *(p<0.005*) for the 22 mutant colonic cases (Figure 6-10).

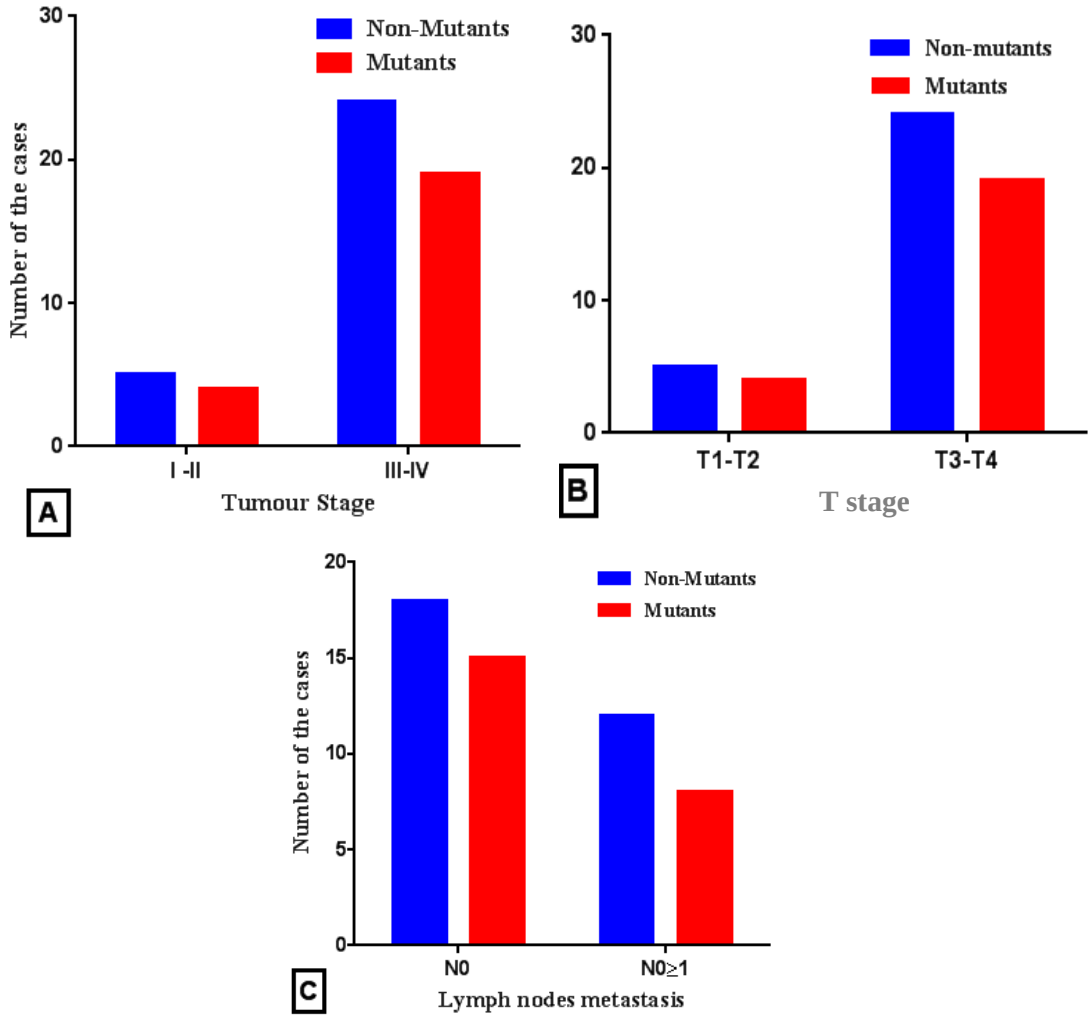


Figure 6-9 Comparison between the colonic mutant (n=22) and non-mutant cases (n=30) in relation to tumour stage (A), size (B) and lymph node status (C). There is no difference in the distribution of pathological tumour features between the mutant and non-mutant cases (p>0.05, Fisher's exact test).

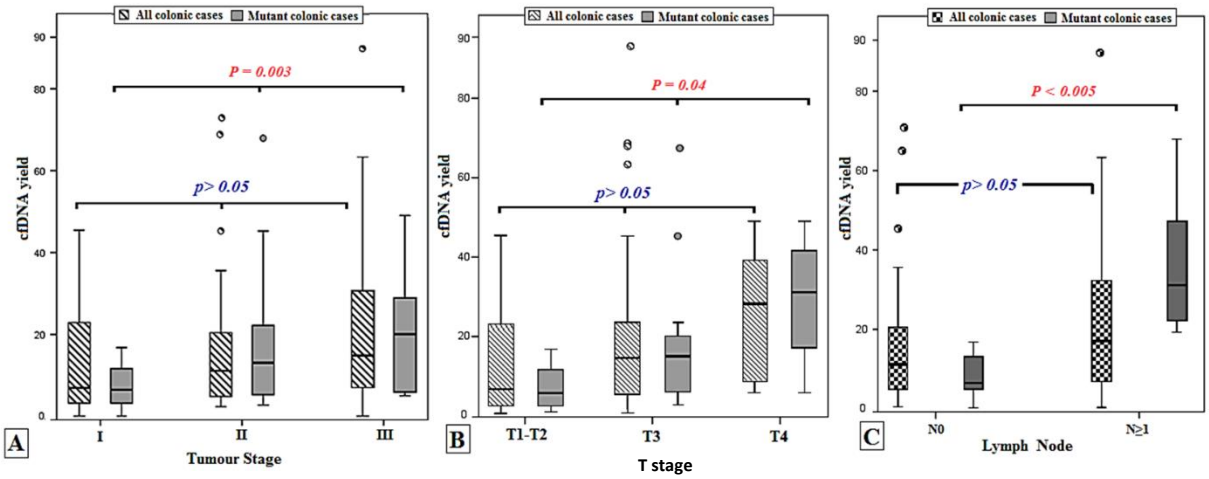


Figure 6-10 Figure show box and whisker plot for actual total cfDNA yield. CfDNA yield and the relation to the A tumour stage, B: T stage and C lymph node metastasis: only mutant colonic cases showed a significant relationship between tumour stage, size and lymph node (LN) ($p=0.003$) ($p= 0.04$) and ($P < 0.005$) respectively. cfDNA yield values in ng/µl.

6.3.6.2 **Lymph node (LN) analysis:**

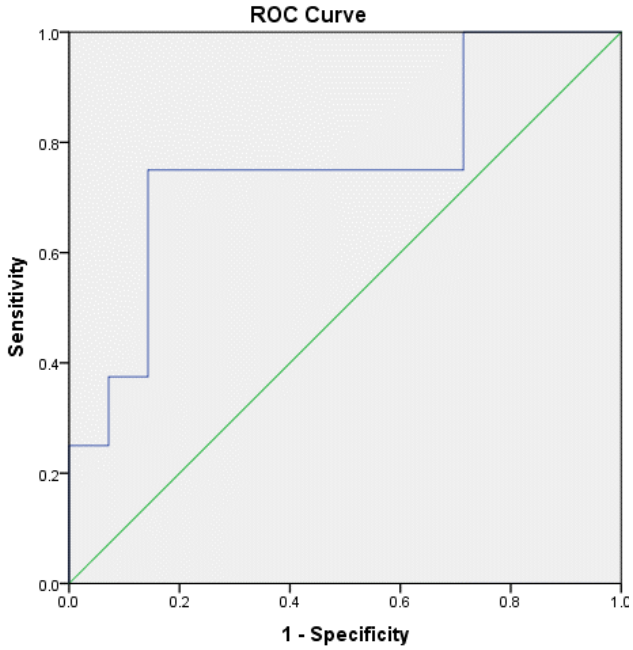
There was a significate relationship between the total mutant cfDNA levels and the LN status; the cfDNA yield was higher in cases with lymph node involvement (N≥1) than the cases without LN metastasis (N0) (Mann–Whitney U = 120, $p <0.005$) (Figure 6-10c).

Receiver Operating Characteristic (ROC) analysis of cfDNA yield for discriminating patients with local LN metastasis from patients without LN metastasis had an AUC of 0.76 (95% CI, 0.53 to 0.98) (Figure 6-11). When the sensitivity was set to 75%, specificity for LN metastasis prediction was 86% ($p = 0.04$), and the cut-off value of cfDNA yield was 18.04ng/µl.

Figure 6-11 Receiver operating characteristic curve for discrimination of patients with LN metastasis by cfDNA yield. Area under the curve is 0.76 (95% CI, 0.53 to 0.98).

6.3.7 **cfDNA analysis in rectal cancer cases**

Plasma cfDNA levels of 14/29 rectal cancer cases with mutations for the genes *KRAS*, *BRAF* and *PIK3CA* in their tissue were compared with clinicopathological



features including the T stage, lymph nodes metastasis and type of treatment (Table 6-6).

There was no significant difference between the rectal cases with mutations in the primary tumour tissue (n=14) and non-mutant cases (n=13) in relation to total cfDNA levels (Mann–Whitney U=48, p>0.05), (Figure 6-12). There was no statistically significant difference between the rectal cancer cases with lymph nodes status, tumour stage and tumour stage in relation to the total cfDNA levels (p>0.05) (Figure 6-13).

