The Utility of Mutations in Circulating Free DNA as Potential Predictive Biomarkers for Mechanism Based Therapeutics in Cancer Treatment

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Kyaw Lwin Aung

School of Medicine

Cancer Research UK Manchester Institute

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THESIS ABSTRACT

Background Tumour specific somatic mutations can be detected in circulating free DNA (cfDNA) of patients with cancer. Lack of qualification hampers the routine use of cfDNA based assays in clinic. Next generation sequencing (NGS) of cfDNA may allow real time monitoring of genetic evolution in human cancers.

Methods Analytical validation of *BRAF* c.1799T>A (p.V600E) mutation testing in cfDNA by allele specific real time PCR was performed using serum/plasma samples from 221 patients with advanced melanoma. Prognostic and predictive significance of cfDNA *BRAF* mutation detection was examined within the context of a MEK inhibitor trial in tumour *BRAF* mutant advanced melanoma. Targeted NGS of cfDNA was also performed in 8 patients with advanced colorectal cancer (CRC).

Results Plasma contains higher proportion of tumour derived mutant DNA than serum. Using mutation calling criteria optimized for cfDNA improves sensitivity of *BRAF* c.1799T>A mutation detection by an allele specific PCR. cfDNA *BRAF* mutation status was an independent predictor of progression free survival in advanced melanoma patients with *BRAF* mutant tumours, of whom those with no mutation in cfDNA may derive better clinical benefits from MEK inhibition with selumetinib. In advanced CRC, cfDNA mutation profiling is complementary to tumour mutation profiling and *FBXW7* mutation may play critical role in development of resistance to 5-Fluouracil based combination chemotherapies.

Conclusions Plasma should be the clinical matrix of choice for cfDNA mutation testing. Biological significance of cfDNA mutation status should be studied and understood within specific clinical contexts before cfDNA based assays are clinically qualified. Targeted NGS of cfDNA could advance our understanding of treatment resistance mechanism in individual patients with cancer.

DECLARATION

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PUBLICATIONS ARISING FROM THIS THESIS

Submitted Manuscripts

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Aung KL, Donald E, Ellison G, Liptrot T, Bujac S, Sharpe A, Cantarini M, Orr M, Walker J, Clack G, Ranson M, Hughes A, Brady G, Dive C. *Detection of BRAF mutation in circulating free DNA provides additional predictive information on response to MEK Inhibition with Selumetinib in patients with a BRAF Mutant Melanoma* (submitted to 'Molecular Cancer Therapeutics' in February 2014)

Article in Press

Aung KL, Donald E, Ellison G, Bujac S, Fletcher L, Cantarini M, Brady G, Orr M , Clack G, Ranson M, Dive C, Hughes A. *Analytical Validation of BRAF V600E Mutation Testing from Circulating Free DNA using the Amplification Refractory Mutation Testing System* (accepted for publication by 'Journal of Molecular Diagnostics' in December 2013)

Published Article

Aung KL, Board RE, Ellison G, Donald E, Ward T, Clack G, Ranson M, Hughes A, Newman W, Dive C. *Current status and future potential of somatic mutation testing from circulating free DNA in patients with solid tumours*. The Human Genome Organization Journal. HUGO J (2010) 4:11-21

ABOUT THE AUTHOR

Kyaw Lwin Aung was born and raised in Rakhine State, Burma (Myanmar). He studied medicine in the Institute of Medicine I, Yangon, Burma and graduated in 1999. In 2001, after completing one year of internship training, he immigrated to the UK to continue postgraduate studies in Internal Medicine. He started working as a junior doctor in the National Health Service from 2002 and became a member of Royal College of Physicians (UK) in 2004. The following year he was appointed as a Specialist Registrar in Medical Oncology, first in Yorkshire Deanery and then in Mersey Deanery where he is currently completing his specialist training. In 2008, he was awarded a Cancer Research UK research bursary to conduct a biomarker study in rectal cancer for a duration of 12 months. Following this, as a CRUK-AstraZeneca Clinical Research Fellow in Clinical Pharmacology, he conducted full time research from February 2010 to February 2013 in Clinical and Experimental Pharmacology Department of the Cancer Research UK Manchester Institute, Manchester, UK. The results from the research conducted during that three years period embody the current thesis. He aspires to become a clinician scientist in experimental cancer therapeutics.

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ABBREVIATIONS

ABL	Abetalipoproteinemia gene
AF	Allelic frequency
ALK	Anaplastic lymphoma kinase
AKT	v-akt murine thymoma viral oncogene homolog
APC	Adenomatous polyposis coli
ARMS	Amplification Refractory Mutation Testing System
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
BAX	BCL2-associated X protein
Bcl-2	B-cell lymphoma 2
BCR	Breakpoint cluster region gene
BEAMing	Beads, Emulsions, Amplification, and Magnetics
BHQ1	Black Hole Quencher 1
BRAF	v-raf murine sarcoma viral oncogene homolog B
CAST PCR	Competitive Allele Specific Taqman PCR
CDC4	Cell division control protein 4
CEA	Carcino-embryonic antigen
CI	Confidence interval
CIN	Chromosomal instability
CML	Chronic myeloid leukaemia
CNAs	Circulating nucleic acids
COLD PCR	Co-amplification at lower denaturation temperature PCR
COT	Carnitine octanoyltransferase
COX-2	Cyclooxygenase-2

CRC	Colorectal cancer
CRAF	v-raf murine sarcoma viral oncogene homolog C
CR	Complete response
CSGE	Sequencing after conformation sensitive gel electrophoresis
CT	Computed Tomography
CTCs	Circulating Tumour Cells
Cy5	Indo-dicarbo cyanine
DAG	Dystroglycan 1
DDQ2	Deep Dark Quencher
DNA	Deoxyribonucleic acid
cfDNA	Circulating free or cell free DNA
ctDNA	Circulating tumour DNA
DTIC	Dacarbazine
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EML4	Echinoderm microtubule-associated protein-like 4
EpCAM	Epithelial cell adhesion molecule
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ERBB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FA	Folinic acid
FBXW7	F-box and WD repeat domain containing 7
FDA	Food and Drug Administration

FFPE	Formalin fixed paraffin embedded
FISH	Fluorescence in-situ hybridization
FOLFIRI	5-Fluouracil, Folinic acid, Irinotecan
FOLFOX	5-Fluouracil, Folinic acid, Oxaliplatin
5-FU	5-Fluouracil
GIST	Gastrointestinal stromal tumour
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio
IGF-1R	Insulin like growth factor 1 receptor
IHC	Immunohistochemistry
IPASS	Iressa Pan-Asia Study
JAK	Janus kinase
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
KRAS	Kirsten rat sarcoma viral oncogene homolog
LAPC	Locally advanced pancreatic cancer
LDH	Lactate dehydrogenase
LNA	Locked nucleic acid
LOD	Limit of detection
MAPK	Mitogen-activated protein kinases
MAP3K8	Mitogen-activated protein kinase 8
MCL1	Myeloid cell leukemia sequence 1
MEK	Mitogen-activated protein kinase kinase
MET	Met proto-oncogene
MKP1	Mitogen-activated protein kinase phosphatase 1
MLH1	mutL homolog 1

MMR	Mismatch repair gene
MSI	Microsattellite instability
MT	Mutant
NGS	Next Generation Sequencing
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
NSCLC	Non-small cell lung cancer
NTC	No template control
OS	Overall survival
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
PD	Progressive disease
PDGFRA	Alpha-type platelet-derived growth factor receptor
PDGFRB	Beta-type platelet-derived growth factor receptor
15-PDGH	15-hydroxyprostaglandin dehydrogenase
PFS	Progression free survival
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	p110α catalytic subunit of the class 1A phosphatidylinositol 3-kinase
PKC	Protein kinase C
PLCγ	Phispholipase C gamma
PNA	Protein nucleic acid
PR	Partial response
PTEN	Phosphatase and tensin homolog
RAF	v-raf murine leukaemia viral oncogene
RECIST	Response Evaluation Criteria in Solid Tumour

RFLP	Restriction fragment length polymorphism
RTKs	Receptor tyrosine kinases
RTPCR	Reverse transcriptase PCR
SD	Stable disease
SE	Standard error
SLE	Systemic lupus erythematosus
SMAD4	SMAD family member 4
SNP	Single nucleotide polymorphism
SOX7	SRY (sex determining region Y) - box 7
SRC	Proto-oncogene tyrosine-protein kinase Src
STAT	Signal transducer and activator of transcription
SSCP	Single strand conformation polymorphism
TGF-α	Transforming growth factor-α
TGF-β	Transforming growth factor-β
TGFBR2	Transforming growth factor, beta receptor II
TKIs	Tyrosine kinase inhibitors
TP53	Tumour protein p53
VEGF	Vascular endothelial derived growth factor
WGA	Whole genome amplification
WHO	World Health Organization
WT	Wild type

1. INRODUCTION

1.1. Historical Perspective

The presence of free circulating nucleic acids (CNAs) in human was first discovered by two French plant biologists, Mandel and Metais, in 1948[1]. From a crucial observation that in crown gall tumour of plants, caused by *Agrobacterium tumefaciens*, incorporation of bacterial plasmid nucleic acids into plant cells might be responsible for tumour initiation, they set out to look for CNAs in human as a possible culprit for cancer. Although little attention was paid to their discovery of CNAs in humans at the time, the effort has been proven worthwhile decades later by the ever expanding field of CNAs research, stemming from their original discovery (Figure 1).

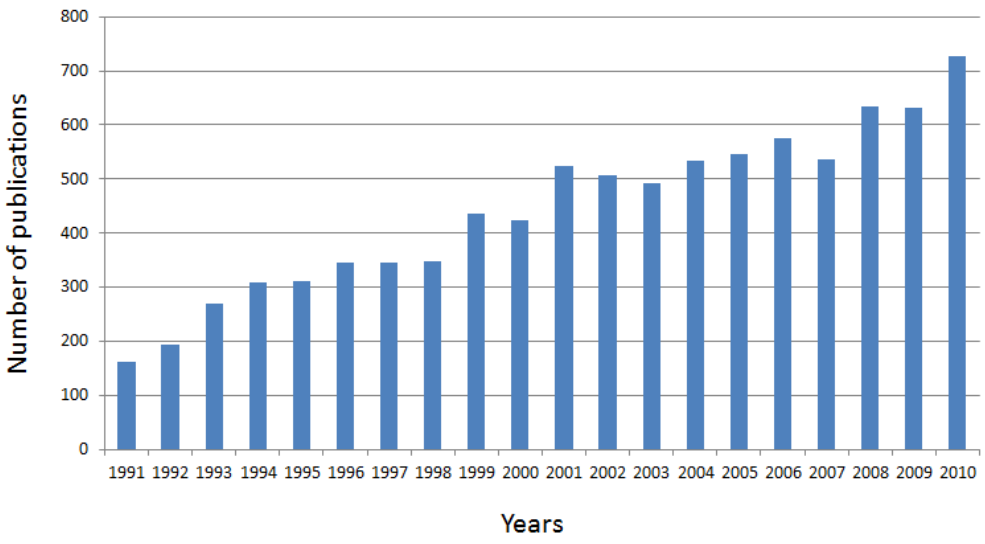

Figure 1. Number of CNAs related publications cited on the PubMed from 1991-2010

In April 1953, James Watson and Francis Crick presented the double helix structure of deoxyribonucleic acids (DNA)[2] and were awarded, together with their colleague Maurice Wilkins, the Noble Prize in Physiology or Medicine nine years later in 1962. In 1966, Tan *et al.* from the Rockefeller University of New York reported that DNA and antibodies to DNA were detected in the serum of patients with systemic lupus

erythematosus (SLE) using the diphenylamine reaction[3]. In 1975, Frederick Sanger from Cambridge University invented the 'dideoxy' technique for DNA sequencing and was awarded the Nobel Prize in Chemistry in 1980 together with Paul Berg and Walter Gilbert for their innovative work in determining base sequences in nucleic acids[4]. However, only after the polymerase chain reaction (PCR), was developed by Kary Mullis in 1983, who received the Nobel Prize in Chemistry in 1993, became widely available in the late 1980s[5], was it possible to characterize DNA from the minute amount present in human blood. The last two decades have seen increasing efforts to elucidate the nature, biology and potential use of CNAs in clinical medicine.

1.2. The Nature, Biology and Source of Circulating Free DNA in Patients with Cancer

It is now recognised that a proportion of patients with cancer have significantly higher level of circulating free DNA (cfDNA) compared to healthy controls. This was first demonstrated in 1977 by Leon *et al.* using serum samples from 173 cancer patients and 55 healthy controls using a radioimmunoassay[6]. In their study, the majority of healthy individuals (51 out of 55) had 0-50ng of DNA per ml of serum; in contrast 50% of the cancer patients had DNA levels ≥50ng/ml. Moreover, patients with metastatic disease had significantly higher levels of cfDNA compared to those with non-metastatic disease. In contrast, no correlation between cfDNA levels and the size and location of the primary tumour was found. In another study Stroun *et al.* reported that double stranded DNA was detected in plasma of 27% (10 out of 37) of patients with advanced cancers but was undetectable in 50 healthy controls[7]. The method employed in this study, however, can only detect DNA above 100ng/ml of plasma and as such the results should be interpreted with caution. Shapiro *et al.*, on the other

hand, demonstrated that patients with malignant gastrointestinal disease had significantly higher mean serum cfDNA levels than those with benign gastrointestinal disease (412ng/ml vs. 118ng/ml, P<0.001)[8]. Levels of cfDNA in patients with benign disease (mean 118±14 (SE) ng/ml) in this study almost exclusively overlapped with those of the healthy controls studied by Leon *et al.*[6]. These findings, taken together, indicated that a higher level of cfDNA was detected in the blood of patients with advanced cancer even though mechanisms of release of DNA into the peripheral circulation were uncertain.

Maurice Stroun and co-workers were among the most active in pursuing the theory that naked DNA can be released by living cells into the circulation and this could be the origin of cfDNA. They based their hypothesis on the biological phenomenon that bacteria released genetically active plasmid DNA, which could be taken up by other cells and incorporated into host genome[9]. This observation reported in crown gall tumour of plants[10] had sparked the hunt for CNAs in human in the first place. In further studies, frog's auricles and human lymphocytes excreted naked DNA in an in-vitro system[11-14]. However, strong in-vivo evidence to support this hypothesis is still lacking up to the present day.