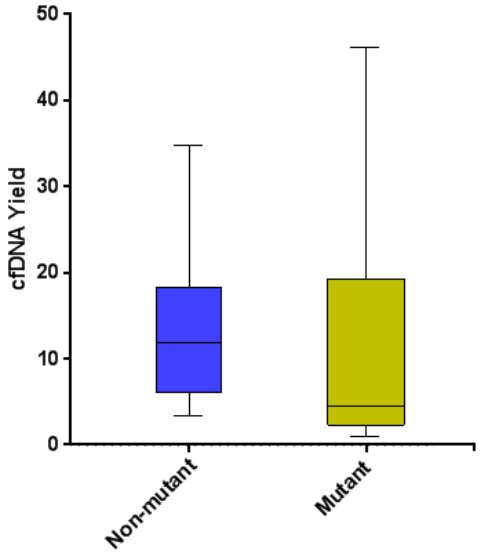


Figure 6-12 Comparison between the rectal cases with any mutation in the primary tumour tissue (*n*=14) and non-mutant cases (*n*=13) in relation to total cfDNA levels (Mann–Whitney *U = 48, P>0.05*), Mutant= Mutation found in the FFPE, non-mutant=no mutation in the FFPE.

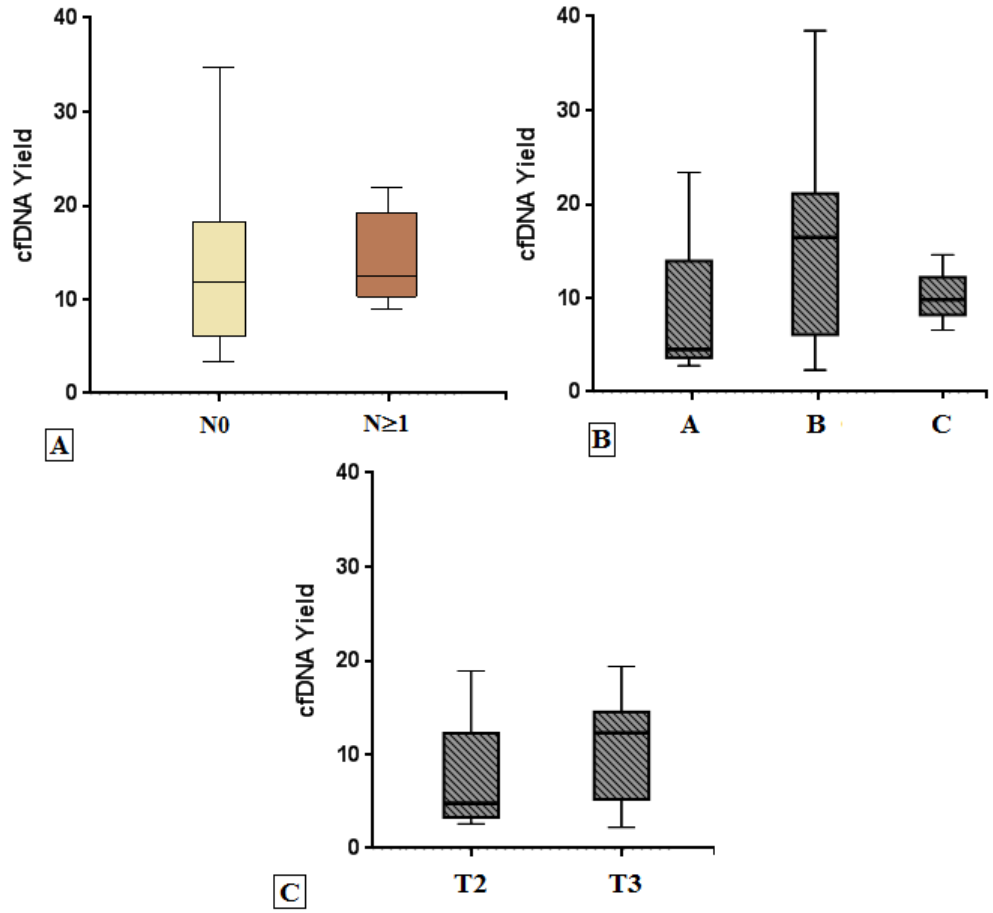


Figure 6-13 Figure show box and whisker plot for actual total cfDNA yield. cfDNA yield values in ng/µl. Total CfDNA levels and the relation to the tumour stage (TS), T stage and lymph node (LN) metastasis in rectal cases A: Lymph node status (*NS*). B Dukes' stage (*NS*). C: T stage (NS).

6.3.7.1 **The correlation to the neoadjuvant treatment:**

There was no statistically significant difference in total cfDNA levels between the rectal cancer cases that received neoadjuvant treatment and cases treated with surgery (Mann–Whitney test, U=17, $p > 0.05$) (Figure 6-14). In nine cases, cfDNA was extracted before the surgery, while, blood samples for 16 cases were obtained after the neoadjuvant therapy.

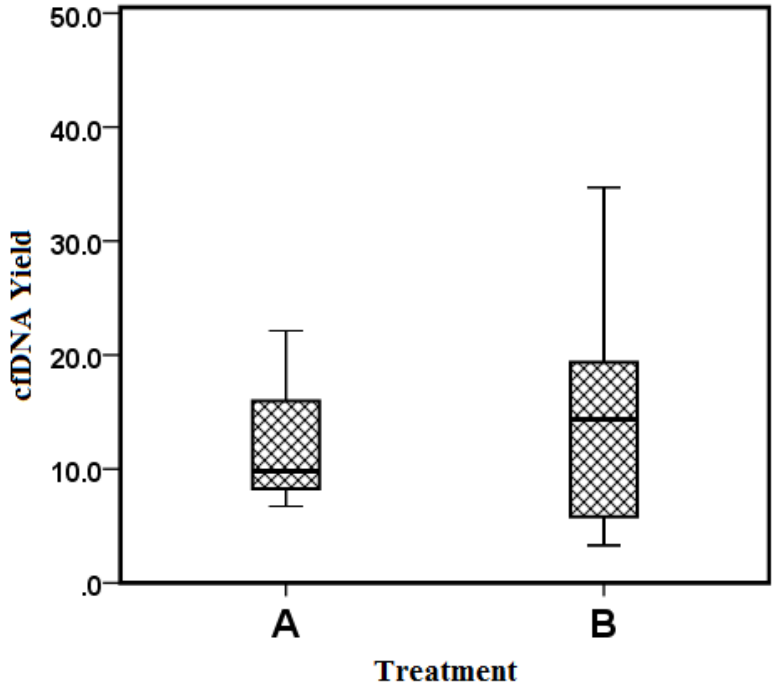


Figure 6-14 Figure show box and whisker plot for actual total cfDNA yield. cfDNA yield values in ng/µl. Total CfDNA levels and the relation to the type of treatment in rectal cancer cases, group A: treated with surgery and group B: treated with neoadjuvant therapy.

6.3.8 **Post-treatment mutation analysis**

There were only fourteen cases who returned for follow-up, and their blood samples were taken after their surgery: 7 rectal cases treated with neoadjuvant therapy and 7 colonic cases treated with surgery. The mutation analysis of cfDNA

in those cases before and after treatment showed that there were three mutant cases for *PIK3CA* in cfDNA, these cases had the mutations in the FFPE but not in pre-treatment cfDNA samples, and no history of relapse and all of them were Dukes' C stage. None of *BRAF* and *KRAS* mutations was detected after treatment (Table 6-7).

Patient ID	FFPE Mutation	Additional Mutation	PrTcfDNA Mutation	Location	PoT cfDNA Mutation	PoS	ChTh	RaTh	Duke's staging	Relapse
H467/14	PIK3CA H1047R	PIK3CA E545K	-	Left Colon	PIK3CA H1047R	Yes	Yes	No	C	No
H470/14	PIK3CA H1047R	-	-	Rectum	PIK3CA H1047R	Yes	Yes	Yes	C	No
H435/14	KRAS G12D	PIK3CA H1047R	KRAS G12D	Right colon	PIK3CA H1047R	Yes	No	No	C	No

Table 6-7 CRC cases with post-treatment cfDNA mutation analysis. PrT=Pre-treatment, PoT=Post-treatment, M= mutation, ChTh= Chemotherapy, RaTh= Radiotherapy, PoS= post-surgical blood sample.

6.3.9 **Mutation and survival analysis**

Overall survival analysis carried out using Kaplan-Meier method for colonic cancers, mutations in cfDNA were associated with significantly poor overall survivals (p<0.05) (Figure 6-16). No relation to the patient's survival was observed in the whole CRC cohort neither in connection with the mutations in FFPE nor cfDNA, and the same case was in the rectal cases (Figure 6-15).

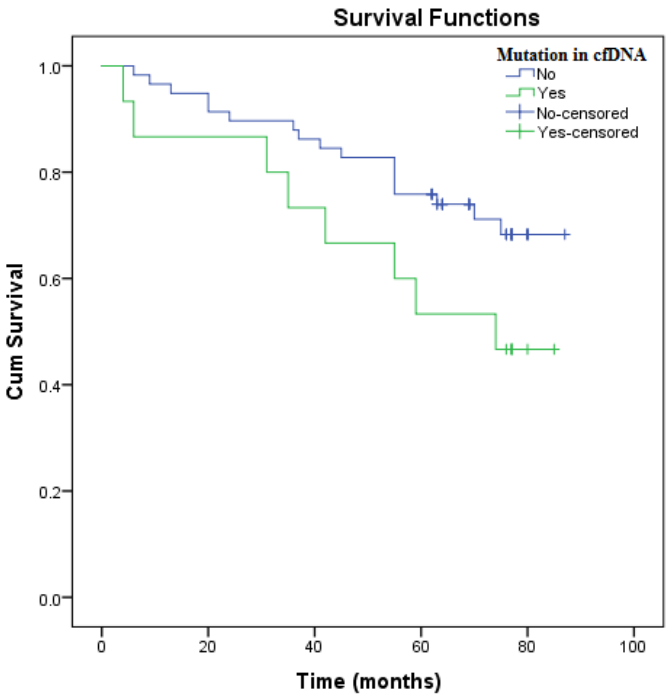


Figure 6-15 Overall survival analysis for CRC cancer cases in relation to cfDNA mutations (p >0.05).