In 1990, Rumore and Steinman demonstrated that cfDNA in plasma of patients with SLE formed discrete bands using 6% polyacrylamide gel electrophoresis closely resembling the 200bp ladder found with oligonucleosomal DNA[15]. Similarly, in patients with pancreatic cancer, stepwise increase in fragment length of plasma derived cfDNA, from 185bp to 370bp, 555bp, 740bp and 925bp, was seen consistent with the nucleosomal DNA pattern[16]. This suggests that the main source of cfDNA is most likely to be cellular apoptosis. In 2001, by using methylation specific PCR in 55 non-selected patients with advanced cancer, Jahr *et al.* demonstrated that cfDNA

fragments' size peaked at 180bp although high molecular weight DNA fragments, which could be released from necrosis of tumour cells, were found in some samples[17]. Taken together, the main source of cfDNA in patients with cancer is most likely to be from apoptotic cell death with perhaps necrosis contributing to a smaller proportion of cfDNA.

With recent technological advances allowing measurement of circulating tumour cells (CTCs) from whole blood, two studies have reported the correlation between CTC number and total cfDNA concentration or level of methylated cfDNA in patients with breast cancer[18,19]. More recently, another study demonstrated a strong correlation between the *SOX7* promoter methylation patterns in CTCs and in cfDNA from patients with breast cancer[20]. These findings raised the possibility that lysis of CTCs could contribute to cfDNA levels. However, in patients with major epithelial solid tumours, median CTC counts, measured by the FDA approved Veridex Cell Search System are usually below 5 cells/7.5ml of blood[21-24]. In contrast patients with advanced cancer usually have cfDNA level >10ng/ml of plasma (~10ng/2ml of whole blood), which is equivalent to 32.5ng/7.5ml or approximately 5000 genomes or cells/7.5ml[25]. Assuming a cancer patient has total cfDNA level 10ng/ml of plasma and only 1% of cfDNA is derived from tumour cells, approximately 50cells/7.5 ml of blood will be needed to explain the amount of DNA typically detectable in plasma. One of the plausible reasons that could potentially explain the discrepancy between CTC number and the amount of cfDNA detectable in circulation of cancer patients is that Cell Search System, which only captures EpCAM positive cells, is perhaps only able to detect a minor proportion of tumour cells present in the blood. However, it is plausible that a proportion of cfDNA does actually come from CTC lysis and further studies are needed to prove or refute the validity of results reported in these studies.

It is now known that as well as tumour specific DNA, wild type DNA circulates in the blood and makes up the major proportion of total cfDNA[26]. The main sources of this wild type circulating DNA, however, remain obscure. In CRC, total cfDNA level was shown to correlate positively with stage of disease. Of 22 patients studied by Diehl *et al.,* 16 had stage I and II disease and their average level of cfDNA was 12ng/ml in contrast to 158ng/ml in 6 patients with stage IV disease[26]. This correlation suggests that this wild type DNA is mostly likely to be tumour related. It could be hypothesized that the sources of wild type cfDNA could include apoptosis and necrosis of tumour associated stromal cells or endothelial cells of tumour vasculature as they do not harbour tumour specific genetic aberrations. Moreover, at least in theory, in patients with aggressive disease, when tumours are rapidly growing, there will be substantial destruction of normal surrounding tissues which could comprise the main source of cfDNA in these patients. This is probably why results from recent studies[27-29] consistently indicate that total cfDNA level is prognostic of overall survival (OS) in cancer patients. In patients with serous epithelial ovarian cancers, cfDNA level was correlated positively with tumour stage and grade and median OS was significantly shorter in patients with detectable cfDNA in plasma compared to those without (21 vs. 52 months, $P = 0.022$)[27]. Another study has demonstrated that in a homogenous population of 108 patients with advanced CRC who underwent third line chemotherapy (Irinotecan/Infusional 5FU with cetuximab), patients with cfDNA level above 75th percentile had worse prognosis compared with those below 75th percentile[28]. Receiver operating characteristic curve analysis showed that patients with a cfDNA level above 90th percentile had the worst prognosis[28]. Similar findings were reported in patients with non small cell lung cancer (NSCLC) where patients with a cfDNA level above 75th percentile had shorter progression free survival (PFS)

and OS compared to those below[29]. Although it could be argued that the percentile cut offs employed in these studies were somewhat arbitrary, these findings do indicate that cfDNA level is prognostic and might reflect tumour burden and/or aggressive tumour biology.

Thanks to advances in PCR based technologies, it is now possible to measure the proportion of tumour specific mutant DNA in a given cfDNA samples. Using a technique called BEAMing (Beads, Emulsion, Amplification, Magnetic), Diehl *et al.* reported that the mutant fraction of *APC* genes in circulating DNA was very low in Dukes' A and Dukes' B colorectal cancer (CRC) patients, with just 0.04% (range 0.01-0.12%) and 0.9% (range 0.03-1.75%) respectively, and in contrast to much higher levels in patients with Dukes' D disease, 11.1% (range 1.9-27%)[26]. It was estimated that approximately 600 million *APC* mutant fragments were shed into the blood stream in Dukes' D patients on a daily basis accounting for approximately 3.3% of the total tumour DNA. This calculation, however, assumed that the weight of the patient was 70kg, that the tumour weighed 100g and that the half-life of cfDNA was 16 hours. The cfDNA half life was extrapolated from the half-life of fetal DNA in maternal circulation but had not been extensively studied in cancer patients but found to be significantly shorter than 16 hours in a later study (approximately 1.5 hours)[30] suggesting that more mutant fragments were shed into the circulation than the amount originally calculated by Diehl *et al.*.

In 1999, Garcia-Olmo and co-workers put forward the controversial hypothesis of geno-metastasis that states that "metastases might develop as a result of transfection of susceptible cells in distant target organs with dominant oncogenes that are present in the circulating plasma and are derived from the primary tumour"[31]. Although attractive, concrete evidence to support this hypothesis is scarce with the exception of

one study conducted by the same investigators[32]. In this study, the development of carcinomas in non-obese diabetic severe combined immuno-deficient mice was seen after the injection with NIH-3T3 cells that had been cultured with plasma from patients with CRC suggesting that these cells were oncogenically transformed. Moreover, detection of human *KRAS*, *TP53* and β-globin-encoding sequences in plasma treated NIH-3T3 cells indicated that the transfer of human DNA had indeed occurred. Although results were interesting, it remains premature to draw any conclusions at present.

Overall, since the discovery of CNAs in 1948, a great deal has been learnt about the nature and biology of cfDNA in patients with cancer. More importantly, several studies have demonstrated that tumour specific molecular characteristics, including somatic mutations, microsatellite instability, loss of heterozygosity, gene amplification and DNA methylation, can be investigated in cfDNA (reviewed by Schwarzenbach *et al.*[33] and Crowley *et al.*[34]). Somewhat disappointingly, the progress made in cfDNA research field has not so far been translated into diagnostic and/or therapeutic medical advancements in cancer medicine. The primary reason for this is most likely lack of standardised blood collection, processing and analysis protocols that have reached an appropriate regulatory accreditation status.

1.3. Somatic Mutations as Predictive Biomarkers for Mechanism Based Cancer Therapeutics in Patients with Solid Tumours

Cancer is now widely recognised as a complex genetic disease. All cancers are caused by abnormalities (mutations) in DNA sequence[35]. In familial cancer syndromes, mutations are inherited as germ line mutations when mutations in sporadic cancers are caused by endogenous genomic instability or exogenous carcinogens[36]. Sporadic

cancers usually originate by clonal expansion of a transformed normal cell that has acquired hallmarks of cancer through accumulation of serial somatic mutations (Figure 2)[36]. Currently 487 genes associated with cancer and known as cancer candidate genes have been identified[37].

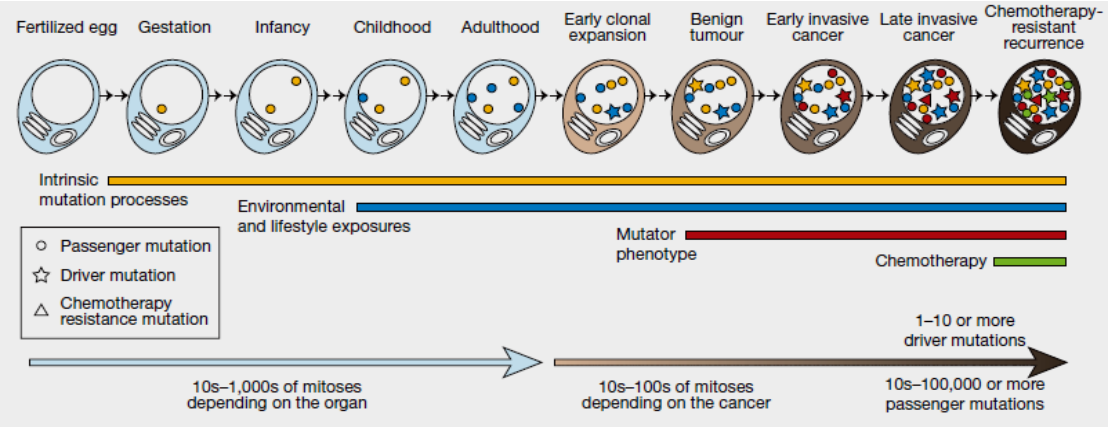

Figure 2. The lineage of mitotic cell division from the fertilized egg to a single cell within a cancer showing the timing of the somatic mutations acquired by the cancer cell and the process that contribute to them *(Figure and legend from Stratton et al.)*[36]

In addition to their clear causal role in carcinogenesis, some somatic mutations are also now proven to be valid drug targets. The fact that mutant proteins in cancer cells could be potential drug targets originated from the premise of oncogene addiction theory proposed by Bernard Weinstein in the early 1990s[38]. He argued that a cancer cell's intracellular circuitries critical for its survival and proliferation are built upon one or a very few initial molecular events that initiated oncogenesis and targeting these early molecular aberrations, coined as 'Achilles' heels of cancer', will produce selective cancer cell deaths sparing normal cells that do not harbour these aberrations. This concept effectively allows selecting patients for treatment with targeted drugs based on genetic characteristics of their tumours.

Understanding the genetic basis of cancer has indeed been crucial for developing mechanism based cancer therapeutics. This is exemplified by the success story of trastuzumab, which, undeniably, is one of the most successful targeted anticancer treatments to date. The pivotal studies in early 2000s demonstrated that treatment with trastuzumab significantly improves OS of patients with early and advanced breast cancer whose tumours overexpress HER2 receptor protein[39,40]. The amplification of *HER2* gene, which is seen in approximately 25% of breast cancers[41,42], predicts patient's response to trastuzumab. Here, clear understanding of tumour biology provided a well-qualified predictive biomarker for treatment response allowing appropriate patient selection in clinical trials that tested and confirmed the therapeutic efficacy of the drug. If it had been tested in a non-enriched breast cancer population, it is very likely that its true therapeutic efficacy will not be as apparent due to a dilution effect. Trastuzumab was also later found to improve survival in patients with HER-2 overexpressed advanced gastric cancer when it was used in combination with chemotherapy[43].

Another 'poster child' of mechanism based therapeutics is imatinib for the treatment of chronic myeloid leukaemia (CML) and gastrointestinal stromal tumour (GIST). CML is typically characterised by the Philadelphia Chromosome which arises through translocation of chromosomes 9 and 22, t(9;22)(q34;q11). This translocation produces the *BCR-ABL* fusion gene that encodes the Bcr-Abl oncogenic tyrosine kinase. Imatinib, the tyrosine kinase inhibitor (TKI) designed to inhibit the constitutively elevated tyrosine kinase activity of Bcr-Abl protein, has proven highly effective in the management of CML[44]. Subsequently imatinib was also found to inhibit the KIT and PDGFRA receptor tyrosine kinases. Gain of function mutations in genes encoding these proteins are the molecular culprits of GIST[45,46]. Imatinib is highly effective in

treating GIST with overall response rates of approximately 80% and a median response duration of 2-3 years[46-48]. During the last ten years, mutations in genes encoding the key proteins of EGFR and components of the downstream signalling effector pathways (Figure 3) have been increasingly being recognised as predictive biomarkers for mechanism based cancer therapeutics in patients with solid tumours.

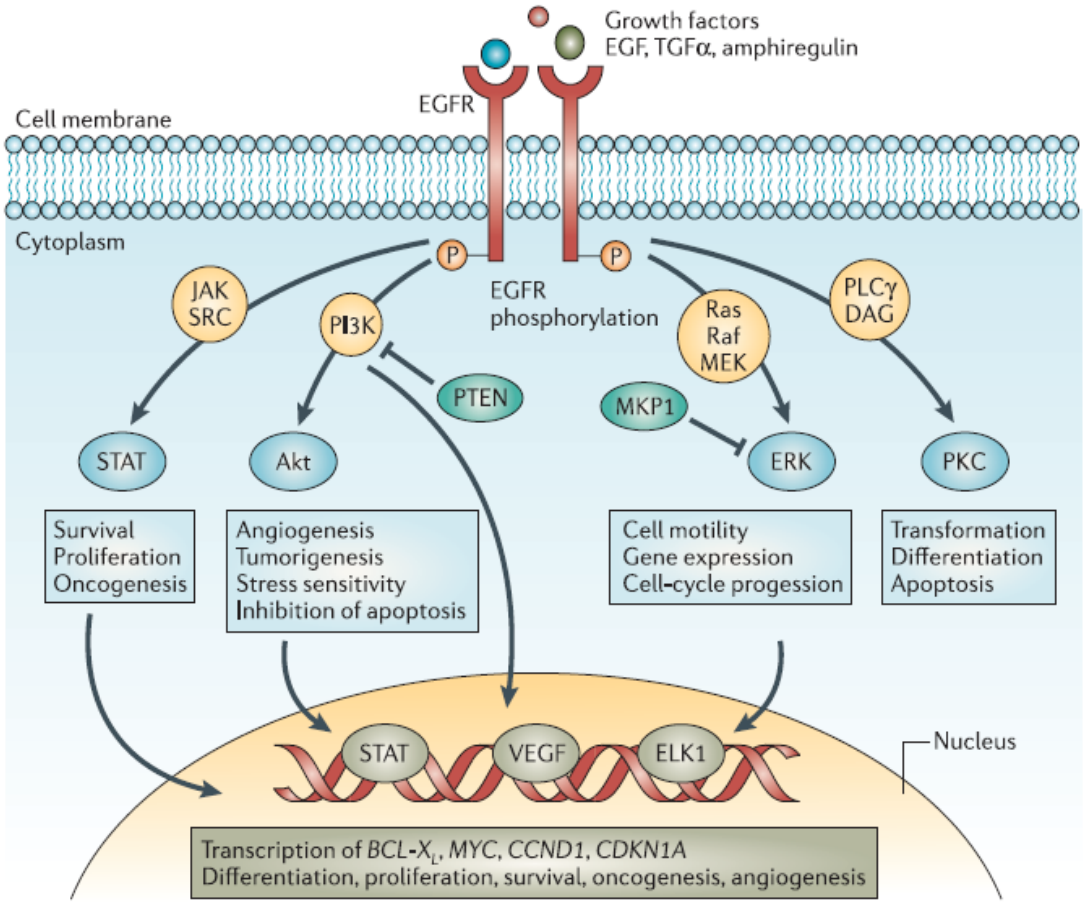

Figure 3. EGFR signalling through MAPK (EGFR/RAS/RAF/MEK/ERK) and PI3K (EGFR/PI3K/AKT) pathways. *It is also important to note that activation of RAS could also activate PI3K pathway. (Figure and adapted legend from Pao et al.)*[49]

In 2005, erlotinib, a TKI of the EGFR receptor, was found to improve survival of patients with chemo-refractory advanced NSCLC when compared to best supportive care[50]. However, the OS benefit was modest (median OS 6.7 vs. 4.7 months) and

gefitinib, a second EGFR TKI, did not significantly improve survival in a very similar group of patients[51]. Further analysis of the molecular and clinical predictors of outcome in erlotinib study showed that patients who were never-smokers, of East Asian origin, female and with adenocarcinoma histology achieved significantly better objective responses to the drug where the presence of an *EGFR* mutation predicted drug response[50]. Mutation in *EGFR* occurs in approximately 35% of NSCLC patients of East Asian origin and 16% of Western populations[52,53]. Multiple in-frame deletions in exon 19 and the p.L858R missense mutation in exon 21 comprise approximately 90% of the mutations detected (Figure 4)[54]. Several other studies reported the presence of *EGFR* mutations in tumour as a predictive biomarker of response to gefitinib[55-60]. The results from IPASS trial demonstrated an increased efficacy of gefitinib as first line treatment in NSCLC compared to carboplatin/paclitaxel combination chemotherapy in a selected East Asian population of non-smokers or former light smokers where mutation in *EGFR* is a strong predictor of a better treatment outcome with gefitinib[52]. More recently the presence of *EML4* and *ALK* fusion gene was identified in a small subgroup of NSCLC patients (approximately 5%) and found to be a strong predictor of response to crizotinib (PF-02341066), a small-molecule inhibitor of the ALK tyrosine kinase[61]. Forty seven of 82 patients (57%) treated with the drug achieved objective response and 27 patients (33%) had stable disease. The estimated probability of 6 months progression free survival (PFS) was 72%[61].

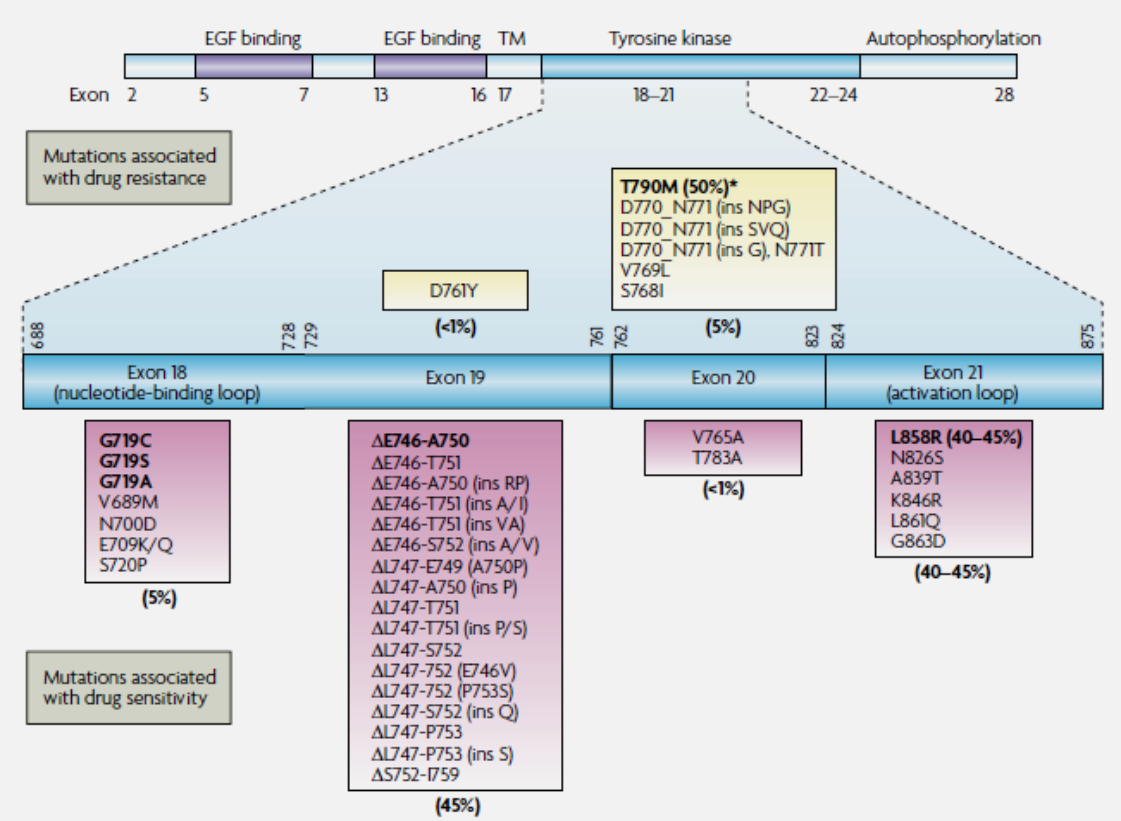

Figure 4. *EGFR* mutations in lung cancer. *Drug-sensitive mutations include L858R mutations (exon 21) and a deletion in exon 19 and infrequent point mutations in exon 18 and exon 20. T790M (exon 20) and D761Y (exon 19) mutations confer acquired resistance to TKI. *EGFR p.T790M mutation is found in 50% of the patients who developed acquired resistance to EGFR TKIs. (Figure from Sharma et al.)*[62]

In cutaneous melanoma, *BRAF* mutation is found in ~50% of cases and p.V600E (c.1799T>A) accounts for more than 90% of the *BRAF* mutations[63,64]. Similar to EGFR story in NSCLC, in patients with *BRAF* mutant advanced cutaneous melanoma, a targeted drug vemurafenib (PLX4032), which inhibits mutated BRAF (p.V600E) in an ATP competitive manner, has improved overall survival[65]. Objective response rate (ORR) of patients to single agent vemurafenib in this study was 48% compared to 5% with standard chemotherapy dacarbazine. The median PFS was longer in the vemurafenib group (5.3 vs. 1.6 months) and the OS rate at 6 months was also significantly better in the vemurafenib group (84% vs. 64%). These findings were

unprecedented in advanced cutaneous melanoma and validate the oncogene addiction theory in that cancer cell deaths was achieved by inhibiting mutated BRAF protein even in late stage disease and despite *BRAF* mutation being an early event in its oncogenesis and was previously shown to be a founder event in a mouse model[66,67]. In another randomised phase III trial, when combined with chemotherapy, inhibition of MEK, which is downstream to BRAF in MAPK pathway, with an oral selective MEK1/2 inhibitor, trametinib, was shown to significantly improve median PFS and OS of *BRAF* mutant melanoma when compared to the standard chemotherapy, dacarbazine[68]. Combined BRAF and MEK inhibition has been reported to yield better survival outcomes than *BRAF* inhibition alone in patients with *BRAF* mutant tumour[69].

However, cancer biology is complex and compelling evidence has now also emerged that presence of somatic mutations in a tumour could also be responsible for primary resistance to targeted drugs. In NSCLC, presence of *KRAS* mutations in tumour was recognised as the main culprit for primary resistance to EGFR TKIs in approximately 20% of the patients[70]. Similarly, in metastatic colorectal cancer, *KRAS* and *BRAF* mutations confer resistance to the EGFR antibody targeted treatments, cetuximab and panitumumab, and only patients with *BRAF* and *KRAS* wild type tumours derive clinical benefits from treatment with these antibodies[71-77]. Although the role of *PIK3CA* and *PTEN* mutations in resistance to these agents is not completely clear at present, the hypothesis is that they can confer resistance to treatment in patients who have *KRAS* and *BRAF* wild type tumours[78]. However, only 10%-15% of patients with advanced CRC will respond to single agent cetuximab or panitumumab and even in 'quadruple negative' (negative for *KRAS* (exon 2 mutations), *BRAF*, *PIK3CA* and *PTEN* mutations) group, 20-25% are non-responders and molecular mechanisms of

EGFR targeted treatment resistance in these patients are still under investigations[78]. Results from a recent exploratory study suggest that additional *RAS* mutations (mutations in exon 3 or 4 of *KRAS* and 2, 3 or 4 of *NRAS*) are likely to confer primary resistance to cetuximab or panitumumab in a subset of these quadruple negative patients[79].

Cancer is heterogeneous and the evolution of a tumour is a dynamic process. In each individual tumour, multiple clones of cancer cells are competing for survival and a particular clone that has survival advantage will be naturally selected over the others[80]. As such, it could be argued that growth of a tumour might be driven by different clones of cancer cells at different stages. For example, emergence of cancer cells with secondary gate keeper *EGFR* mutation, p.T790M, which inhibits binding of drugs to the ATP binding pocket of EGFR, was seen in approximately 50% of NSCLC patients who develop secondary resistance to EFGR TKIs[81-84] and the presence of p.T790M mutant cells as a minor subclone in the primary tumours has been demonstrated[85]. Moreover, an acquired genetic alteration, *c-Met* amplification, was found to be associated with drug resistance in another 20% of the patients[86]. In vitro, in response to gefitinib treatment, NSCLC cell lines underwent amplifications of chromosomal region 7q31.1-33.3 containing the *c-Met* gene, allowing c-Met mediated activation of the PI3K/AKT pathway in an ERBB3-dependent manner, but independent of either EGFR or ERBB2 activation[87]. Similarly emergence of cancer cell clones with secondary mutations in ALK kinase was found and thought to be responsible for development of drug resistance to crizotinib, an ALK kinase inhibitor[88,89].

Almost all patients with advanced cutaneous melanoma, who initially responded to BRAF inhibitor vemurafenib, will also develop acquired resistance to vemurafenib

and this acquired resistance is recognised as a major stumbling block for achieving long term tumour control in these patients. Both MAPK kinase pathway dependent and MAPK kinase pathway independent mechanisms of resistance to BRAF inhibitors have been reported (reviewed by Alcala and Flaherty)[90]. COT was identified as a kinase that activates MAPK pathway and mediates resistance to vemurafenib[91]. Compensatory CRAF signalling was also shown to enable the *BRAF* mutant cells to escape from BRAF dependency conferring resistance to BRAF inhibition[92]. Moreover, over expression of MAP3K8 can activate MEK, which is downstream to RAF, in a RAF independent manner and mediate resistance to BRAF inhibitors[91]. Up-regulation of NRAS through an activating mutation, p.Q61K, or over expression of PDGFRβ was also shown to confer resistance to vemurafenib[93]. Moreover, Villanueva *et al.* demonstrated that melanoma cells switch RAF kinase signalling to phosphorylate ERK and also receive pro-survival signals through other RTKs such as IGF-1R[94].

Not unexpectedly, all patients with metastatic CRC who initially responded to cetuximab or panitumumab will also succumb to drug resistance. Currently, the underlying mechanism of this acquired resistance is still not fully understood although two recent studies demonstrated that the emergence of *KRAS* mutant clones is responsible for development of resistance to EFGR monoclonal antibodies in approximately 30% of the patients[95,96].

In summary, it is apparent that mechanism based targeted treatments have made a significant impact on survival outcomes in some subgroups of cancer patients thanks to the availability of predictive biomarkers, and many of these biomarkers are somatic mutations in cancer candidate genes for treatment response (summarised in Table 1). A lack of predictive biomarkers prevents patient stratification i.e. selecting those who

have best chance of responding to the drugs, and a higher chance of failure in proving therapeutic efficacy. Negative predictive biomarkers such as *KRAS* mutations for EGFR targeted treatment in CRC also prevent toxic side effects in patients who are not going to benefit from the treatment and save unnecessary health care expenditure. However, in patients treated with targeted agents, secondary drug resistance currently hampers the long term control of cancer and a greater understanding of drug resistance mechanism is required in order to improve clinical outcomes. It is also important to note that most of the treatment resistance mechanisms to the targeted agents described to date have a genetic basis and further progress in understanding the genotypic evolution of cancer cells during and after anti-cancer treatment will be critical in the struggle to overcome secondary drug resistance.

Table 1. Clinically proven predictive biomarkers for mechanism based anti-cancer therapeutics in solid tumours

Cancer	Biomarker	Prevalence	Detection Methods	Clinical Relevance
Breast	ER/PR expression	70%[97,98]	IHC	Predicts clinical benefits from tamoxifen[99,100]
	HER2 gene amplification	25%[41,42]	IHC/FISH	Predicts clinical benefits from HER2 targeted treatment[39]
GIST	*c-KIT* mutations	88%[45]	DNA sequencing/RT-PCR	Predicts clinical benefits from Kit receptor TKIs[46,48]
	PDGFRA mutations	5%[45]	DNA sequencing/RT-PCR	Predicts clinical benefits from Kit receptor TKIs[46]
NSCLC	*EGFR* mutations (p.L858R or Exon 19 deletions)	35/16%*[52,53]	DNA sequencing/RT-PCR	Predicts clinical benefits from EGFR TKIs treatment[50,55,59]
	EGFR p.T790M	50%†[86]	DNA sequencing or RT-PCR	Mediates secondary resistance to EGFR TKIs[81]
	EML4-ALK gene fusion	5%[101,102]	Reverse transcription PCR	Predicts clinical benefits from crizotinib[61]
CRC	*KRAS* mutations	35-40%[103]	DNA sequencing or RT-PCR	Predicts primary resistance to EGFR targeted treatments[71,72,96]
Melanoma	*BRAF* p.V600E mutation	50%[104]	DNA sequencing or RT-PCR	Predicts clinical benefits from BRAF and MEK inhibitors[65,68,69]
Gastric	*HER2* gene amplifications	20%[43]	FISH	Predicts clinical benefits from HER2 targeted treatments[43]

Abbreviations: GIST, Gastrointestinal Stromal Tumour; NSCLC, Non-Small Cell Lung Cancer; CRC, colorectal cancer; ER, oestrogen receptor; PR, progesterone receptor; IHC, immunohistochemistry; FISH, fluorescence in-situ hybridization; RT-PCR, real time PCR; TKIs, tyrosine kinase inhibitors. *Prevalence is 35% and 6% East Asian and Western Populations respectively. †EGFR p.T790M mutation is found in 50% of the patients who developed acquired resistance to EGFR TKIs.

1.4. Current Status of Somatic Mutation testing from cfDNA in Solid Tumours

For the last ten years, the main focus of cfDNA research has been assessing feasibility of testing somatic point mutations in genes encoding key proteins of MAPK and PI3K pathways using PCR based technologies. Although recent advent of next generation sequencing and rapid progress in this technology have now started to change this research landscape, only a small number of research groups so far have applied this technology to cfDNA mutation testing and it remains an exploratory research tool at present (discussed in details in the section 1.6). Current status of somatic mutation testing from cfDNA using PCR based techniques in patients with solid tumours is summarised below within the context of specific cancer types.