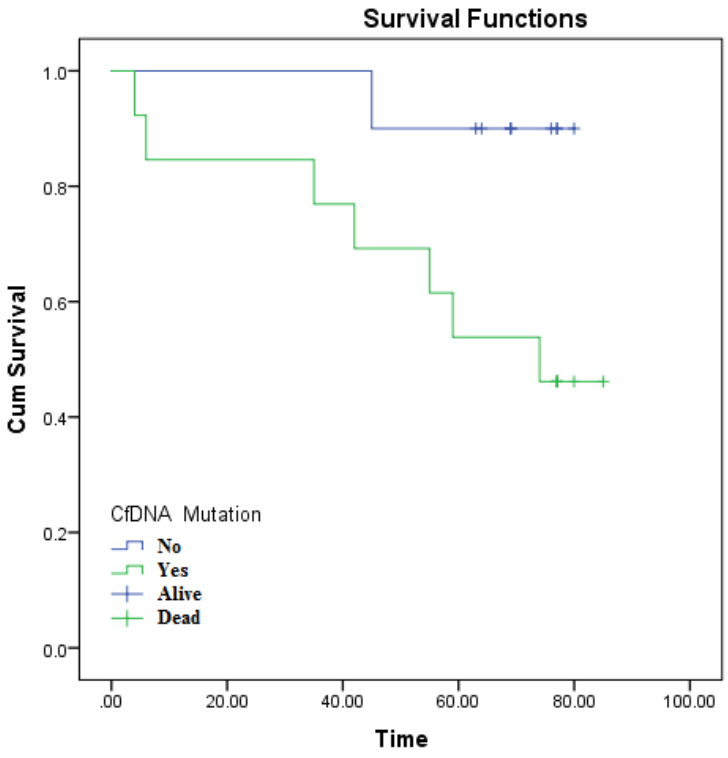


Figure 6-16 Overall survival analysis for colonic cancers in relation to cfDNA mutations *(p* <0.05).

There was a significant relation between the mutation in cfDNA and mortality in colon cancer (Fisher's Exact test *p*=0.01) (Figure 6-17). On the other hand, there was no such a relation in rectal cancer cases.

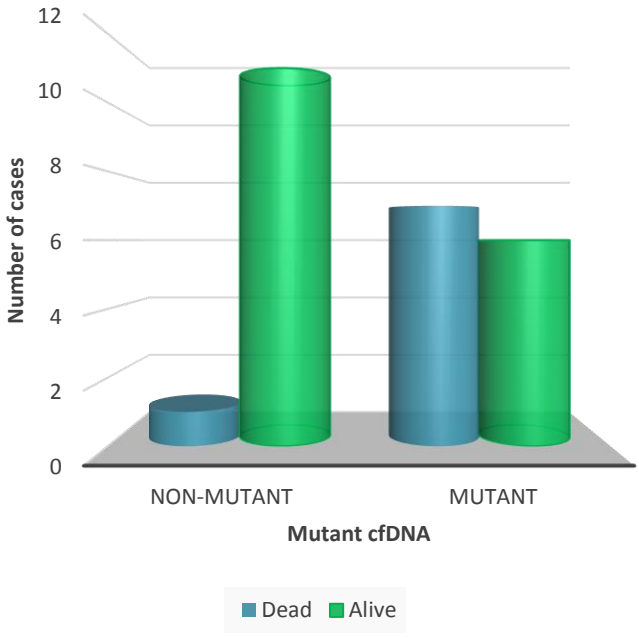


Figure 6-17 Relation between mutations in cfDNA and mortality in colon cancer cases *(p=0.01)*.

6.4 Discussion

Circulating free DNA has been at the core of molecular research especially in the diagnostic and early detection applications. It carries genetic alterations of primary tumours. Thus, it has been investigated as a potential biomarker of tumours such breast, lung, colorectal, liver, head, and neck (Ziegler et al., 2002) (Frattini et al., 2006) (Frattini et al., 2008) (Dawson et al., 2013).

Some research, which developed mutations detection assays, have also investigated the level of cfDNA and correlated it to the clinicopathological features of the CRC patients (Mouliere et al., 2014). However, many other studies quantified the cfDNA separately from the mutations detection and in early stages of the disease (Flamini et al., 2006) (Frattini et al., 2006) (Mead et al., 2011) (Lin et al., 2014). In this study, both mutations detected and cfDNA levels were investigated in both tissue and plasma.

As some studies used serum to trace genes mutations instead of plasma (Pu et al., 2013) (Frattini et al., 2006). Many publications illustrated that wild-type DNA levels are higher in serum because of white blood cells lysis during the clotting process. Therefore, detected mutant cfDNA in a high background of wild-type DNA is more challenging in serum than plasma (Lee et al., 2001) (Lui et al., 2002) (Lam et al., 2004).

Various techniques have utilised different strategies to detected gene mutations in plasma and quantify cfDNA, these methods are mainly qPCR-based techniques and focused on suppressing the background of wild-type and enriched the mutant cfDNA. Nested PCR, Intplex QPCR PNA-PCR, Mass Array-based and digital PCR and LNA-PCR have shown considerable and variable levels of sensitivity and also based on preventing non-specific amplification of undesirable amplicons (Yung et al., 2009) (Pu et al., 2013) (Mouliere et al., 2014) (Miyano et al., 2012) (Lin et al., 2014). Nevertheless, the cost and practicality of developing each for use in the clinical setting is a significant concern and poses various technical challenges (Crowley et al., 2013). This project has an advantage of combining two sensitive mutation detection techniques (AS-LNA and PNA-qPCR clamping) in addition to the Touchdown profile of the qPCR assays to optimise the efficiency.

This combination is considerably cheap, reliable and sensitive in comparison to other detection methods (Robin et al., 2016).

Previously, *KRAS* and *BRAF* mutations were widely investigated in early stages as it was associated with resistance to the monoclonal antibody (mAb) (Bokemeyer et al., 2011) (Sartore-Bianchi et al., 2009),. Therefore, early discovery, especially in matched cfDNA samples, of the gene mutations would offer a better choice of treatment. In this project, oncogenic mutations of *BRAF*, *KRAS*, as well as *PIK3CA*, were discovered in localised primary CRCs. *PIK3CA* is an important oncogene, which identified as a potential factor in mCRC resistance for anti- EGFR (Sartore-Bianchi et al., 2009) and it would be a great value if these mutations discovered at an earlier stage in cfDNA.

Recent studies have focused on studying gene mutations in CRC pre-operatively (Pu et al., 2013) (Mouliere et al., 2014) (Miyano et al., 2012). While others performed the analysis pre and post-operatively (Ryan et al., 2000) (Frattini et al., 2008) (Lindforss et al., 2005) (Lin et al., 2014). Our study also involved some cases that came for follow up after they started the treatment. In addition, this study focuses on the characterisation of the molecule-pathological features in colon and rectum separately as both have different prevalence of genetic alterations and various types of treatment.

In the present study, mutations in the CRC, especially in colonic tumours, was frequently detectable in the plasma and they have the highest yield of cfDNA, mutant cfDNA levels have a significant relation to tumours stages and size than cases without mutations. The level of cfDNA was proportionally correlated with lymph node status in the presence of oncogenic driver mutations. These findings were in disagreement with other studies that failed to confirm the correlation between oncogenic mutations in cfDNA of CRC patients and the tumour stage, (Jan-Sing et al., 2005) (Wang et al., 2006) (Uen et al., 2008) (Frattini et al., 2008) (Lecomte et al., 2010) (Lu et al., 2011).

In section 6.3.1, analysis of the whole cohort showed a significant difference between the cases that had mutations in both tissue and plasma and cases that cases harboured the mutations only in tissue in relation to total cfDNA level. The loss of this significance when subset of analysis was carried out on the colonic

and rectal cases might be referred to the small sample size and the fact that two third of the rectal cases treated with neoadjuvant therapy.

In this study, cfDNA promises a molecular indicator about the LN metastasis. 59% (13/22) colonic cancer cases have no LN metastasis but carried mutations of the most important oncogenes in addition to measurable cfDNA yield, which shows a potential screening test with 75% sensitivity 86% specificity (P= 0.04). Although a larger cohort is required for validation, the test could offer a triage for patients with a positive faecal occult blood test or to monitor post-operative cases as a useful molecular test, which comes in support of use ALU repeat as a biomarker in CRC management (Agostini et al., 2011) (Umetani et al., 2006B).

The data generated in this study partially matched the results from a study by Lin et al., 2014 who found cfDNA levels increase proportionally with stage and LN invasion and were significantly higher in advanced disease (Lin et al., 2014). This indicates that tumour cells lysis deposited in the lymphovascular lumens of the tumour and release tumour cfDNA into the blood circulation and causing high levels of cfDNA (Anker et al., 1999) (Schwarzenbach et al., 2011) (Cancer Genome Atlas, 2012). Hence, cfDNA levels and genetic alterations that are carried by cfDNA might serve, as a biomarker for LN status in addition to the histopathological examination. These results would improve the overall management and survival in CRC.

Two-third (54/84) of the cases have no lymph node (LN) metastasis. CRCs that have no nodal involvement at diagnosis have a good prognosis (Sarli et al., 2005), whereas, CRCs with detectable LN metastasis have a poor prognosis especially with stage Dukes' B (Caplin et al., 1998). The number of nodes affected can also determine prognosis particularly in advanced stages such as Stage II and III (Kotake et al., 2011). However, nearly 20% of node negative cases relapse (Haince et al., 2010).

The assessment of cfDNA concentration does not appear to be useful alone in the diagnostic setting because of the overlapping levels in cfDNA found in healthy people as well as patients with cancer and benign lesions (Schwarzenbach et al., 2011). Therefore, the quantification of cfDNA may be more accurate if combined with other molecular biomarkers such as oncogenic driver mutations. In this study, the presence of genes mutations related to higher levels of cfDNA and also cfDNA

was significantly higher with some clinical parameters. However, the techniques of cfDNA detection and the cut off cfDNA level has not been clearly determined and needs a large cohort to strengthen the diagnostic and prognostic properties of the cfDNA in combination with other biomarkers (Lin et al., 2014).

Regarding the overall patient survival, these data have shown that poor survival was associated with colonic cases harbouring key gene mutations in their cfDNA. Other studies indicated that genes such as *KRAS* and *BRAF* are strongly associated with poor prognosis when they found in the cfDNA of the CRC patients (Yokota et al., 2011).

Post-surgery surveillance is one of the important applications to recruit tumour cfDNA analysis; Reinert et al., studied tumour cfDNA in two groups of relapsing and non-relapsing CRC patients, digital droplet PCR used to quantify the cfDNA and the findings correlated well with the clinical data. The disease status was efficiently evaluated, and the response to the treatment was monitored over time. Relapse anticipated months in advance as compared to routine follow-up (Reinert et al., 2015).

A mutation analysis panel of 74 genes was conducted in cfDNA of patients with stages I-III by Lin et al. They performed a univariate analysis and reported that the 5-year OS among cases who harboured a mutation in cfDNA was significantly poorer than those without a cfDNA mutation (Lin et al., 2014). Other studies also found a poor prognosis with the presence of oncogenic mutations such as *KRAS* (Lecomte et al., 2002).

In summary, this study is one of few studies, which investigated cfDNA in localised primary CRCs including early stages of CRC in both plasma and tissue samples, utilised a combination of robust, sensitive and cheap techniques. The outcome has revealed the diagnostic and prognostic potential of cfDNA, especially in colonic cancers. Key driver genes mutations have boosted the power of cfDNA levels in predicting the early phase of tumour metastasis starting with lymph nodes invasion. cfDNA genetic makeup was significantly related to tumours characteristics and patient survival in colonic more than rectal cases, confirming the genetic diversity that previously shown in colonic and rectal solid tumours.

Chapter 7

General Discussion, Conclusions and Future Direction

7.1 Discussion

Colorectal cancer is one of the leading causes of cancer-related mortality in the Western world. Patient survival is highly reliant on the tumour stage at the time of diagnosis and CRC is mostly asymptomatic until it advances to incurable stages. Treatment of advanced disease is hindered by diminishing sensitivity to chemotherapy (Gonzalez-Pons and Cruz-Correa, 2015) Hence, development of screening programs aimed at early detection is fundamental to improve survival. Contemporary screening and diagnostic approaches vary from semi-invasive procedures such as colonoscopy to non-invasive stool-based tests. Therefore, suboptimal accuracy of these techniques has led to late diagnosis of the disease (De Roock et al., 2010a).

Over the last two decades, research has focussed on establishing biomarkers with high sensitivity and specificity to detect early stage cancers non-invasively. Alterations in gene expression leading to colorectal carcinogenesis are represented in dysregulated levels of nucleic acids (Gonzalez-Pons and Cruz-Correa, 2015). Therefore, cfDNA "liquid biopsy" in plasma of cancer patients and the mutations detected in them have gained tremendous significance in this field, which can be used as non- invasive molecular biomarkers. With the rapid advances and the decrease in the cost of high throughput methods, cfDNA levels and mutations may lead to a new era of cancer diagnostics and therapy (Singh N, 2015).

However, due to lack of consistency in laboratory approaches, variability in cancer progression, low number of samples in various studies, high costs of the method used or the lack of sensitivity for detecting circulating nucleic acids, has led to reluctance in the clinical routine practice to approve this strategy (Qin et al., 2016) (Singh N, 2015).

In this context, this study was carried out to clarify and investigate using cheap and robust technique in detecting cfDNA. Moreover, this study aimed at identify common oncogenic drives in localised primary CRCs and confirm that cfDNA is a potential clinical biomarker for early detection and screening of CRC. Also, as cfDNA harbours important genetic makeup of the primary tumour, so it would be useful tool in treatment monitoring, and early prediction of the metastatic capability of CRC.

The advancement in the nucleic acid purification techniques has allowed a purification of small fragments (approximately 20bp and more) of cfDNA. These DNA fragments have been recognised as a significant component of cfDNA in cancer patients (Thierry et al., 2010). Nevertheless, increased levels of cfDNA recorded in patients with inflammatory conditions as a result of raised apoptosis in non-neoplastic conditions (Margraf et al., 2008). Thus, elevated cfDNA levels cannot be regarded as cancer specific (Wang et al., 2003), so, it is beneficial to combine its assessment with other biomarkers.

As the ALU is the most abundant repeated sequence (Gu et al., 2000), they were found significantly higher in patients with CRC than healthy individuals and more interestingly, ALU-qPCR has sufficient sensitivity for direct assessment of serum in CRC cases due to the abundance of the ALU repeats (Umetani et al., 2006B) (da Silva Filho et al., 2013). The technique has been successfully applied in previous cfDNA studies in breast and prostate cancer (Umetani et al., 2006A) (Fawzy et al., 2016). In this study, an ALU-qPCR method was established and optimised for cfDNA quantification, using a short amplicon (69bp, ALU69). The ALU69 assay detects multiple copies and was found more sensitive for detecting low concentration DNA compared a single copy GAPDH reference assay.

High levels of cfDNA deposited during inflammation and injury may interfere with the mutation detection and dilute cfDNA (Kin et al., 2013). Therefore, three different techniques were integrated to qPCR-based on suppressing the wild-type allele amplification and increasing of the mutation detection sensitivity as well as optimising the assay efficiency. The assays are an innovative form of qPCR-based on allele specific discrimination. It is worth stating that this method has not been combined or evaluated by any other studies because of its novelty. Hence, it was challenging for producing and enhancing the assay strategy. Moreover, Touchdown-qPCR method has been applied for the first time on real-time PCR to allow annealing of PCR oligonucleotides at the most appropriate temperature to improve the sensitivity and specificity of the assays.

Kwon et al., reported that PNA- clamping qPCR increased the detection levels of *BRAF*, *KRAS* and *PIK3CA* mutations in FFPE tissues in advanced CRC compared to direct sequencing (Kwon et al., 2011). Combined PNA-mediated PCR clamping with a melting curve analysis detected *KRAS* mutations in a ratio of

1:1000 wild-type molecules (Oh et al., 2010). The AS-LNA qPCR is an allele-specific assay using forward or reverse mutation-specific primers modified together with LNA nucleotides for the 3′-end sequence that recognises and quantifies oncogenic mutations having higher specificity and sensitivity (Morandi et al., 2012). The previously recorded sensitivity of PNA clamping qPCR was 0.1% (Wilson and Lackner, 2014). This was increased to 0.001% by combining it with AS-LNA method it becomes equivalent to and more practical than digital droplet PCR. Combining PNA and AS-LNA in one assay has produced a robust, sensitive practical and cheap method to detect minority DNA species such as cfDNA, which could pave the way towards transferring the technique into the clinical setting.

The results that can concluded from this thesis as following:

Total cfDNA levels analysis:

- New sensitive assay has been established to quantify highly fragmented cfDNA.
- There was a heterogeneity in the total cfDNA levels due to many factors mainly due to long storage of the plasma samples and variety in tumour stages.

Whole cohort analysis

- There was a significant relation between total cfDNA levels and tumour stage for the whole cohort (*p=0.03*).
- cfDNA total levels are more significant in cases where there were mutations in tissue and plasma than cases which had mutations only in tissue (*p=0.02*).
- The number of gene mutations including *KRAS, BRAF* and *PIK3CA* had no relation with the cohort clinicopathological features.
- No relation between the cfDNA levels with or without gene mutations and patient survival for the whole cohort.

Subset analysis according to the tumour location

- There was no significant difference between the colonic and rectal cases in term of cfDNA levels whether there were plasma mutation positive or negative.
- However, the number of plasma mutation positive colonic cases were significantly higher than plasma mutation positive rectal cases (*p=0.01*).
- Gene mutations frequency were variable as following:
    - *BRAF* and *PIK3CA* mutations were only detected in the cfDNA of colonic cases.
    - *KRAS* mutations were detected in both colonic and rectal cfDNA.
    - There was a heterogeneity of gene mutation between pre and post therapeutic cfDNA samples confirming the usefulness of cfDNA in monitoring tumour heterogeneity.

Colonic tumours analysis:

- Total cfDNA levels were significantly higher in plasma mutation positive colonic cases than cases were negative (*p=0.01*).
- cfDNA levels in plasma mutation positive colonic cases had a significant relation to tumour stage (*p=0.03*) (Dukes'), T stage (*p=0.04*) and lymph node metastasis (*p=0.005*).
    - There was as significant relation between the cfDNA levels of plasma mutation positive colonic cases and patient survival (*p<0.05*).
    - There was a significant relation between number of cases with gene mutation in cfDNA and mortality in colon cancer (*p=0.01*).