1.4.1. Non-small cell lung cancer

Mutation in *EGFR* occurs in approximately 35% of NSCLC patients of East Asian origin and 16% of Western populations[52,53]. Multiple in-frame deletions in exon 19 and the p.L858R missense mutation in exon 21 comprise approximately 90% of the mutations detected[54]. Studies have confirmed *EGFR* mutations as a predictive biomarker of treatment response to the TKIs, gefitinib and erlotinib in NSCLC[62].

Kimura *et al.* first reported the feasibility of detection of *EGFR* mutations in cfDNA extracted from serum of patients with NSCLC[105]. However, this study was limited by lack of paired tumour tissues. In 2007, the same group published results from *EGFR* mutation analysis of 42 paired tumour and serum samples using allele-specific amplification refractory mutation testing system combined with scorpion probes (Scorpion-ARMS)[106]. It was demonstrated that *EGFR* mutation status was consistent in 39 (93%) of the 42 paired samples tested. However, this encouraging result was not confirmed in another study that reported *EGFR* mutations in only 33% of plasma-

derived cfDNA samples from 12 patients whose tumour was positive for *EGFR* mutation by using Scorpion-ARMS[107]. Better sensitivity of *EGFR* mutation detection in serum was seen in another study conducted by Spanish Lung Cancer Group where of 164 patients with *EGFR* mutations in tumours, 97 (59%) had *EGFR* mutation in serum tested by protein nucleic acids mediated PCR analysis[53].

Yung *et al.* used a novel technology platform, microfluidics digital PCR, in 35 patients with stage III and IV non-small cell lung cancer to detect *EGFR* exon 19 and 21 mutations in plasma[108]. *EGFR* mutations in tumours were analysed by sequencing after confirmation sensitive gel electrophoresis (CSGE) and also by digital PCR. Of 12 patients, who had EGFR mutations in tumour detected by CSGE and sequencing, 11 (92%) had the corresponding mutation detected in plasma. In 4 patients in whom CSGE analysis in tumour was unsuccessful but mutations were detected by digital PCR, corresponding mutations were detected in plasma in all of them. High sensitivity (73%) of *EGFR* activating mutations detection from plasma was also achieved by using BEAMing in 44 patients with EGFR mutant NSCLC[109]. Two exploratory studies have also demonstrated that p.T790M mutation, which confers resistance in approximately 50% of the cases, can be tested from plasma derived cfDNA highlighting the potential role of mutation testing from cfDNA in monitoring secondary resistance to anticancer treatment[109,110].

Ramirez *et al.* analysed *KRAS* mutation in serum and tumours of 50 resected NSCLC using combined PCR-RFLP and sequencing[111]. Twelve mutations were found in serum and 9 in tumour. High discordance between mutations detected in tumour and serum was thought to be the main limitation of the study. However considering more up to date understanding of tumour heterogeneity, the discordance could potentially be explained by spatial intra-tumour heterogeneity of primary tumours especially

considering lung biopsies usually contain a small amount of tumour material. Another study reported that *KRAS* codon 12 mutations could be detected in 9% of the 180 plasma samples tested using the same technique employed by Ramirez *et al.*[112]. However the study was severely limited by the fact that paired tumour tissue was available only in 9 patients and as a result no firm conclusions can be made based on these results. More recently, Wang *et al.* reported their findings from *KRAS* mutation analysis of DNA extracted from 273 plasma samples and matched tumour tissues from advanced NSCLC patients of East Asian origin. PCR-restriction fragment length polymorphism (PCR-RFLP) combined with denaturing high performance liquid chromatography was used for mutational analysis[113]. *KRAS* mutation was found in 35 (13%) plasma samples and 30 (11%) tumours. Concordance of mutations between plasma and tumour was 77%. The fact that more mutations were found in plasma compared to tumour raises the possibility of false positive results in plasma by the technique employed. However, as discussed earlier, the spatial heterogeneity of the primary tumours could also potentially explain these discordant results as it is possible that a biopsy did not contain all mutant sub clones. Moreover, cfDNA could also be shed from metastatic disease sites and heterogeneity between primary and metastatic tumours should also be taken into consideration in interpreting the results of the study.

1.4.2. Colorectal cancer

The genetic basic of colorectal cancer is well characterized and mutations in tumour suppressor genes, *APC* and *TP53*, and proto-oncogene, *KRAS*, are all implicated in adenoma-carcinoma sequence of colorectal carcinogenesis (Figure 5)[114]. Several studies have tried to establish the presence of mutations of these genes in plasma or

serum and prognostic value of circulating tumour DNA (ctDNA). Most of the earlier studies produced variable results because of the small sample sizes and variability of the mutation detection methods employed (comprehensively reviewed by Fleischhacker and Schmidt)[25]. However, these earlier studies did establish some important facts with respect to mutation testing from cfDNA in patients with colorectal cancer. They demonstrated that cfDNA can be detected in plasma of CRC patients and its level increases with stage of the disease[26]. *APC*, *TP53* and *KRAS* mutations were all shown to be detectable in cfDNA highlighting their potential as circulating biomarkers in CRC[115]. Diehl *et al.* also reported the potential prognostic value of ctDNA in resected colorectal cancer during postoperative follow up and demonstrated that ctDNA can be used to assess tumour dynamics in patients undergoing multimodality therapies for CRC in a subsequent study[30].

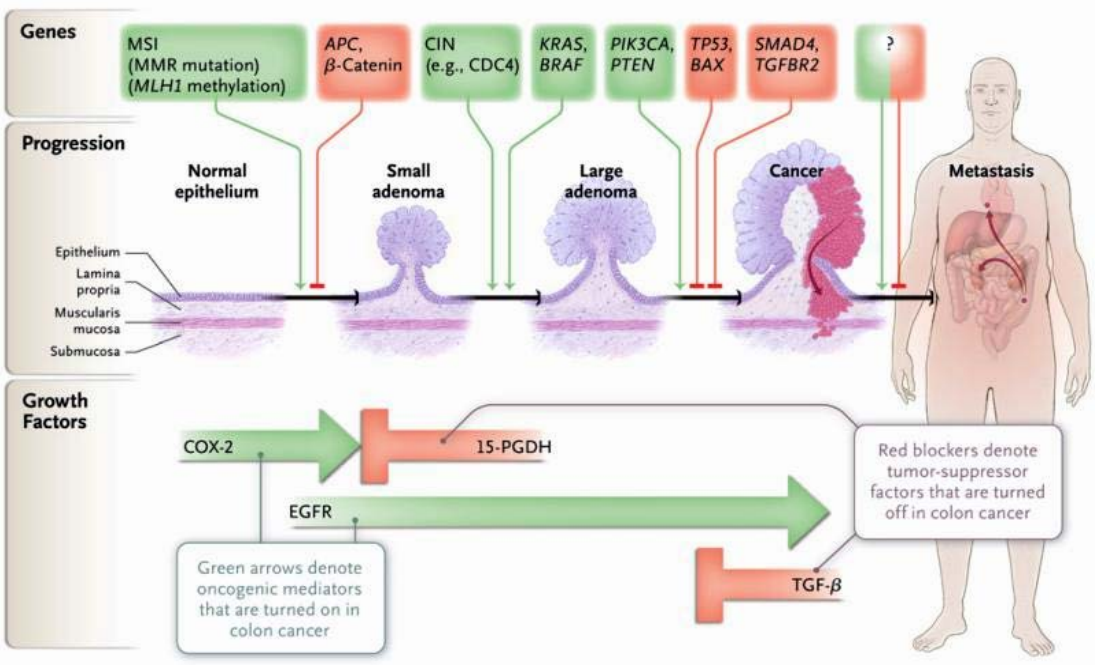

Figure 5. Genes and growth factor pathways that drive the progression of colorectal cancer *(figure and legend from Markowitz and Bertagnolli)*[114]

More recently, *KRAS* mutation was shown to be detectable in plasma of patients with advanced CRC with 78% sensitivity and 100% specificity using ARMS with a wild type blocker[28]. Moreover, the result from this study showed that mutation fraction in plasma predicts patients' survival outcomes and their response to chemotherapy. Although increasing clinical relevance of *BRAF* and *PIK3CA* mutations in CRC is currently being recognized, there is no study reporting significance of detecting *BRAF* and *PIK3CA* mutations in plasma or serum of CRC patients. However, cfDNA mutation analysis are increasingly being applied to study EGFR targeted treatment resistance mechanisms using cutting edge technologies such as BEAMing and digital PCR. Two studies have demonstrated that emergence of *KRAS* mutant clones is responsible for acquired resistance to anti-EGFR targeted treatments as evidenced by detectable *KRAS* mutations by BEAMing in plasma of patients undergoing treatment with cetuximab or panitumumab well before radiological progression[95,96]. Moreover, heterogeneous and concomitant mutations in *KRAS* and *NRAS* were also detected in plasma of patients with resistance to anti-EGFR antibodies[116]. In an innovative study, MET amplification was shown to confer resistance to EGFR targeted therapies in advanced CRC and was found to be detectable in ctDNA using a breakpoint digital PCR detecting a translocation specifically associated with MET amplification[117].

1.4.3. Pancreatic Cancer

Although mutation in codon 12 of *KRAS* is a very common and early event, occurring in up to 90% of pancreatic cancers, preliminary studies that assessed the feasibility of using *KRAS* mutations in plasma or serum of pancreatic cancer patients as a diagnostic tool produced disappointing results. Castells *et al.* studied *KRAS* mutations in 44 consecutive patients with histologically confirmed primary pancreatic ductal

carcinoma using PCR-RFLP in both tumours and plasma samples[118]. Out of 39 patients in whom both plasma and tissue samples were available, 28 patients (72%) had *KRAS* mutations in their primary tumours and 9 (23%) had mutations detectable in plasma[118]. In a separate study, Dianxu *et al.* assessed the diagnostic value of codon 12 *KRAS* mutations in plasma combined with serum CA19-9 in 58 consecutive patients with a suspected pancreatic mass[119]. Forty one patients were subsequently diagnosed with pancreatic adenocarcinoma. Mutations in *KRAS* codon 12 were found in 29 (71%) patients in plasma using PCR-RFLP whereas elevated CA19-9 was found in 30 patients with pancreatic adenocarcinoma demonstrating that plasma *KRAS* mutation does not have an advantage over CA 19-9 as a diagnostic marker in this setting. A recent study showed that ctDNA quantities are lower in patients with pancreatic cancer when compared to those with colorectal cancer or breast cancer or melanoma[120]. There are two plausible explanations for why ctDNA levels are lower in this disease group. Pancreatic cancer is unique in that it has unusually dense fibrous stroma and is poorly vascularised[121]. Those anatomical conditions are unfavourable for shedding of ctDNA into the peripheral circulation. Moreover clinical context is also important for interpreting these studies as a significant proportion of advanced pancreatic cancer has locally advanced inoperable disease without metastasis. In those patients, one could expect that pick up of mutations in ctDNA would be lower than that of those with metastatic disease. It is demonstrated in a recent study that number of enumerable CTCs in patients with locally advanced pancreatic cancer (LAPC) is low (11% had detectable CTCs)[122] and the clinical utility of CTC as biomarkers in patients with LAPC remains doubtful[123]. On the other hand, CTC number was higher in those with metastatic disease[124]. Further larger studies with

detailed clinical annotation will be needed before we could make firm conclusions on the merits of studying mutation testing from ctDNA in patents with pancreatic cancer.

1.4.4. Breast Cancer

A relatively high frequency of mutations of the *PIK3CA* gene in human cancers was reported in 2004[125]. Subsequent studies confirmed that somatic *PIK3CA* mutations occur in approximately 25% of breast cancer[126-128]. As such, mutated PI3K has become an attractive therapeutic target in breast cancer therapy. Parallel with this development, Board *et al.* demonstrated the feasibility of *PIK3CA* mutation testing from cfDNA[129]. DNA was extracted from plasma and serum samples of 46 patients and four hot spot mutations in *PIK3CA* gene, p.H1047R, p.H1047L, p.E545K and p.E542K, were analysed with Scorpion-ARMS. Matched tumour and plasma data was available for 41 cases. Ten (24%) mutations were detected in tumour and of those 10 patients, 8 (80%) had mutations in cfDNA isolated from plasma and 6 (60%) had mutations in cfDNA isolated from serum. Concordance between matched tumour and cfDNA data was 95% (95%CI: 83-99%) and 88% (95% CI: 73-95%) for plasma derived cfDNA and serum derived cfDNA respectively. More recently, Higgins *et al.* reported that, in 41 patients with metastatic breast cancer, 100% concordance between *PIK3CA* mutation results from plasma and that from tumour was achieved by using BEAMing[130]. However, intriguingly, only 57% sensitivity and 82% specificity was seen when cfDNA mutation results by BEAMing were compared to tumour pyrosequencing results in 51 patients[130]. Although, the difference in sensitivities of the two techniques (limit of detection (LOD) of BEAMing was previously shown to be 0.01% [131,132]and that of pyrosequencing is 5%[133]) might explain the discordant results between BEAMing and pyrosequencing, the possibility of getting false

positive results in plasma or tumour by BEAMing cannot be completely excluded at this stage.

1.4.5. Cutaneous Melanoma

BRAF mutation is found in ~50% of cases of cutaneous melanoma. Daniotti *et al.* were the first to report the feasibility of *BRAF* mutation testing from plasma and serum derived cfDNA in an exploratory study using an allele specific PCR[134]. Tumour *BRAF* mutation was detected in 13 of the 20 patients studied and 5 (38%) had *BRAF* mutations in plasma. Two patients who had cfDNA plasma mutation did not have mutation in tumours. Yancovitz *et al.* also reported detection of mutant *BRAF* alleles in plasma of 14 (54%) out of 26 patients using mutant specific PCR[135]. Subsequently, Board *et al.* investigated the clinical utility of cfDNA from serum as an alternative source of *BRAF* mutation testing in 126 metastatic melanoma patients who participated in a phase II study testing the efficacy of a MEK inhibitor selumetinib (AZD6244)[136]. Mutation testing in both tumours and serum were performed by ARMS. Matched tumour and serum samples were available in 96 cases and 45 (47%) patients have *BRAF* mutation in tumour and 25 (27%) patients have *BRAF* mutation in serum derived cfDNA. Based on those data, concordance in *BRAF* mutation detection was 76% (95% CI 66-84%) and pick up rate in cfDNA was 56% (95% CI 40-70%). Key studies that reflect the current status of somatic mutations testing from cfDNA by PCR based techniques are summarized in Table 2.