Although, this study was conducted on localised primary CRCs, it resulted in a considerable levels of cfDNA that can be detected with sufficiently sensitive assays. Moreover, small tumours were able to yield significant cfDNA levels especially in colonic cancer cases (Diehl et al., 2005). Interestingly, at early stages there is no lymph node or distal metastasis and patients have a good 5-year survival rate at 75-80%. Whereas, most previous cfDNA studies focused on

advanced-stage cancers with relatively high concentrations of cfDNA (Frattini et al., 2005) (Diehl et al., 2008) (Karapetis et al., 2008) (Spindler et al., 2012) (Mostert et al., 2013) (Tie et al., 2015).

Evaluation of cfDNA quantity did not seem to be useful alone in the diagnostic setting because of the overlapping levels in cfDNA found in healthy controls as well as patients with cancer and benign lesions (Schwarzenbach et al., 2011), which may explain the poor correlation with cfDNA total levels to the clinicopathological features in this CRC cohort. As a part of the study and to increase the sensitivity and specificity of cfDNA from CRC patient, genetic alterations of common oncogenic drivers were investigated in order to assess reliability of cfDNA as surrogate for the primary tumour.

In several studies, *KRAS, BRAF* and *PIK3CA* mutations were widely investigated as these are associated with resistance to the monoclonal antibody (mAb) (Bokemeyer et al., 2011) (Sartore-Bianchi et al., 2009), those mutations showed adverse effects on the response to treatment and predicted poor outcomes . In this study, oncogenic mutations of *BRAF*, *KRAS* and *PIK3CA*, were identified in cfDNA of approximately 40% of early stage CRCs. Therefore, early discovery of gene mutations using a dynamic monitoring tool such as cfDNA could offer a better choice of treatment and to avoid the resistance to anti-EGFR therapy especially in mCRC.

The diversity between solid colonic and rectal tumours confirmed that they are no longer single organ but two separate entities (Cucino et al., 2002) (Rabeneck et al., 2003), they differ epidemiologically, genetically, different therapeutic approaches and clinical outcome (Li et al., 2012). This project also revealed distinctive features of cfDNA in both colonic and rectal cancers. The total cfDNA levels correlated to the clinicopathological features of mutant colonic cases including tumour stage and size and LN status and also associated with poor patient survival, however, no such relation was observed with the rectal cases.

Assessment of tumour stage is one of the most dominant predictors of prognosis, and the relationship between levels of tumour specific genetic alterations and stage was evaluated. Mutations in the CRC cohort, particularly in colonic tumours, were frequently detectable in the plasma. These cases had the highest levels of cfDNA, and more significant higher levels of cfDNA with tumours stages and size

than cases without mutations, but, that was not the case in rectal cancer cases. Some studies have revealed a statistically significant correlation between tumour stage and the presence of tumour-associated genetic alterations (including in *TP53, KRAS,* and *APC* mutations) in cfDNA of CRC cases (Diehl et al., 2008) (Wang et al., 2004). However, our findings were in disagreement with other studies that failed to confirm this correlation (Frattini et al., 2008) (Lecomte et al., 2010) (Lu et al., 2011). Conflicting results associated with such studies is usually due to limited sample sizes as well as the technical differences and methods were used (Crowley et al., 2013).

The data in this study illustrate a significant relationship between the T stage and the cfDNA level indicating a potential use of cfDNA in early cancer detection. In colon cancer, the level of cfDNA increased with increasing T stage (p= 0.04). This comes in line with findings in similar study by Tie et al., who reported that cfDNA levels correlated to T stage s as assessed by CT-scan with sensitivity of 92% in the baseline samples. cfDNA had at least one gene mutation screened by sequencing panel of 15 genes in their study, which included early stage CRC cases and also illustrated that early alterations in cfDNA could predict later radiological response in CRC cases having chemotherapy (Tie et al., 2015).

The resolution limit of diagnostic imaging studies to detect tumour is a tumour-size of approximately 7–10 mm, which contains about 1 billion cells (Francis and Stein, 2015). In contrast, tumour carrying about 50 million tumour cells discharges sufficient DNA that can be identified in the blood (Diaz Jr et al., 2012), indicating that cfDNA may also be better than radiological imaging in detecting disease recurrence (Yamada et al., 1998). This advantage of cfDNA could promise early management of small non-detectable tumours by conventional imaging (Diaz Jr et al., 2012) (Bettegowda *et al.*, 2014). Despite, previous studies investigated the stage rather than the size in relation to the cfDNA integrity or levels in CRC.

Overall 5-year survival (OS) for CRC is excellent for tumours confined to the colon (95% for stage I and 82% for stage II), but the OS declines to 61% for patients with regional spread to the lymph nodes (stage III). Indeed, about 25% of patients with histopathology-negative lymph nodes die from metastatic cancer. CRC with stage I and II may just undergo surgical resection with no adjuvant

therapy, usually without LN metastasis (Hyslop and Waldman, 2013) (Benson et al., 2014) (Aldecoa et al., 2016).

In this study, the level of cfDNA was proportionally correlated with lymph node status in the presence of oncogenic driver mutations. Therefore, cfDNA promises a molecular indicator about the LN metastasis. 59% (13/22) colonic cancer cases have no LN metastasis but carried mutations of the most common oncogenes in addition to measurable cfDNA yield, which shows a potential screening test with 75% sensitivity 86% specificity (p= 0.04). These findings suggested that tumour cells accumulated in the lympho-vascular lumens of the tumour may discharged into the systemic circulation (Schwarzenbach et al., 2011).The lysis of circulating cancer cells or micrometastases released by the tumour, as well as tumour necrosis or apoptosis, might cause in higher cfDNA level, as described in previous studies (Cancer Genome Atlas, 2012) (Spindler et al., 2012). Although a larger cohort is required for validation, the test could offer a triage for patients with a positive faecal occult blood test or to monitor post-operative cases as a useful molecular test, which comes in support of use ALU repeat as a biomarker in CRC management (Agostini et al., 2011) (Umetani et al., 2006B). Therefore, early detection of LN involvement could narrow down the therapeutic choices and give a better prognosis.

BRAF, KRAS and *PIK3CA* mutations were more in colonic cases than rectal ones. The cfDNA the concordance rates were 75%, 35% and 20% respectively. In comparison, there were higher concordance rates in previous study with more metastatic stages up to 78%-98% for *KRAS* as it was the most investigated oncogene in matched cfDNA-tDNA studies (Kuo et al., 2014) (Thierry et al., 2014) (Lecomte et al., 2002). *KRAS* mutations are considered to be an early event in CRC tumorigenesis and are present in between 20 and 50% of all CRCs. In this study the $BRAF^{V600E}$ mutation was detected exclusively in the cfDNA of the right colonic cases matching many studies described that $BRAF^{V600E}$ mutation dominantly present in proximal colonic cancers and associated with MSI (Thierry et al., 2014) (Kuo et al., 2014). *KRAS* and *BRAF* mutations are associated resistance to EGFR-targeted therapy and poor prognosis (Lièvre et al., 2008) (Gonzalez-Pons and Cruz-Correa, 2015). *PIK3CA* mutations were found exclusively in the cfDNA of three colonic cases and also identified in three post-

treatment cfDNA samples with concordance at 20%. In comparison to a study by Guedes et al., in 2013, who reported higher frequency of *BRAF* and *PIK3CA* in colonic than rectal metastatic cases (Guedes et al., 2013).

PIK3CA was investigated more in the cfDNA of breast cancer than any cancer (Board et al., 2010) (Higgins et al., 2012). In CRC, *PIK3CA* was only investigated in single CTCs (circulating tumour cells) (Kin et al., 2013). *PIK3CA* mutations were widely described in relation to the primary tumours and metastatic tissues (Guedes et al., 2013) (De Roock et al., 2010a). Nevertheless, to the best of our knowledge, no study has investigated *PIK3CA* mutations in matched cfDNA-tDNA in early stages CRC before this study. In this project, *PIK3CA* mutations were detected in early stages of both colonic and rectal cancer cases, but they were exclusively identified in cfDNA of colonic cases. Moreover, *PIK3CA* mutations were found in cases after treatment and weren't identified in pre-treatment cfDNA samples indicating the heterogeneity that was confirmed previously in tumour mutation analysis (Baldus et al., 2010). *PIK3CA* has been linked to EGFR-targeted therapy resistance and also attributed to poor outcome (Sartore-Bianchi et al., 2009).

Although, the total cfDNA levels in this cohort were not correlated to the patient's survival, the data showed that poor survival is associated with colonic cases harbouring key gene mutations in their cfDNA, this is in agreement with the findings by Frattini who reported a higher incidence of oncogenic alteration in the colon than in the rectum (Frattini et al., 2004). cfDNA level were independent from the OS in a study by Lecomte et al. (Lecomte et al., 2002). However, Schwarzenbach et al. reported a significant correlation between the OS and the level of the cfDNA in advanced stages of the CRC cohort (Schwarzenbach et al., 2008). Bettegowda et al. stated that patient survival decreases when concentration of the cfDNA increase and *KRAS* mutations in cfDNA was correlate to shorter survival (Bettegowda et al., 2014). Most of studies analysed the patient survival in relation to the mutant cfDNA such as mutations of *BRAF* and *KRAS* rather total cfDNA levels (Kin et al., 2013). Other studies indicated that genes such as *KRAS* and *BRAF* are strongly associated with poor prognosis when they found in the cfDNA of the CRC patients (Yokota et al., 2011). High cfDNA levels were

correlated to poor outcome in metastatic CRC in a study by Spindler et al. (Spindler et al., 2012).

In summary, this work proved the feasibility of detecting cfDNA in cases with localized colorectal cancer and this detection is associated with patient prognosis, the data reported are preliminary study on a small sample size and this will be promoting results empower further prospective studies in a larger cohort including appropriate healthy, non-cancerous controls and working on more fresh samples.

7.2 Conclusion and future direction

In conclusion, results of this thesis have confirmed that mutations were successfully detected in cfDNA of some patients with localised primary tumours including early stage CRC and this related to clinical parameters such as tumour stage, T stage and lymph node status and poor prognosis, particularly in colonic cancer cases. This study had the advantage of using a novel combination of three techniques (AS-LNA, PNA clamping and Touch-down qPCR profile), some of which were examined individually in previous studies and shown to have significant sensitivity (Oh et al., 2010) (Morandi et al., 2012). To the best of our knowledge, no study has used the touchdown method with qPCR, also this is the first study to join the three described techniques to suppress wild-type alleles, and optimise the sensitivity of detection of mutant alleles. The uniqueness of this study is in investigating the utility of cfDNA as biomarker in early stages of CRC. I was able to examine the feasibility of genetic material in cfDNA to be analysed as a surrogate for the primary tumours, clarifying the importance of previously studied mutations but in early stage disease. In particular a novel finding was identifying *PIK3CA* mutations in cfDNA in early stages, this could broaden horizons to more confirmatory research.

Total cfDNA levels did not show significance in relation to clinical parameters in the whole cohort. However, we found a significant correlation between the major key gene mutations in the cfDNA and total cfDNA levels and a significant relation to clinicopathological features of the colonic cases. Preliminary results showed that total cfDNA levels were associated with presence of mutations and can be used to screen for early identification of potential lymph nodes metastasis. Nevertheless, this should be validated in larger cohort.

Fundamentally, cfDNA may anticipate any drug-resistant cancers. Several studies have indicated that cfDNA analysis could be successfully combined with diagnostic imaging, moreover, in many situations, liquid biopsies seemed to be able to monitor response to anticancer treatments and probably anticipate tumour advancements as compared to CT-scan evaluation. A recent study has revealed that early tumour shrinkage (ETS) in the treatment of mCRC associated with better survival (Tie et al., 2015), this indicates that radiological studies and cfDNA

evaluations could be joined to assess therapeutic strategies as a clinical model in oncology, which, has been shown in this study when cfDNA levels and oncogenic mutations in cfDNA were related to the T stage and stage of the tumours,. This potentially means an early improvement in treatment, avoiding undesirable side effects, improving efficacy and reducing costs (Surani and Poterlowicz, 2016).

It is doubtful that cfDNA would replacement conventional diagnostic applications shortly, as imaging will always be required to visualise the anatomic locality of metastatic cancers, the resectability of tumours or the invasion of surrounding organs. Eventually, the study of cfDNA may provide a more extensive evaluation of the molecular heterogeneity of CRC, which may also guide a more personalised treatment with targeted cancer therapies. A unique utility of cfDNA analysis is that it enables following tumour molecular evolution in time (Siravegna and Bardelli, 2016).

cfDNA can be investigated regularly and non-invasively at different time points throughout the treatment course. For instance, real-time monitoring for *PIK3CA*, *BRAF* and *KRAS* mutations in cfDNA could be employed to create dynamic therapeutic protocols of EGFR-targeted antibodies. Well-designed prospective studies, adequately powered validation studies are now required to establish a clinical validity and applicability of cfDNA analysis and how cfDNA will reflect the heterogeneity and evolution of the tumour.

Insufficient plasma samples and unavailability of plasma for some cases was a major obstacle in this work, which could be overcome in a future study. Improvements in this study could be made by designing a wider multigene panel to include tumour suppressor genes such as *p*53 and *APC* to increase the specificity of the mutational analysis of cfDNA. Genotyping cfDNA in plasma samples can be applied to personalise the molecular profile of CRC and to closely observe its progression during treatment. This strategy can also be utilised to discover minimal residual disease after surgery and to create effective therapeutic objects, and exhibit mechanisms of therapeutic resistance.

Appendix

Appendix I

Publication related to this thesis

I. Conference abstract NCRI Liverpool November 2014

Circulating free DNA: a tumour biomarker for early detection and follow-up in colorectal cancer

Abdlrzag Ehdode, Imran Aslam, Baljit Singh, David Guttery, Jacqui Shaw, Howard Pringle

ABSTRACT

Background: Circulating free DNA (cfDNA) in plasma constitutes a "liquid biopsy" for monitoring colorectal cancers (CRC) as a surrogate for the primary tumour. The aim of the study was to evaluate the potential of cfDNA as a liquid biomarker in CRC.

Methods: 62 CRC (prior to surgery) and 22 rectal carcinomas (after neo-adjuvant therapy) were investigated comparing formalin-fixed, paraffin embedded (FFPE) tumour tissue and cfDNA. DNA was quantified using an ALU repeat qPCR assay and a PNA-LNA approach was used to detect 3 hot spot mutations in *KRAS* and *BRAF (V600E)*, optimised using cell lines with known mutation status.

Results: Total cfDNA levels correlated with tumour stage (*P=0.003*) and T stage (*P<0.04*). Cell lines studies demonstrated a limit of mutant DNA detection of 6pg, or one cell equivalent for each mutation. In 62 CRC cases, 7 tumours had the *BRAF* V600E mutation, and 6 of these also had the mutation in matched cfDNA. Ten tumours were positive for *KRAS* mutations, six of which were detected in matched cfDNA. Mutations in cfDNA were found in all 4 cases with nodal involvement and 8/13 cases without spread, suggesting early detection of cancer prior to metastasis. The presence of mutations in cfDNA was also related to tumour stage (*P=0.05*). 22 rectal carcinomas received neo-adjuvant treatment prior to surgery. Eight of these had either a *BRAF* or *KRAS* mutation in FFPE tissue, but none persisted post-surgery and neo-adjuvant treatment.

Conclusion: This pilot study has shown that cfDNA can potentially be used as a surrogate for the primary tumour in CRC, and has potential for early detection of cancer prior to metastasis. In rectal cancer cfDNA may also reflect response to neo-adjuvant therapy. However, further study in a larger cohort is required.

II. Accepted oral presentation in ASCRS scientific session

TITLE: ***Liquid Biopsy for Colonic Cancer: Utility of Circulating cell free DNA as Biomarker***

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ABSTRACT:

Background: Circulating cell-free DNA (cfDNA) is a mixture of DNA from malignant and normal cells, and can be used as a liquid biopsy to detect tumour specific mutations. The aim of this study was to evaluate the potential utility of cfDNA as a liquid biomarker for colonic cancer. We investigated the clinical utility of common genetic mutations when detected synchronously in colonic cancer tissue and cfDNA isolated from blood samples.

Methods: Matched plasma and formalin fixed paraffin embedded (FFPE) tissue samples, collected preoperatively from 55 patients with colonic cancer were analysed for total cfDNA levels and common genetic mutations. Total cfDNA was quantified from 1ml of plasma using an ALU repeat qPCR assay and a PNA-LNA approach was used to detect hot spot mutations in KRAS, BRAF V600E, and PIK3CA. Total cfDNA quantity and mutation status were compared to clinic-pathological features of colonic cancers and overall survival.

Results: Total cfDNA levels correlated with tumour stage ($p=0.003$) and T stage (pT) ($p=0.04$). In 23 (42%) FFPE cancer tissue samples, 30 mutations (KRAS n=10, BRAF V600E n=7, PIK3CA n=13) were detected. BRAF V600E mutation was detected only in right colonic cancers. For mutant colonic cancers, 6 (86%) with BRAF, 6 (60%) with KRAS and 3 (23%) with PIK3CA mutations were detected in cfDNA isolated from matching plasma samples. The presence of mutations in cfDNA was also related to tumour T stage ($p=0.05$). Circulating cfDNA mutations were found in all 4/13 cases with lymph node involvement and in 9/13 cases without lymph node involvement ($p<0.005$). No significant survival differences were observed for mutant left sided colonic cancers for the presence of cfDNA mutations. For right sided colonic cancers, cfDNA mutation were associated with significantly poor overall survivals ($p<0.005$).

Conclusions: This study has shown that cfDNA is a potential surrogate biomarker for colonic cancers. However, a further study on a larger cohort is required.

Appendix II

Clinicopathological characteristics of cohorts' participants:

	FFPE/ID	Age	Gender	Patient survival	Tumour Location	Duke's staging	T	N	no of LN	Duke's staging	Differentiation	THERAPHY
1	**H497/14**	66	F	Alive	Right Colon	A	T2	N0	17/0+	A	MODERATE	Rt HCt
2	**H436/14**	74	F	Alive	Right Colon	A	T2	N0	18/0+	A	MODERATE	Lap Rt HCt
3	**H453/14**	75	F	Dead	Right Colon	A	T1	N0	11/0+	A	MODERATE	Rt HCt
4	**H475/14**	79	F	Alive	Right Colon	A	T2	N0	12/0+	A	MODERATE	Rt HCt
5	**H478/14**	71	M	Dead	Right Colon	B	T3	N0	20/0+	B	MODERATE	Rt HCt with hepatectomy
6	**H456/14**	82	M	Alive	Right Colon	B	T3	N0	19/0+	B	MODERATE	Rt HCt
7	**H912 /13**	77	M	Dead	Right Colon	B	T3	N0	21/0+	B	MODERATE	Rt HCt
8	**H464/14**	75	F	Alive	Right Colon	B	t3	n0	15/0+	B	MODERATE	Rt HCt
9	**H913/13**	77	F	Dead	Right Colon	B	T3	N0	8/4+	B	MODERATE	Rt HCt
10	**H460/14**	86	M	Dead	Right Colon	B	T3	N0	13/0+	B	MODERATE	Rt HCt