Table 2. Summary of studies that reflect the current status of somatic mutation testing in cfDNA in solid tumours by PCR based techniques

Cancer	Mutation	Study	Matched Samples	Technique	Matrix	Sensitivity	Specificity
Non-small cell lung	*EGFR* (Exon 19 deletions and p.L858R)	Kimura *et al.*[106]	42	Scorpion-ARMS	Serum	75%	97%
		Maheswaran *et al.*[107]	12*	Scorpion-ARMS	Plasma	33%	NA
		Rosell *et al.*[53]	164*	PNA mediated PCR	Serum	59%	NA
		Goto *et al.*[137]	86	ARMS	Serum	43%	100%
		Yung *et al.*[108]	12*	Digital PCR	Plasma	92%	NA
		Taniguchi *et al.*[109]	44*	BEAMing	Plasma	73%	NA
	KRAS	Wang *et al.*[113]	273	PCR-RFLP	Plasma	76%	95%
Breast	*PIK3CA*	Board *et al.*[129]	41	Scorpion-ARMS	Plasma	80%	97%
		Higgins *et al.*[130]	41	BEAMing	Plasma	100%	100%
			51	Pyrosequencing in tumour, BEAMing in cfDNA	Plasma	57%	82%
Colorectal	*KRAS*	Spindler *et al.*[28]	95	ARMS in tumour, ARMS with wild type blocker in cfDNA	Plasma	78%	100%
		Morgan *et al.*[138]	70	Scorpion-ARMS	Plasma	31%	97%
Melanoma	*BRAF*	Pinzani *et al.*[139]	56	Allele specific PCR	Plasma	72%	88%
		Board *et al.*[136]	96	ARMS	Serum	56%	94%
Pancreatic	*KRAS*	Castells *et al.*[118]	39	PCR-RFLP	Plasma	23%	100%

Abbreviations: ARMS, amplification refractory mutation testing system; PNA, protein nucleic acid; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; BEAMing, Beads Emulsions Amplification and Magnetics.*****All cases have *EGFR* mutation in tumour

1.5. Challenges in cfDNA Mutation Testing and Critical Appraisal of Currently Available PCR Techniques

Mutation testing from cfDNA has clear logistical advantages over tumour mutation testing in cancer types where tumour biopsy is difficult to perform because of the anatomical positions of the tumour such as in lung cancer and pancreatic cancer and especially when longitudinal multiple samplings are necessary for disease monitoring. Moreover, emerging evidence suggest that tumours are heterogeneous and a single biopsy at one time point from one tumour site is unlikely to provide comprehensive information needed for making personalised therapeutic decisions. Problems posed by spatial heterogeneity of primary tumours and intertumoural heterogeneity between primary tumour and metastatic tumours (also between metastatic tumours at different organ sites) in a patient could potentially be addressed by profiling of ctDNA to catalogue all somatic mutations shedding from all tumour sites in a patient (see detailed discussion in section 1.8.). However mutation detection from cfDNA also has important limitations imposed by the biology and nature of cfDNA.

A wide variation of cfDNA levels is seen in patients with different types of cancer[120] and studies have demonstrated that not all cancer patients have higher level of cfDNA compared to healthy controls[25]. Moreover, often, the major proportion of cfDNA that circulates in the blood is wild type DNA and the proportion of tumour derived mutant DNA is, in actual fact, relatively small[26]. In addition, cfDNA is fragmented and most of the DNA molecules measure below 180bp in length[17]. This minute quantity and fragmented nature of cfDNA impose difficulties in analysing tumour specific molecular characteristics from cfDNA. For example, cfDNA is not suitable for standard dideoxy DNA sequencing because this needs high input DNA. Although whole genome amplification (WGA) can increase the amount of starting material

DNA, WGA of cfDNA can result in loss of minor mutant alleles as fraction of ctDNA in total cfDNA can be very low in some patients (unless amplification is uniformly linear) and amplified samples might not fully represent original samples. This is why most of the research groups have used PCR based mutation detection techniques, which work better with smaller DNA input and shorter DNA fragment size compared to standard DNA sequencing. However, variability in sensitivities (Table 2) still hampers its clinical application. It is not completely clear whether this is attributable only to technical limitations or to other biological factors such as amount of tumour intrinsic apoptosis and necrosis or the degree of leakiness of the tumour vasculature which may determine the amount of tumour DNA released into the circulation.

One of the most widely used methods for cfDNA mutation testing is an allele specific real time PCR ARMS. It essentially allows rapid analysis of any known point mutation in genomic DNA[140]. The technique is based on the principle that extension is efficient when 3′ terminal base of a primer matches its target, whereas extension is inefficient when the terminal base is mismatched[140]. There are two pre-requisites for ARMS; the absence of a 3′-exonucleolytic proof reading associated with the DNA polymerase employed and 3′-OH terminal mismatched primers are refractory to extension by the chosen polymerase[140]. Although Taq-polymerase meets the first requirement, amplification of the normal allele may occur at variable degrees depending on assay conditions and could limit sensitivity and specificity. This technique was subsequently modified by Whitcombe *et al.* using a scorpion probe so that PCR products could be detected in real-time by fluorescent signals[141]. The scorpion probe is a bi-functional molecule containing a PCR primer covalently linked to a probe, the fluorophore of which interacts with a quencher that reduces the fluorescence. During a PCR reaction, the fluorophores and quenchers are separated

leading to an increase in light output from the reaction well. Commercial kits are currently available for *KRAS, BRAF, PIK3CA* and *EGFR* mutations testing and LOD of this method is claimed to be 1% provided that input DNA per PCR reaction is 1ng (~300 copies of DNA). This means that the absolute limit of detection is ~3 copies of mutant DNA. In most cases, however, it might not be possible to use input DNA of 1ng and therefore a higher mutation fraction will be needed to have 3 mutant DNA copies per reaction. For example, if the input DNA is 0.1ng (~30 copies), then to have 3 mutant copies in the reaction, at least 10% of the input DNA must be mutant. Otherwise, the assay will not detect the mutant signal. This is probably one of the reasons why this technique, even though highly specific, has limited sensitivity of 30-75% reported for cfDNA mutation detection in most of the tumour types[106,107,136-138] with an exception of breast cancer, where sensitivity PIK3CA mutation detection in cfDNA was reported to be 80%[129].

Several groups have tried to improve LOD of PCR based techniques with an aim to detect a very low level of mutant alleles. In 1991, Kahn *et al.* first described the mutant enriched PCR[142]. Essentially, it is a two-step PCR with an intermittent restriction enzyme digestion of wild type sequences in between. Kahn *et al.* used this method to detect *KRAS* codon 12 mutations and reported that it could identify a mutant allele in the background of 10,000 wild type alleles (LOD 0.01%)[142]. However, when the method was adopted by Asano *et al.* to detect *EGFR* exon 19 and 21 mutations, LOD was one mutant allele in the background of 2000 wild type alleles (LOD 0.2%)[143]. There is a single report of this method to detect *EGFR* mutations in NSCLC where concordance of 94% was seen between tumour and cfDNA mutation status in 18 patients in whom matched tumour and plasma samples were available[144]. Modified versions of this technique were also used by other groups to analyse cfDNA

mutations in patients with cancer[113,115]. The major caveat of this technique is that false positive results could arise from incomplete digestion of wild type alleles. Moreover, it is a multistep procedure and as such it is more error prone and there is a substantial risk of contamination. It also bears an increasing risk of Taq polymerase borne infidelity as soon as the total number of PCR cycles involved exceeds 70-80 cycles.

Sequence specific clamping by protein nucleic acids (PNAs) has also been used in combination with different PCR techniques to improve detection of mutant alleles in the presence of excess amount of background normal genomic DNA[145-147]. PNAs are oligonucleotide mimics that bind complementary DNA strands by hydrogen bonding according to the standard base paring rule producing PNA-DNA hybrids and this blocks amplification of the complementary DNA strand. PNAs, however, cannot serve as primers as they lack ribose sugar backbone. Even though PNA-DNA hybrids have higher thermal stability compared to DNA-DNA hybrid, they are destabilised by single base pair mismatches allowing selective amplification of the mutant allele in the PCR reaction[145]. Using a two-step protocol, an allele specific PCR clamping followed by a PCR-RFLP, Behn *et al.* reported a LOD of 0.1% using DNA from *KRAS* and *TP53* mutations harbouring cell lines[146]. In comparison with unmodified allele specific PCR technique, improved sensitivities of mutation detection in cfDNA were seen by using PNA mediated PCR in both *EGFR* mutation testing from cfDNA in NSCLC[53] and *KRAS* mutation testing in CRC[28]. However, whether these methods will be robust and reproducible in the hands of other investigators remains to be seen.

An alternative to using PNAs to reduce non-specific noise from amplification of the wild type allele is single molecule PCR in which mutant DNA molecules are amplified in separate compartments at a single molecule level effectively reducing background noise from wild type allele amplification to zero. Physical separation of

DNA molecules at a single copy level is achieved using a microfluidic device or the creation of micro bubbles using water in oil emulsions. Yung *et al.* reported *EGFR* mutation testing using microfluidic digital PCR (Biomark System)[108]. Biomark Digital Array Chip consisted of twelve panels and each panel was further partitioned into 765 reaction chambers. All together one chip can perform 9180 reactions in a single PCR run. PCR reaction from each chamber will give a fluorescent signal; the colour of which differs depending on whether mutant or wild type molecule was amplified in the chamber. This also allows counting of mutant and wild type copy numbers using the different colour coding. Yung *et al.* developed an assay for detection of *EGFR* exon 19 deletions using this technique and demonstrated that this technique could detect one mutant copy in the background of 1000 wild type copy (LOD 0.1%). A major limitation of this technique is that it is exquisitely sensitive and contamination could be a major issue in non-expert hands. Furthermore, it is labour intensive as the number of samples that can be analysed on a single run is relatively limited.

BEAMing uses water in oil emulsions to perform single molecule PCR. It was first described by Dressman *et al.* in 2003[148] and subsequently modified by Li *et al.* and Diehl *et al.*[131,132]. In this procedure, after amplifying the regions of interest in a gene by PCR, water-in-oil emulsions are formed in which single DNA molecules are amplified by primers bound to beads. Subsequently the beads are recovered from the emulsion and interrogation of wild type or mutant sequence is performed by allele-specific hybridization using probes labelled with specific coloured fluorescence. The colour and quantity of the magnetic beads were investigated by flow cytometry. The LOD was reported to be 0.01% (1 mutant copy in the background of 10000 wild type copies). Diehl *et al.* used this method for detection and quantification of mutations in

plasma of colorectal cancer patients and to assess tumour dynamics[26,30,149]. Recently it was shown that *PIK3CA* mutations could reliably tested by using this methods in patients with metastatic breast cancer patients achieving 100% concordance between mutation detected in plasma and that in tumour[130.] It has also been used to detect EGFR mutations in NSCLC with encouraging results[109]. The technique, however, is complex and it is not likely to be reproducible in the hands of other groups who have less experience and expertise. Cost also seems to be a limiting factor for widespread adoption of this technology into clinical practice unless further modifications of the technique allow easy and affordable access. More importantly, cross validation with another technique will be necessary to demonstrate unequivocally that this technique does not produce false positive results.

Overall, it has been difficult to improve LOD for PCR based technologies to consistently detect <1% of mutant allele in a reproducible manner and allele specific PCR remains the most widely available technique for somatic mutation testing from cfDNA. More sophisticated techniques such as digital PCR and BEAMing offer better sensitivity but application of these technologies to cfDNA on a larger scale and in multisite trials has been limited so far because of the expense, complexity and accessibility. Moreover, PCR based technologies only allow to testing of candidate mutations and for novel mutation detection in cfDNA, ultimately sequencing of cfDNA will be necessary.

1.6. Next Generation Sequencing of cfDNA

The advent of massively parallel DNA sequencing, known as Next Generation Sequencing (NGS), has changed the landscape of cancer genomics research. Sequencing has an advantage over PCR based methods in that it can be used to detect

both known and novel mutations. Moreover, NGS platforms can be used to study chromosome rearrangements and also provide copy number variation. However, direct sequencing of cfDNA using NGS is technically challenging. NGS generally requires and performs best with higher amount of input DNA, preferably in microgram range. As cfDNA is normally detected within the nano gram range, input DNA quantity is one of the rate limiting factors for sequencing cfDNA. Furthermore, as cfDNA is fragmented and ctDNA only constitutes a very small fraction of total cfDNA, target enrichment of tumour specific DNA is problematic. Sequencing error rate also determines the limit of detection of the NGS platforms[150] and currently sensitivity of mutation detection is relatively limited compared to PCR based methods critically appraised in the earlier section.

Nevertheless, feasibility of deep sequencing of tumour DNA in plasma has now been reported[151]. In this study, target enrichment was performed by a two-step amplification process. During the first step, regions of interest in *TP53* genes were amplified in parallel (15 cycles of PCR) in overlapping amplicons (150-200bp). At this step of limited cycle pre-amplification, both mutant and wild type molecules were amplified at the same time. In the following second step, regions of interest in the pre-amplified material were separately amplified in parallel single-plex PCRs using a microfluidic system to avoid biased coverage of mutant and wild type alleles. The products from single-plex PCR were then pooled and sequenced using 100-base single end sequencing on Illumina Genome Analyser IIx platform. Average read depth of 3250 for 48 amplicons of 6 genes (*PIK3CA*, *EGFR*, *BRAF*, *PTEN*, *KRAS* and *TP53*) was achieved for 96 samples tested. It was reported that mutations with allele frequency as low as 2% were detected with greater than 97% specificity. This method, however, was purely amplicon-based and only predefined mutations were analysed in

the study. It was not designed for screening of novel mutations. Its clear advantage over PCR methods is its multiplex capacity.

More comprehensive genome-wide sequencing of circulating DNA was first performed in maternal plasma by Lo *et al.* in 2010 to genotype a fetus[152]. After genotyping both parents for single nucleotide polymorphism, paired end sequencing of maternal plasma derived DNA was performed. Combined analyses of these data allow constructing a genome-wide genetic map to determine the mutational status of the fetus. More recently, whole genome sequencing of plasma DNA (paired end sequencing on Illumina HiSeq platform) from 10 patients with breast and colorectal cancer was reported[153]. Genomic structural alterations, chromosomal copy number changes, rearrangements and amplifications of known driver oncogenes, were detected in all patients with cancer but not in healthy controls (Figure 6)[153].