11	**H462/14**	80	M	Alive	Right Colon	B	T3	N0	23/0+	B	MODERATE	Rt HCt
12	**H465/14**	76	M	Alive	Right Colon	B	T3	N0	13/0+	B	MODERATE	Rt HCt
13	**H915 /13**	72	M	Alive	Right Colon	B	T3	N0	14/0+	B	MODERATE	Rt HCt
14	**H483/14**	74	F	Alive	Right Colon	B	T1	N0	14/0+	B	MODERATE	Rt HCt
15	**H918 /13**	78	F	Alive	Right Colon	B	T3	N0	15/0+	B	MODERATE	Rt HCt
16	**H447/14**	70	M	Dead	Right Colon	B	T3	N0	17/0+	B	MODERATE	Rt HCt
17	**H917 /13**	59	M	Alive	Right Colon	B	T3	N0	37/0+	B	MODERATE	Rt HCt
18	**H911/13**	49	F	Alive	Right Colon	B	T3	N0	34/0+	B	MODERATE	Extended Rt HCt
19	**H493/14**	92	F	Dead	Right Colon	B	T3	N0	14/0+	B	POOR	Rt HCt
20	**H485/14**	48	M	Alive	Right Colon	B	T3	N0	21/0+	B	POOR	Extended Rt HCt/ChTh
21	**H451/14**	26	M	Alive	Right Colon	C	T2	N1	60/1+	C	MODERATE	Rt HCt
22	**H468/14**	69	F	Alive	Right Colon	C	T3	N2	17/3+	C	MODERATE	Rt HCt
23	**H461/14**	64	M	Alive	Right Colon	C	T3	N1	20/1+	C	MODERATE	Rt HCt/post-op ChTh
24	**H473/14**	69	M	Dead	Right Colon	C	T3	N1	12/1+	C	MODERATE	Rt HCt
25	**H928 /13**	82	F	Dead	Right Colon	**C**	T4	N0	11/0+	**C**	MODERATE	Rt HCt
26	**H914 /13**	73	M	Alive	Right Colon	C	T3	N1	16/2+	C	MODERATE	Rt HCt

27	**H435/14**	73	M	Alive	Right Colon	C	T4	N2	31/6+	C	MODERATE	Rt HCt
28	**H910/13**	60	M	Alive	Right Colon	C	T4	N2	18/13+	C	MODERATE	Lap sigmoid HCt
29	**H933 /13**	90	F	Dead	Right Colon	C	T4	N2	7/6+	C	POOR	Extended Rt HCt
30	**H519/13**	85	M	Dead	Right Colon	C	T4	N1	5/0+	C	POOR	Rt HCt
31	**H440/14**	73	F	Alive	Right Colon	C	T3	N1	19/2+	C	POOR	Rt HCt
32	**H495/14**	86	M	Alive	Left Colon	A	T2	N0	19/0+	A	MODERATE	Hartmana procedure
33	**H442/14**	57	M	Alive	Left Colon	A	T2	N0	20/0+	A	MODERATE	Lt HCt
34	**H441/14**	75	M	Alive	Left Colon	A	T2	N0	10/0+	A	MODERATE	Lt HCt
35	**H474/14**	79	M	Alive	Left Colon	A	T2	N0	12/0+	A	MODERATE	Low Hartmans
36	**H439/14**	68	M	Alive	Left Colon	A	T2	N0	_	A	WELL Differentiation	Lt HCt
37	**H499/14**	62	F	Alive	Left Colon	B	T3	N0	9/0+	B	MODERATE	AS
38	**H486/14**	66	M	Alive	Left Colon	B	T3	N0	11/0+	B	MODERATE	Lt HCt
39	**H514/13**	75	F	Alive	Left Colon	B	T3	N0	15/0+	B	MODERATE	AS
40	**H919 /13**	64	M	Alive	Left Colon	B	T3	N0	24/0+	B	MODERATE	Extended Rt HCt
41	**H446/14**	73	M	Alive	Left Colon	B	T4	N0	17/0+	B	MODERATE	Lt HCt
42	**H916 /13**	80	M	Alive	Left Colon	B	T3	N0	10/0+	B	MODERATE	Lt HCt
43	**H927 /13**	67	M	Alive	Left Colon	B	T3	N0	13/0+	B	MODERATE	Lt HCt
44	**H443/14**	81	M	Alive	Left Colon	B	T3	N0	22/0+	B	MODERATE	Lt HCt
45	**H452/14**	69	M	Alive	Left Colon	B	T3	N0	15/0+	B	MODERATE	Sigmoid colectomy/CheTh
46	**H459/14**	69	M	Alive	Left Colon	C	T3	N2	19/5+	C	MODERATE	Sigmoid colectomy/CheTh

47	**H455/14**	69	M	Alive	Left Colon	C	T3	N2	16/4+	C	MODERATE	Lt HCt
48	**H469/14**	60	M	Alive	Left Colon	C	T3	N2	12/9+	C	MODERATE	AS/CheTh/RaTh
49	**H481/14**	64	F	Dead	Left Colon	C	T4	N2	17/9+	C	MODERATE	APR /CheTh
50	**H467/14**	67	M	Alive	Left Colon	C	T3	N1	14/3+	C	MODERATE	AS/CheTh
51	**H433/14**	69	M	Dead	Left Colon	C	T3	N1	7/2+	C	MODERATE	AS
52	**H929 /13**	71	M	Dead	Left Colon	C	T3	N2	24/8+	C	MODERATE	Rt HCt with hepatectomy
53	**H480/14**	65	M	Dead	Left Colon	C	T3	N2	12/7+	C	MODERATE	AS/CheTh
54	**H518/13**	85	F	Dead	Left Colon	C	T3	N2	17/8+	C	POOR	AS
55	**H438/14**	70	F	Alive	Left Colon	**C**	T3	N2	13/4+ AND 19/6+	**C**	WELL Differentiation	Lap subtotal colectomy/CheTh
56	**H472/14**	48	F	Alive	Rectum	A	T2	N0	10/0+	A	MODERATE	Lt HCt/RaTh
57	**H515/13**	87	F	Dead	Rectum	A	T2	N1	_	A	MODERATE	TEMS
58	**H476/14**	83	F	Alive	Rectum	A	T2	N0	12/0+	A	MODERATE	Hartmans and AS
59	**H454/14**	73	F	Alive	Rectum	A	T2	N0	22/0+	A	MODERATE	Lap AS
60	**H488/14**	71	M	Dead	Rectum	A	T2	N0	14/0+	A	MODERATE	AS/CheTh
61	**H931 /13**	59	F	Alive	Rectum	A	T2	N0	7/0+	A	MODERATE	AS/RaTh
62	**H450/14**	60	M	Dead	Rectum	A	T2	N1	5/0+	A	MODERATE	AS/CheTh/RaTh
63	**H449/14**	72	M	Alive	Rectum	A	T2	N0	14/0+	A	MODERATE	AS/CheTh/RaTh
64	**H479/14**	82	M	Alive	Rectum	A	T2	N0	11/0+	A	MODERATE	AS/RaTh
65	**H932 /13**	51	M	Alive	Rectum	B	T0	N0	8/0+	B	_	AS/CheTh/RaTh
66	**H494/14**	66	M	Alive	Rectum	B	T3	N0	14/0+	B	MODERATE	AS
67	**H490/14**	69	F	Dead	Rectum	B	T3	N0	4/0+	B	MODERATE	AS/RaTh
68	**H471/14**	75	M	Alive	Rectum	B	T3	N0	12/0+	B	MODERATE	AS/RaTh

69	**H484/14**	67	M	Dead	Rectum	B	T3	N0	10/0+	B	MODERATE	CheTh/RaTh
70	**H487/14**	73	M	Alive	Rectum	B	T3	N0	14/0+	B	MODERATE	AS+ loop ileostomy
71	**H463/14**	63	M	Dead	Rectum	B	T3	N0	8/0+	B	MODERATE	APR/CheTh/RaTh
72	**H448/14**	74	M	Alive	Rectum	B	T3	N0	20/0+	B	MODERATE	AS
73	**H434/14**	62	F	Dead	Rectum	B	T3	N0	13/0+	B	MODERATE	AS
74	**H492/14**	81	F	Dead	Rectum	**B**	T3	NX	_	**B**	MODERATE	TEMS
75	**H458/14**	86	M	Dead	Rectum	B	T3	N0	17/0+	B	MODERATE	Hartmana procedure
76	**H482/14**	69	M	Alive	Rectum	B	T3	N0	26/0+	B	MODERATE	AS
77	**H489/14**	63	M	Alive	Rectum	**C**	T3	N1	6/1+	**C**	MODERATE	AS/ChTh
78	**H470/14**	73	M	Alive	Rectum	C	T2	N1	10/1+	C	MODERATE	APR/CheTh/RaTh
79	**H1030 /13**	81	M	Dead	Rectum	C	T4	N1	12/1+	C	MODERATE	AS/ loop ileostomy
80	**H445/14**	82	M	Alive	Rectum	C	T3	N1	13/1+	C	MODERATE	Lap APR/RaTh
81	**H930 /13**	50	F	Alive	Rectum	C	T3	N1	7/1+	C	MODERATE	AS/RaTh
82	**H431/14**	41	F	Dead	Rectum	C	T1	N1	13/0+	C	MODERATE	AS,resection of ileostomy
83	**H457/14**	71	M	Dead	Rectum	**C**	T3	N1	2/2+	**C**	MODERATE	AS
84	**H432/14**	82	F	Alive	Rectum	NO STAGE	_	_	_	_	_	Excision EMR technique/RaTh

Appendix III

The results of tDNA and cfDNA quantifications:

	FFPE/ID	Cт	Quantity	DNA conc *ng*/µl	Plasma samples ID	Cт	Quantity Mean	DNA Conc ng/ µl	Yield
1	**H497/14**	19.2	0.72	**7.15**	**H1106/10**	33.97	0.00	0.00	0.02
2	**H436/14**	19.0	0.86	**8.55**	**H43/09**	26.16	0.01	0.06	6.49
3	**H453/14**	19.6	0.55	**5.52**	**H298/09**	26.05	0.01	0.07	6.97
4	**H475/14**	21.3	0.04	**0.41**	**H669/10**	NO SAMPLE			
5	**H478/14**	18.5	0.29	**2.86**	**H680A/10**	27.62	0.00	0.02	2.37
6	**H456/14**	20.1	0.19	**1.92**	**H300B/09**	26.57	0.00	0.05	4.94
7	**H912 /13**	19.7	0.63	**6.32**	**H180B/09**	25.89	0.01	0.08	7.82
8	**H464/14**	20.6	0.14	**1.38**	**H274/10**	25.62	0.01	0.09	9.38
9	**H913/13**	20.1	0.51	**5.06**	**H180C/09**	25.20	0.01	0.13	12.60
10	**H460/14**	17.3	1.28	**12.83**	**H265/10**	24.89	0.02	0.16	15.58
11	**H462/14**	17.9	0.84	**8.39**	**H269/10**	24.85	0.02	0.16	16.04
12	**H465/14**	20.5	0.14	**1.41**	**H275/10**	24.53	0.02	0.20	19.99
13	**H915 /13**	18.1	1.48	**14.80**	**H278/09**	24.49	0.02	0.20	20.47
14	**H483/14**	18.2	1.41	**14.11**	**H677/10**	23.98	0.03	0.29	29.16
15	**H918 /13**	17.6	1.96	**19.62**	**H281/09**	23.34	0.05	0.45	45.29

16	**H447/14**	21.5	0.15	**1.48**	**H164/09**	22.76	0.07	0.68	67.91
17	**H917 /13**	19.7	0.62	**6.21**	**H280/09**	22.75	0.07	0.69	68.63
18	**H911/13**	16.2	4.29	**42.89**	**H176/09**	NO SAMPLE			
19	**H493/14**	16.6	4.09	**40.85**	**H957/10**	27.31	0.02	0.20	20.00
20	**H485/14**	18.1	1.50	**14.99**	**H676/10**	NO SAMPLE			
21	**H451/14**	23.0	0.05	**0.53**	**H289/09**	26.52	0.01	0.05	5.03
22	**H468/14**				**H278/10**	25.22	0.01	0.12	12.35
23	**H461/14**	17.3	0.14	**1.4**	**H268/10**	24.96	0.01	0.15	14.85
24	**H473/14**	18.3	0.64	**6.41**	**H664/10**	24.29	0.02	0.24	23.54
25	**H928 /13**	20.4	0.36	**3.55**	**H286/09**	23.79	0.03	0.28	28.25
26	**H914 /13**	18.5	1.19	**11.86**	**H277/09**	23.91	0.03	0.31	30.57
27	**H435/14**	19.3	0.75	**7.45**	**H513/08**	23.76	0.03	0.34	34.02
28	**H910/13**	18.5	1.17	**11.71**	**H175/09**	23.33	0.04	0.44	44.38
29	**H933 /13**	20.4	0.35	**3.47**	**H296/09**	25.50	0.01	0.10	10.23
30	**H519/13**	20.1	0.51	**5.09**	**H511/08**	23.02	0.05	0.49	49.05
31	**H440/14**	23.2	0.05	**0.46**	**H53/09**	22.35	0.09	0.90	89.80
32	**H495/14**	20.9	0.22	**2.23**	**H958/10**	31.72	0.00	0.00	0.10
33	**H442/14**	21.5	0.15	**1.51**	**H60A/09**	23.29	0.05	0.45	45.47
34	**H441/14**	20.1	0.40	**4.03**	**H56/09**	NO SAMPLE			
35	**H474/14**	21.1	0.10	**0.97**	**H668/10**	NO SAMPLE			
36	**H439/14**	20.6	0.27	**2.73**	**H45/09**	24.68	0.02	0.17	16.78
37	**H499/14**	18.6	1.08	**10.81**	**H1111/10**	27.13	0.00	0.03	2.63
38	**H486/14**	18.3	1.34	**13.40**	**H675/10**	27.04	0.00	0.03	2.77
39	**H514/13**	21.0	0.30	**3.00**	**H51/09**	26.66	0.00	0.04	4.08

40	**H919 /13**	23.6	0.04	**0.43**	**H282/09**	26.24	0.01	0.06	5.50
41	**H446/14**	21.7	0.13	**1.26**	**H163/09**	26.16	0.01	0.06	5.82
42	**H916 /13**	21.9	0.19	**1.87**	**H279/09**	25.27	0.01	0.11	11.17
43	**H927 /13**	18.6	1.15	**11.47**	**H283/09**	24.84	0.02	0.15	15.02
44	**H443/14**	19.0	0.86	**8.63**	**H60B/09**	23.63	0.04	0.36	35.64
45	**H452/14**	19.7	0.51	**5.12**	**H297/09**	NO SAMPLE			
46	**H459/14**	23.0	0.03	**0.28**	**H264/10**	26.29	0.01	0.05	5.31
47	**H455/14**	18.4	0.65	**6.52**	**H300A/09**	26.05	0.01	0.06	6.28
48	**H469/14**	21.7	0.06	**0.63**	**H279/10**	25.80	0.01	0.07	6.74
49	**H481/14**	16.5	4.52	**45.21**	**H673/10**	25.73	0.01	0.07	7.09
50	**H467/14**	18.3	0.63	**6.28**	**H277/10**	24.33	0.02	0.19	19.30
51	**H433/14**	20.5	0.33	**3.34**	**H397/08**	24.46	0.02	0.21	20.90
52	**H929 /13**	20.3	0.38	**3.77**	**H287/09**	24.10	0.03	0.25	25.49
53	**H480/14**	18.8	0.94	**9.40**	**H671/10**	NO SAMPLE			
54	**H518/13**	22.0	0.17	**1.74**	**H399/08**	25.01	0.01	0.14	14.34
55	**H438/14**	22.5	0.07	**0.72**	**H44/09**	22.86	0.06	0.63	63.32
56	**H472/14**	21.0	0.05	**0.48**	**H662/10**	28.19	0.00	0.01	1.22
57	**H515/13**	22.2	0.15	**1.54**	**H333/08**	27.66	0.00	0.02	2.31
58	**H476/14**	22.3	0.02	**0.21**	**H680/10**	26.82	0.00	0.03	3.25
59	**H454/14**	18.4	0.61	**6.13**	**H299/09**	26.79	0.00	0.04	3.70
60	**H488/14**	19.0	0.82	**8.19**	**H959/10**	26.16	0.01	0.05	5.20
61	**H931 /13**	21.1	0.22	**2.22**	**H292/09**	26.21	0.01	0.06	5.63
62	**H450/14**	22.9	0.06	**0.55**	**H276/09**	20.24	0.03	0.27	21.60
63	**H449/14**	24.4	0.02	**0.20**	**H165/09**	23.27	0.05	0.46	46.25

64	**H479/14**	19.7	0.25	**2.54**	**H670/10**	NO SAMPLE			
65	**H932 /13**	18.2	1.59	**15.91**	**H293/09**	23.67	0.03	0.35	34.71
66	**H494/14**	18.4	1.24	**12.41**	H960C/10	27.06	0.00	0.03	2.74
67	**H490/14**	20.6	0.07	**0.67**	**H960/10**	27.28	0.00	0.02	3.30
68	**H471/14**	18.8	0.47	**4.67**	**H660/10**	26.35	0.00	0.05	4.55
69	**H484/14**	19.8	0.48	**4.82**	**H678/10**	25.87	0.01	0.06	6.42
70	**H487/14**	18.3	0.93	9.25	**H952/10**	25.80	0.01	0.07	6.73
71	**H463/14**	21.8	0.06	**0.60**	**H270/10**	24.87	0.01	0.15	14.73
72	**H448/14**	20.2	0.37	**3.65**	**H166/09**	24.72	0.02	0.16	16.43
73	**H434/14**	24.7	0.02	**0.21**	**H512/08**	24.75	0.02	0.17	17.12
74	**H492/14**	21.9	0.12	**1.19**	**H960A/10**	24.14	0.02	0.22	22.13
75	**H458/14**	18.2	0.70	**7.01**	**H263/10**	NO SAMPLE			
76	**H482/14**	16.2	5.50	**55.03**	**H674/10**	NO SAMPLE			
77	**H489/14**	17.9	1.73	**17.27**	**H951/10**	28.46	0.00	0.01	1.01
78	**H470/14**	18.7	0.25	**2.50**	**H280/10**	25.75	0.01	0.07	7.00
79	**H1030 /13**	18.3	1.43	**14.32**	**H284/09**	25.43	0.01	0.10	9.81
80	**H445/14**	21.6	0.14	**1.40**	**H160/09**	25.30	0.01	0.11	10.77
81	**H930 /13**	22.3	0.10	**1.00**	**H290/09**	24.94	0.01	0.14	13.96
82	**H431/14**	23.6	0.04	**0.43**	**H336/08**	24.58	0.02	0.19	19.27
83	**H457/14**	22.0	0.05	**0.51**	**H262/10**	NO SAMPLE			
84	**H432/14**	23.7	0.04	**0.41**	**H332/08**	24.56	0.02	0.20	19.59

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