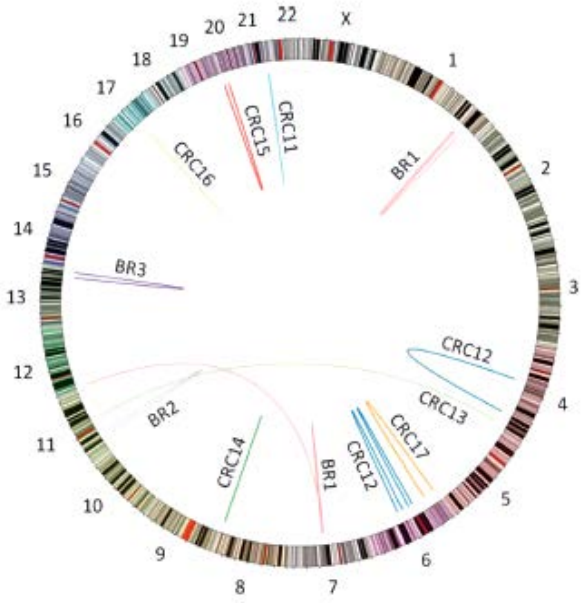

Figure 6. Detection of tumour specific rearrangements in plasma samples from cancer patients (CRC11-CRC17 and BR1-BR3) *Abbreviations: CRC, colorectal cancer; BR, breast cancer. (Adapted figure and legend from Leary et al.)*[153]

Although these results were encouraging, so far only a handful of research groups have applied next generation sequencing to cfDNA. Further optimizations of the technique will be necessary before it can become an easily accessible and affordable tool for smaller research groups. However, with rapid progress recently seen in DNA sequencing technologies, it has already become an indispensable research tool in cfDNA research field.

1.7. Potential Clinical Utilities of Somatic Mutations testing from cfDNA

In this era of mechanism based therapeutics, it is going to be inevitable that screening of molecular characteristics of patients' tumours will be necessary to stratify patients into the most appropriate clinical trials with new targeted drugs to achieve the main goal of personalized medicine that is to deliver the right drug to the right patient. As such, importance of efficient and standardized testing for somatic mutations cannot be overemphasized at present. However, significant problems remain in testing somatic mutations in clinical practice.

Tumour biopsies are not always readily available for genotyping for several biological, technical, ethical and logistical reasons. For example, diagnosis in significant number of patients with lung cancer is based purely on sputum cytology and as a result there may be insufficient tumour material available for comprehensive molecular profiling including mutation testing[154]. This imposes immense difficulties if patients are stratified by mutation status before entering into clinical trials with new biological targeted agents[49]. Even if biopsy material is available, the quality and quantity of tissue specimen could be variable. Archival formalin fixed paraffin embedded tumour tissue from diagnostic biopsies usually contain small amount of tumour materials mixed with normal stromal tissue and DNA is usually degraded to

various degrees from formalin fixation[155]. Whether material from diagnostic biopsy can truly represent intra-tumour heterogeneity is also debatable[156,157]. As biopsy is usually taken from one small part of a tumour, potentially mutant clones could be missed during biopsy. On the other hand, it has been argued that analysis of cfDNA might yield information about all sub clones in the tumour. For patients with multiple metastases- all of which may shed DNA into the circulation, cfDNA may represent tumour heterogeneity more comprehensively than a small, single tumour biopsy (see detailed discussion in section 1.8.). More importantly it is minimally invasive and readily accessible.

Secondly, real time monitoring of evolution of a tumour is desirable for understanding of genotypic changes responsible for emergence of various drug resistant phenotypes. It could also be argued that, metastatic lesions could have different molecular characteristics compared to the primary tumour and targeted treatment of metastatic disease should actually be based on the characteristics of metastatic lesions rather than that of the primary. Although performing serial tumour biopsies is seldom practical or justified in routine clinical practice, from a research perspective, it is highly desirable for developing a better understanding of biological processes which are responsible for cancer recurrence, progression and development of drug resistance. To achieve real time monitoring of tumour dynamics, mutation testing should be simpler, although not less robust, less invasive, readily available and easily repeatable. In this regard, using alternative source of DNA from plasma or serum to test for cancer specific genetic alterations is an attractive proposition if the protocols and assays can be standardised.

Thirdly, it could also be envisaged that, to prove the mechanism of a drug targeted towards a particular clone of mutant tumour cells, it would be necessary to

demonstrate that these mutant cells are markedly reduced or eradicated after treatment. Currently this proof of mechanism is not routinely possible in oncology clinics. However, if a particular tumour derived mutation or mutations can be tested from circulating free DNA using a robust assay, disappearance of these mutations from circulation would at least in part suggest that the drug is hitting the intended target. Moreover, reappearance of these or new mutations in the blood stream could herald disease recurrence and/or indicate mechanism of drug resistance. At the commencement of my PhD project, a few exploratory studies had already reported the pharmacodynamic value of ctDNA in various tumour types highlighting the need for further large scale studies[30,158-160]. Leary *et al.* also reported the development of personalized biomarkers for disease monitoring in patients with cancer using cfDNA[161]. By using next generation mate pair sequencing, it was demonstrated that unique chromosomal rearrangements can be identified in each individual tumour (n=6) and it was possible to detect the rearranged sequences in plasma using digital PCR. As these sequences are unique, digital PCR can detect theses sequences down to a very low level in cfDNA (down to <0.001%). It was shown that changes in levels of these DNA sequences in circulation could be used as a personalized biomarker to monitor individual patient's response to therapy or disease relapse or disease progression.

Lastly, mutations testing in cfDNA might lead to discovery of novel driver mutations in cancer candidate genes. If the whole spectrum of cancer specific mutations in cfDNA can be catalogued by novel technology platforms such as next generation DNA sequencing at multiple longitudinal time points, it could significantly advance our understanding of tumour biology.

1.8. Intra-tumour heterogeneity and personalised cancer medicine in patients with advanced solid tumours

The concept that tumours are heterogeneous is not a new one. It has long been recognised that different cancer types behave clinically differently and even patients with the same cancer type respond differently to the same treatment and their prognosis varies widely. However, understanding of tumour heterogeneity at molecular level is relatively new and underpins the development of personalised medicine. Sub-classification of cancers based on inter-tumour heterogeneity defined by tumours' molecular characteristics allowed the development of personalised therapeutics. This is best exemplified by success stories of targeted treatments in various solid tumour types. In breast cancer, patients with oestrogen receptor positive tumours are selected for treatment with tamoxifen, an oestrogen receptor modulator, or aromastase inhibitors whilst those with *HER2* amplified tumours are treated with HER-2 targeted agents such as herceptin and lapatinib. In NSCLC, patients with *EGFR* mutant tumours are now selected for treatment with EGFR TKIs, erlotinib or gefitinib. On the other hand those with *ALK* translocated tumours benefit from treatment with crizotinib[61]. Similarly, in metastatic CRC, only patients with *KRAS* and *NRAS* wild type tumours benefit from cetuximab and panitumumab[78,79] and more recently, patients with *BRAF* mutant advanced melanoma were shown to benefit from BRAF inhibitors and MEK inhibitors[65,68,69]. However, patients with advanced cancer treated with targeted agents will eventually succumb to drug resistance that hampers achieving long-term disease control.

More in depth understanding of intra-tumour heterogeneity, thanks to recent advances in DNA sequencing technology, now explains why it is so difficult to eradicate or achieve long tern control over advanced cancers and brings challenges for further

development of personalised cancer medicine. The dynamic process of tumour evolution contributes to tumour heterogeneity in space and time. Tumours arise from a single cell that evolves clonally over time[80]. During tumour progression, replication stress causes genomic instability and produces heterogeneous clones of cancer cells[80] that could have different survival potentials under various selection pressures such as chemotherapy and radiotherapy. Multi-region sequencing of tumours in clear cell renal cell carcinoma and glioblastoma demonstrated how tumour subclones could arise within the same tumour through branched evolution causing intra-tumour spatial heterogeneity[157,162]. Intratumour heterogeneity between the primary tumour and a subsequent local or distant recurrence in the same patients (temporal intratumour heterogeneity) has also been demonstrated in breast cancer[163], pancreatic cancer[164] and medulloblastoma[165]. Moreover, tumour microenviroment is heterogeneous and can also contribute to spatial intratumour heterogeneity and clonal diversity[166]. For example, blood supply to different regions of a tumour is not homogeneous and there could be hypoxic regions even within a highly vascularised tumour. Clonal diversity within a tumour is likely to determine primary drug resistance as different clones will have different sensitivity to different types of anticancer treatment. For instance, cancer cells within the hypoxic regions of tumours are resistant to both radiotherapy and chemotherapy[167].

On the other hand, development of secondary drug resistance is rather complex. Ongoing debate is whether secondary drug resistance is caused by emergence of already existing minor clones of cancer cells within the primary tumours or new clones that develop after exposure to selection pressure i.e. anti-cancer treatment. Recent studies have shown that presence of drug resistant minor clones could be detected in primary tumours in multiple cancer types[85,95,168,169]. However, it is also

plausible that tumour cells can acquire new somatic mutations after exposure to drugs and mediate resistance to them. In reality, how tumour cells develop secondary drug resistance is most likely to be context dependent. The main challenge however is how to overcome primary and secondary drug resistance mediated by intra-tumour heterogeneity and develop more effective treatment strategies for patients with cancer to improve their survival.

To win a war, one has to know the enemy. In other words, to develop treatment strategies to overcome challenges posed by intra-tumour heterogeneity, we must have tools to study and chart accurately how this contributes to drug resistance in individual patients. The first challenge then is to how to perform optimal representative tumour samplings to study intra-tumour heterogeneity in cancer patients with high accuracy. Currently most treatment decisions are based on histo-pathological or molecular analysis of single tumour biopsies or tumour resection specimens of primary or metastatic tumours. It is now clear that single tumour biopsy either taken from a primary tumour or a metastatic lesion is unlikely to give us comprehensive picture of a tumour as a whole. Although it is possible to perform multi-region analyses from tumour resection specimens, currently this is not routinely done and how many analyses are needed to perform to get the full picture of intra-tumour heterogeneity within a tumour is unclear. However, encouragingly, the emerging data suggest that clonal diversity and evolution of a tumour could be studied by comparative sequencing approaches[170-172] and the falling cost of next generation DNA sequencing means that it might be feasible to study intra-tumour heterogeneity in individual patients. However, to address temporal intratumour heterogeneity in patients with multiple metastatic lesions, it would be necessary to analyse tumour materials from all the lesions. Moreover, to address spatial heterogeneity within each

metastatic lesion, ideally, multi-region sampling of each and every metastatic lesion would be needed. This clearly is not possible to implement routinely in clinical practice and impose challenges on studying tumour heterogeneity in individual patients.

One alternative approach to multiple tumour samplings is to use ctDNA as an alternative source of tumour derived DNA. ctDNA detectable in circulation could potentially derive from all the sub-clones present in metastatic lesions and reflect genetic heterogeneity in advanced cancer patients more accurately. A proof of concept study has reported that exome sequencing of ctDNA could provide insight into tumour evolution and development of drug resistance mechanisms in individual patients with breast, ovarian and lung cancers[173]. However, sensitivity of mutation testing from ctDNA by next generation sequencing remains low and as such not all the mutations present in tumour lesions will be detected in ctDNA implying ctDNA analysis at present is complementary rather than an alternative to tumour analysis. Moreover these methods and approaches still need to undergo technical and clinical validation before they could be adopted in oncology clinics. Although analysis of CTCs could also provide more insight into intra-patient tumour heterogeneity, technological hurdles are still needed to be overcome before genetic information obtained from CTC analysis could be used for making clinical decisions.

Considering the fact that currently there is no single method for optimum representative sampling to address intra-tumour heterogeneity, one has to consider combining all the available tools to address this issue. The best possible data could be achieved by combined analysis of primary tumour, metastatic tumours and ctDNA collected at the time of diagnosis of metastatic disease and different longitudinal time points of a patient's therapeutic journey thereafter. As mentioned earlier, as it is not

practically feasible or ethical to biopsy all metastatic lesions, information obtained from analysis of a single biopsy or limited number of biopsies from metastatic sites has to be used at present although it is not ideal. By analysing primary and metastatic tumour samples, albeit collected from a limited number of tumour sites, we could at least partially reconstruct how tumour evolved over time from diagnosis of primary tumour to development of metastatic disease. ctDNA analysis could complement tumour analyses by providing genetic information derived from metastatic tumour sites that are not biopsied. In addition, longitudinal samplings of ctDNA can inform changes in genetic landscape of tumours in real time. Eventually, data from CTCs analysis will also be possible to incorporate into and complement this combined analysis potentially allow studying intra-tumour heterogeneity more comprehensively. However, whether more in-depth knowledge of intra-tumour heterogeneity and clonal evolution of tumours in cancer patients will allow us developing new therapeutic strategies that will eventually improve patients' clinical outcomes including survival remains uncertain at this juncture[174].

1.9. Rationale of the Project

Despite the fact that there are real potential clinical utilities of cfDNA in oncology practice, it has to be acknowledged that currently there is no blood based test for somatic mutation testing in oncology clinics. cfDNA research suffers enormously from lack of standardization in pre-analytical and analytical stages of the analysis. Currently, there is no standardisation in which clinical matrix, plasma or serum, should be used for cfDNA analysis. Both were used across multiple studies (Table 2). Although it is known that storage time of plasma and serum could affect the mutation results in cfDNA[134,136], most published studies do not include information on sample

storage time and as such it is difficult to compare the results across the studies. Several different DNA extraction techniques are also currently being used to isolate cfDNA from plasma and serum even though different methods could have different efficiencies. Most studies have been small exploratory studies and as such it is difficult to draw firm conclusions from the results. Moreover, real time PCR techniques that are being used for mutation testing in cfDNA are mainly validated using FFPE tumour DNA even though these assays could have different performance characteristics in cfDNA. These limitations hamper current application of cfDNA based mutation testing in the clinics. To exploit its full potential, somatic mutation testing from cfDNA needs rigorous pre-analytical and analytical validation to make it fit for purpose for clinical use. Addressing these issues was one of the first tasks in my PhD.

Clinically, there is limited knowledge of whether the presence of an oncogenic driver mutation in the circulation of patients with cancer has any biological significance with respect to mechanism based anti-cancer therapeutics. The relationships have been founded on mutation testing of tissue. Theoretically, the biology and clinical behavior of patients with cfDNA detected mutations could be different from those with only tissue detectable mutations. Proving or refuting this hypothesis is important as it could have enormous impact on the development of cfDNA based assays as companion diagnostics. Moreover, although results from recent studies showed that next generation sequencing of the cfDNA is feasible and it could potentially allow multiplex profiling of cancer related somatic aberrations from cfDNA, how this kind of assay could be incorporated into clinical practice to help make personalized treatment decisions for patients with solid tumours is not clear at present.

It is envisaged that concerted effort from cancer physicians and researchers will be necessary to overcome current challenges preventing clinical application of cfDNA based assays in oncology clinics. My PhD project, in effect, represents a rigorous attempt to bring cfDNA mutation testing a step closer to the clinic within the specific clinical context of mechanism based cancer therapeutics.

1.10. Aim and Objectives of the Project

The overall aim of this study was to assess somatic mutations in cfDNA as potential predictive biomarkers for patients' response to mechanism based cancer therapeutics within specific clinical contexts and to explore how testing somatic mutations from cfDNA could help advance in understanding of tumour biology and making real time therapeutic decisions in individual patients.

Specific objectives are as follows:

- To establish the optimum clinical matrix for cfDNA mutation testing (Chapter 2)
- To validate an assay for *BRAF* c.1799T>A mutation testing from cfDNA using ARMS allele specific PCR (Chapter 2)
- To investigate the prognostic value of *BRAF* c.1799T>A mutation detection in cfDNA in patients with advanced melanoma (Chapter 3)
- To investigate the predictive value of cfDNA *BRAF* c.1799T>A mutation detection with respect to MEK inhibition with selumetinib in patients with advanced melanoma (Chapter 3)
- To investigate the clinical utility of somatic mutation testing from cfDNA in patients with advanced colorectal cancer by targeted NGS (Chapter 4)

2. ANALYTICAL VALIDATION OF BRAF MUTATION TESTING FROM CIRCULATING FREE DNA USING ARMS

2.1. Introduction

Circulating free DNA (cfDNA) levels are raised in the blood of patients with advanced cancers compared to healthy controls and tumour specific somatic mutations can be examined in cfDNA[25,33]. *BRAF* is one of the most commonly mutated oncogenes in human tumours and its mutation is present in approximately 50% of cutaneous melanoma[63]. The p.V600E mutation (c.1799T>A transversion) accounts for up to 90% of *BRAF* gene mutations[64] and mutated BRAF has now been proven as a valid drug target. Vemurafenib, an ATP competitive BRAF inhibitor, improves survival in patients with *BRAF* mutant advanced melanoma[65]. Moreover, studies have also shown that *BRAF* mutation is a positive predictor of response to MEK inhibition and combined BRAF and MEK inhibition produced better clinical outcomes than BRAF inhibition alone in this group of patients[68,69]. On the other hand, in colorectal cancer, patients with *BRAF* mutation do not derive clinical benefit from treatment with EGFR monoclonal antibodies, cetuximab and panitumumab[77]. Considering these findings, the importance of establishing *BRAF* mutation status of patients' tumours before they are treated with agents targeting components of the mitogen activated protein kinase (MAPK) pathway cannot be overemphasized as this could have potential impact on their treatment related clinical outcomes.

Conventionally, somatic mutations are detected from archival formalin fixed paraffin embedded (FFPE) tumour tissues obtained at diagnosis and/or from other biopsies or during surgery. However using archival tumour material for mutation testing has inherent problems. The first challenge is when there is lack of tumour material in patients whose tumours are difficult to biopsy and as a result there will be insufficient

tumour material for comprehensive molecular profiling including the somatic mutation testing[60]. A second challenge is that archival tumour tissue from diagnostic biopsies often contains small amounts of tumour material mixed with normal stromal tissue and DNA is usually degraded by formalin fixation[155]. Furthermore, as a biopsy is usually taken from one small part of a tumour, potentially mutant clones could also be missed and it is debatable whether tumour material from a core or needle biopsy adequately represents intratumour heterogeneity[156,157]. Analysis of cfDNA might yield better coverage of sub-clones present in a tumour[156]. Lastly, in a tertiary cancer centre, turn-around time for getting the tumour mutation result can take several weeks unless fresh biopsies are taken because tracing and retrieving archival FFPE tumour material from referral hospitals can take time and occasionally be logistically difficult. Testing the mutations of interest from cfDNA, on the other hand, could significantly shorten the turn-around time.

Previous studies have shown that *BRAF* c.1799T>A mutation status can be assessed in cfDNA using real time quantitative polymerase chain reactions (qPCR) based assays highlighting the potential of using cfDNA mutation testing as a surrogate for tumour testing[134-136]. Considering encouraging data in advanced CRC where resistance mechanisms to EGFR targeted therapies are being elucidated using cfDNA based assays[95,96,116,117], similar approaches could also be adopted in *BRAF* mutant melanoma. One potentially feasible application could be monitoring *BRAF* mutant loads in cfDNA using PCR based assays during treatment with BRAF inhibitors to detect early progression. However, before this cfDNA based approach can be adopted into oncology practice, a number of pre-analytical and analytical questions need to be addressed. For instance, there is no robust data to support which clinical matrix, serum or plasma, is better suited for cfDNA mutation testing. Preliminary evidence

suggests that plasma will be superior because serum contains higher background levels of wild type DNA emanating from white blood cell lysis during the clotting process intrinsic to the preparation of serum[175-177]. However, both plasma and serum are being used widely for cfDNA based mutation testing and as yet there has been no systematic and definitive direct comparison of *BRAF* mutation 'pick up' rate between serum and plasma in an adequately powered study.

Also pertinent to the overall goal of routine implementation of mutation testing from cfDNA, qPCR approaches validated for mutation testing from FFPE tumour tissues are currently being used for cfDNA mutation detection; they employ identical mutation calling criteria even though these assays could have different performance characteristics in cfDNA than in FFPE tissues. DNA cross-linking via formalin fixation of tumour is one of the 'culprits' of mis-priming in PCR reactions causing non-specific amplification of wild type DNA[178] and as such stringent mutation calling criteria are usually employed for FFPE tumour DNA to prevent the introduction of false positive results. However, cfDNA is not formalin fixed and as a result less non-specific amplification should be observed potentially allowing more appropriate and sensitive mutation calling criteria to be applied without affecting the assay specificity.

In this study, two research hypotheses relating to the pre-analytical and analytical phases of *BRAF* p.V600E mutation testing from cfDNA were examined; 1) more *BRAF* p.V600E mutations will be detected in plasma compared to serum because plasma contains less wild type DNA and 2) increased sensitivity of *BRAF* mutation detection from cfDNA using ARMS will be achieved by using mutation calling criteria specific to cfDNA without compromising the assay's specificity. This study was accepted for publication in Journal of Molecular Diagnostics in December 2013.

2.2. Materials and Methods

2.2.1. Patients and Samples

Two hundred and eight plasma and 208 serum samples were available for cfDNA quantification and *BRAF* c.1799T>A mutation analysis from 221 patients who were screened for participation in a randomized phase II study (NCT00936221). This study has evaluated the efficacy of a specific MEK 1/2 inhibitor, selumetinib (AZD6244, ARRY-142886), in combination with dacarbazine versus dacarbazine alone in patients with *BRAF* mutant advanced melanoma in the first line setting. The study is conducted according to Good Clinical Practice and the Declaration of Helsinki.

2.2.2. Study Design

Samples were analyzed in two stages in a blinded fashion and analysts were also blinded to tumour mutation status. In the first stage, 50 serum and plasma samples from patients with *BRAF* mutant tumours and 50 serum and plasma samples from those with *BRAF* wild type tumours were analyzed for *BRAF* c.1799T>A mutation using an ARMS allele-specific PCR. Of the 100 serum and plasma samples analyzed in the first stage, 90 of them were matched. The results from this first analysis stage were employed as a training data set to derive a mutation calling criteria specific to cfDNA. The criteria derived were then validated using the remaining matched 108 plasma and serum samples in the second stage.

2.2.3. Processing of blood to plasma and serum

For plasma, 4ml of whole blood was drawn into a Becton-Dickinson Vacutainer Collection Tube containing EDTA and centrifuged at 2000 x g for 10min at 4°C within 30min of blood collection. The supernatant was transferred to a 15ml falcon

tube and centrifuged at 2000 x g for 10min at 4°C. The resultant plasma supernatant was separated and stored immediately at -80°C. For serum, 4ml blood was drawn into a Becton-Dickinson Vacutainer Serum Collection Tube. After allowing blood to clot for 30min at room temperature, it was centrifuged at 2000 x g for 10min and the resultant serum supernatant was subsequently separated and immediately stored at -80°C.

2.2.4. cfDNA extraction

cfDNA was extracted from 2ml of plasma and 2ml of serum using QIAamp Circulating Nucleic Acids Kit (Qiagen, Hilden, Germany). The principles of DNA extraction are shown in the Figure 7 and the method is described in detail below.

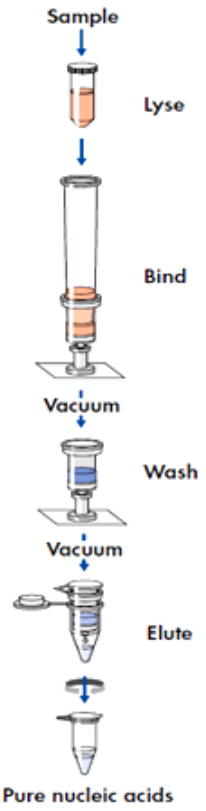

Figure 7. Principles of DNA extraction by using QIAamp Circulating Nucleic Acid Kit

Sample Lysis Serum or plasma samples were thawed at room temperature and centrifuged at 3000rpm for 2 minutes. The supernatant was transferred into a clean 15ml falcon tube containing 200µl of Proteinase K. Subsequently, 1.6 ml of lysis buffer (buffer ACL) that contains 2.6µg of carrier RNA was added to the tube and mixed by pulse vortexing for 30 seconds. The mixture was then incubated in a water bath at 60°C for 60 minutes to lyse the samples.

DNA Binding After the lysis step, 3.6 ml of binding buffer (Buffer ACB) was added to the tube and mixed by pulse vortexing for 15 seconds and incubated on ice for 5 minutes. QIAvac vacuum manifold was set up according to the manufacturer instructions (Figure 8). Briefly, after connecting the QIAvac manifold to a vacuum pump, QIAamp mini columns were connected to the QIAvac manifold using the Vac valves and Vac connectors. The tube extenders were then attached to each mini-column and sample lysates were applied into the tube extender and drawn through the QIAamp mini columns by using the vacuum pump. Binding of DNA to the silica membrane of QIAamp mini columns occurs during this process.

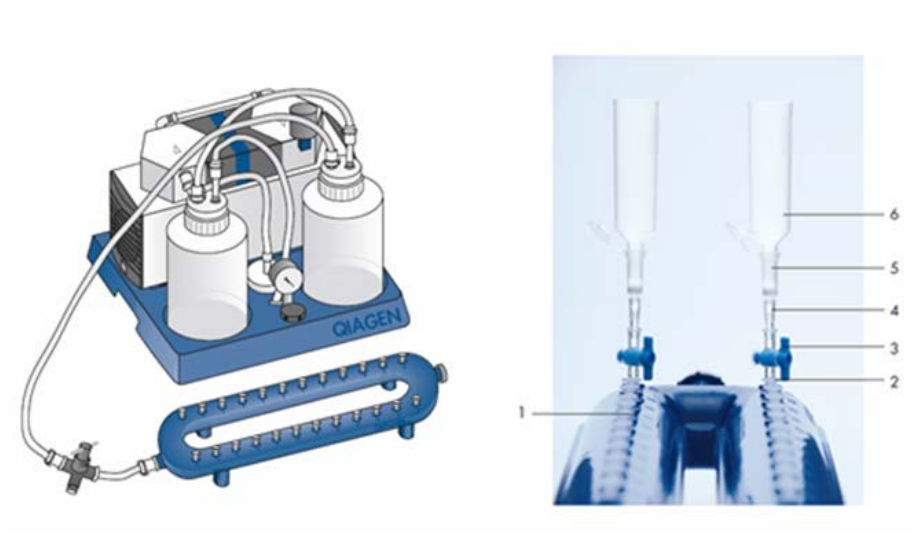

Figure 8. Set up of QIAamp Mini column and tube extender on a QIAamp Vacuum Manifold. *1, QIAvac 24 Plus vacuum manifold: 2, Luer slot of the QIAvac 24 Plus: 3, Vac Valve: 4, Vac Connector: 5, QIAamp Mini column: 6, Tube Extender)*

Washing Steps Once all the lysates were drawn through, the vacuum pump was turned off and 600µl of first wash buffer (Buffer ACW1) was added to the tube extender and drawn through the mini columns using the vacuum pump. The same procedure was repeated by using 750µl of second wash buffer (Buffer ACW 2) and 100% ethanol to complete the washing steps. After the washing steps, tube extenders were removed and mini columns were disconnected from the vacuum manifold and placed in a 2ml collection tube. The columns were then centrifuged at 13000rpm for 3 minutes to remove any residual ethanol. Subsequently columns were transferred into a clean 2ml collection tube and incubated at 56°C for 10 minutes with lids open to dry the membranes completely.

DNA Elution 100µl of elution buffer (Buffer AVE) was applied to the centre of the membrane and the column was incubated at the room temperature for 3 minutes. DNA was eluted by centrifugation of the columns at 13000rpm for 1 minute. Eluted DNA was stored at -20°C until use.

2.2.5. cfDNA quantification

The DNA concentration of the extracted DNA was measured using quantitative PCR, by measuring the copy number of *RNase P* gene in the sample. The reaction mix for PCR was prepared using 2X TaqMan Universal Master Mix PCR (ABI Life Technologies, Foster City, New Jersey, USA), 20X RNase P assay Mix PCR (ABI Life Technologies, Foster City, New Jersey, USA) and nuclease free water. Each reaction comprised 10µl of Universal Master Mix, 1ul of 20X RNase P assay Mix, 4ul of water and 5µl of DNA. A serial dilution set of high molecular weight genomic DNA (50ng/5µl, 25ng/5µl, 12.5ng/5µl, 6.25ng/5µl, 3.125ng/5µl), purchased from Roche (Basel, Switzerland), was used as standards in each PCR reaction. Each

standard was repeated in triplicates in a single PCR run. PCR was performed using Stratagene MX3000 Cycler (Agilent Technologies, Berkshire, UK) using the cycling conditions as shown in the Table 3. The results were analysed using MxPro software version 4.1. The R^2 values of the standard curves should be > 0.985 for results to be of acceptable quality.

Table 3. Thermal cycling conditions for RNase P qPCR assay

Denaturation	Cycles	PCR: denaturation and annealing		Cycles
95°C, 10 minutes	1	94°C, 15 seconds	60°C, 1 minutes	40

2.2.6. *BRAF* c.1799T>A Mutation Analysis by Allele-specific ARMS PCR

Assay system *BRAF* c.1799T>A (p.V600E) mutation in cfDNA was examined by allele specific PCR developed in AstraZeneca's Genetics Team (Alderley Park, Cheshire, UK) based on the ARMS technique. This is a single assay which identifies the *BRAF* c.1799T>A transversion in exon 15 of the *BRAF* proto-oncogene resulting in an amino acid change from valine to glutamic acid in codon 600 of BRAF protein (p.V600E). Less common additional flanking nucleotide changes also result in p.V600K and p.V600D amino acid changes, and although in theory this assay will still detect the c.1799T>A in the presence of the additional changes, it will not distinguish between them. As an internal control, the assay system detects a wild type sequence in exon 17 of *BRAF* gene. The PCR product size from mutant reaction is 91bp and that from control reaction 101bp. The primers and probes used, (synthesized by Eurogentec (Seraing, Belgium)), are summarized in the Table 4.

Table 4. Primers and probes used for BRAF ARMS allele specific real time PCR

Description	5· Modification	Sequence (5·-3·)	3· Modification
BRAF ARMS primer		AAAAATAGGTGATTTTGGTCTAGCTACATA	
Exon 15 common primer		CATCCACAAAATGGATCCAGACAA	
Exon 15 probe	Yakima Yellow	GATGGAPTGGGTCLCATCEG*	BHQ1**
BRAF control forward primer		CTCCAGATCTCAGTAAGGTACGG	
BRAF control reverse primer		GGGAAAGAGTGGTCTCTCATCTC	
BRAF control probe	Cy5**	CATGAEGEGATTAATGGCAGEGTGLC*	DDQ2**

***Probe contains Locked Nucleic Acid (LNA) modified bases**. LNA base nomenclature: A-LNA: E, C-LNA: L, G-LNA: P, T-LNA: Z. ****Abbreviations:** BHQ1-Black Hole Quencher 1, Cy5-Indo-dicarbo Cyanine, DDQ2-Deep Dark Quencher.

PCR Reaction Each reaction contained 12µl of platinum q-PCR master mix (Invitrogen, Paisley, UK), 2µl of 10 mg/ml bovine serum albumin (New England Biolabs, Beverley, MA, USA), 0.5µl of *BRAF* ARMS primer (2µM), 0.5µl of *BRAF* common primer (2µM), 0.125µl of *BRAF* probe (0.5µM), 0.125µl of forward control primer (0.1µM), 0.125µl of reverse control primer (0.1µM), 0.05µl of control probe (0.2µM) and 4.075µl of nuclease free water (summarised in Table 5). DNA (5µl) was added to each reaction and all clinical samples were tested in duplicate. PCR was performed in a 96 well optical PCR plate using Stratagene MX3000 Cycler (Agilent Technologies, Berkshire, UK) using the following thermal cycling conditions; 95°C for 10min, followed by 40 cycles of 94°C for 45 sec, 60°C for 60 sec, and 72°C for 45 sec (summarised in Table 6). Fluorescent data was captured at 60ºC annealing step.

For each PCR run, 3 positive controls and 1 no template control (NTC) were included in duplicate.

Table 5. Components of BRAF ARMS reaction and their concentration

Component	Concentration	Final Concentration	Volume (µl) per reaction
Platinum q-PCR mix	2X	1X	12.5
BRAF ARMS primer	100µM	2µM	0.5
BRAF common primer	100µM	2µM	0.5
BRAF probe	100µM	0.5µM	0.125
Forward control primer	20µM	0.1µM	0.125
Reverse control primer	20µM	0.1µM	0.05
Bovine Serum Albumin	10mg/ml	0.5mg/ml	2
Water	-	-	4.075
Total volume	-	-	20

Table 6. Cycling conditions for BRAF ARMS assay

Denaturation	Cycles	PCR: denaturation and annealing			Cycles
95°C, 10 min	1	94°C, 45 sec	60°C, 1 min	72°C, 45 sec	40

Data interpretation and assay characteristics Data was analyzed with MX Pro software version 4.1 (Agilent Technologies, Berkshire, UK). No logarithmic increase in fluorescent signals should be seen in the NTC for results to be valid. Thresholds were then set manually above the NTC. If there was only control signal in the replicate tested, it was classed as 'mutation negative'. If there were both control and diagnostic signals in the reaction, the delta Cq (ΔCq) value was calculated by subtraction of the control Cq value from the mutation Cq value. Presence or absence of the mutation in a sample was determined by the values of ΔCq in its replicates (described below). LOD of the assay was 2% (20 mutant copies in 1000 wild type copies) when it was assessed by using cell line admixtures (made by HT 29 cell line

DNA and normal genomic DNA). Of the 2% admixtures tested in duplicate in 7 different PCR runs, *BRAF* p.V600E mutation was detected in all with mean ΔCq value of 9.8 (median 9.8, range 7.4-12). Mean standard deviation of the ΔCq between the replicates tested in the same PCR run was 0.9 (median 0.8, range 0.3-2.2).

Establishing mutation calling criteria in FFPE tumour DNA The performance of the assay on FFPE tumour DNA was assessed by using 72 archival melanoma tumour samples with known *BRAF* p.V600E mutation status established by Sanger sequencing. Based on the results from these samples tested in duplicates, mutation calling criteria specific to FFPE tumour DNA was defined. If there were both control and diagnostic signals in the replicate tested and the ΔCq was ≤ 7.5, it was classed as 'mutation positive'. Replicates with no diagnostic signal or those with a ΔCq value of greater than 7.5 were classed as 'mutation negative'. A sample was classed as **'***BRAF* mutant**'** when both replicates of the sample tested were positive for mutation. The same samples were analysed by a separate analyst and all the mutations were identified correctly using the criteria. The assay performance was also assessed on a separate cohort of 48 archival colorectal tumour samples with known *BRAF* p.V600E mutation status established by Sanger sequencing. The concordance between *BRAF* mutation results by ARMS and that by sequencing was 100% using the mutation calling criteria described above achieving 100% sensitivity and 100% specificity.

2.2.7. *BRAF* mutation testing in tumour samples

After confirmation of the percentage of tumour cells in an H&E stained tumour section by a histo-pathologist, DNA from 8 x 5µm unstained sections of FFPE tumour tissue was extracted using a QIAamp FFPE Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. *BRAF* mutation status was evaluated by

an AstraZeneca appointed central laboratory (Cranford, New Jersey, USA) by ARMS allele specific PCR or Sanger sequencing or by AstraZeneca appointed local laboratories using AstraZeneca agreed methods that included Sanger sequencing, allele specific PCR, pyrosequencing and TaqMan PCR. *BRAF* mutation status was established by using ARMS method (*BRAF* ARMS allele-specific PCR designed by AstraZeneca or Qiagen *BRAF* ARMS assay (Qiagen, Manchester, UK)) in 87% of the cases and by other methods in the remaining 13%.

2.2.8. Statistical methods

All statistical analyses including calculation of 95% confidence intervals were performed by R software (version 2.14.1., Vienna, Austria). Comparison of cfDNA concentrations in paired plasma and serum samples were calculated using Wilcoxon signed rank test. Comparison of sensitivities and specificities of *BRAF* mutation detection from plasma and serum was analyzed by Fisher's Exact Test. Mann-Whitney U test was used to assess whether there was any difference in distribution of cfDNA concentration between patients with *BRAF* mutation in cfDNA and those without. All the P values are two sided and considered significant if <0.05.

2.3. Results

2.3.1. DNA quantification

Serum and plasma cfDNA concentrations were compared using the data from 199 paired samples. Median sample storage time (from blood collection to cfDNA extraction) was 467 days (range 107-690 days) for plasma and 478 days (range 97-704 days) for serum. Median cfDNA concentration for plasma was 13.3ng/ml (range 0-247.2ng/ml, mean 23.1ng/ml) and that for serum was 29.7ng/ml (range 4.4-

462.0ng/ml, mean 48.8ng/ml) (Figure 9). Median concentration of cfDNA was 2.2 times higher in serum compared to plasma (P value < 10^{-6}).

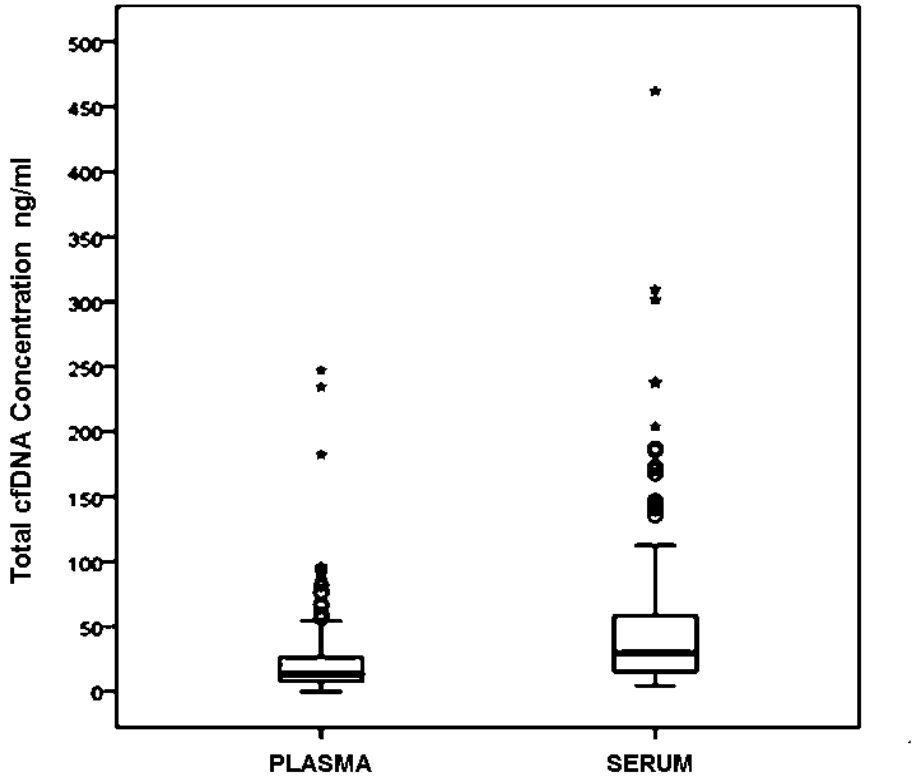

Figure 9. Comparison of total cfDNA concentration (ng/ml) in plasma and serum. *In 199 matched plasma and serum samples analysed, median concentration of cfDNA was 2.2 times higher in serum compared to plasma (29.7 vs. 13.3ng/ml, P value < 10^{-6}).*

2.3.2. *BRAF* c.1799 T>A mutation

Defining optimum cut off for cfDNA BRAF mutation testing using the results from the first stage-training data set. One hundred plasma and serum samples were analysed in this stage. Sensitivities and specificities were calculated by using 5 different ΔCq cut offs for mutation calling in serum and plasma using tumour mutation status as the 'gold standard' comparator (Table 7).

Table 7. Sensitivities and specificities obtained by using different ΔCq cut offs for samples analysed in stage I

ΔCq Cut Offs*	Serum		Plasma	
	Sensitivity	Specificity	Sensitivity	Specificity
≤ 7.5	36%	98%	46%	98%
≤ 8	40%	98%	54%	98%
≤ 9	46%	98%	54%	98%
≤ 10	46%	98%	54%	98%
≤ 11	46%	96%	54%	98%

*To class a sample as "*BRAF* mutant", ΔCq values of both replicates of a sample tested should be ≤ defined cut off

ΔCq cut off of 10 was found to give the best sensitivity and specificity for *BRAF* mutation detection in cfDNA; 27 and 23 mutations were detected in plasma and serum respectively giving sensitivity of *BRAF* mutation detection in plasma of 54% (95% CI, 39-68%) and that in serum 46% (95% CI, 32 -61%). In one patient with a *BRAF* wild type tumour, a *BRAF* mutation was detected in both plasma and serum giving specificity of *BRAF* mutation detection in both matrices 98% (95% CI, 89-99%). By using ΔCq cut off of 7.5, which has been established for mutation calling in FFPE tumour DNA, only 23 and 18 mutations were detected in plasma and serum respectively giving sensitivity of *BRAF* mutation detection in plasma 46% (95% CI, 32-61%) and that in serum 36% (95% CI, 23-51) whilst specificity was 98% (95% CI, 89-99%) for both matrices. Extending the cut off to 11 would not increase the sensitivity in both plasma and serum but would have introduced one more discordant result in serum reducing specificity to 96% (Figure 10). Based on these results, a mutation calling criteria specific to cfDNA where a sample is classed as '*BRAF mutant*' when the ΔCq values of both sample replicates are ≤10 was adopted for validation in the second stage.

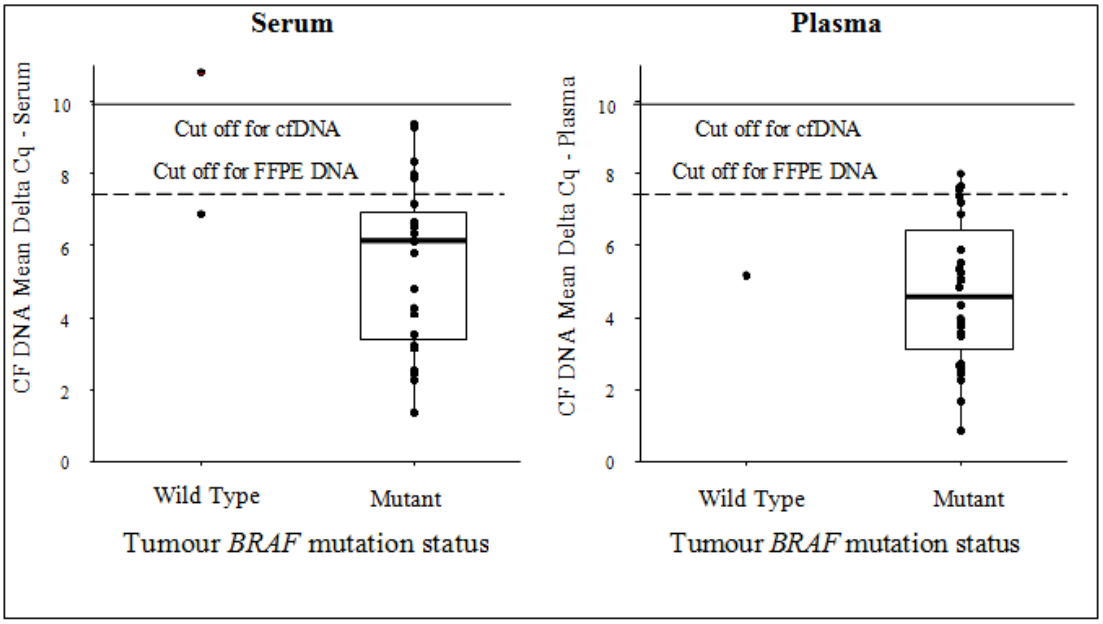

Figure 10. Determining the optimal ΔCq cut off for BRAF mutation calling in cfDNA using training data set. *Two box plots in the figure summarize the distribution of mean ΔCq values for 23 serum and 27 plasma samples where BRAF mutation were detected in cfDNA and the results were concordant with tumour mutation status. Extending the ΔCq cut off from 7.5 (cut off used for FFPE tumour DNA represented by dotted lines in the figure) to cfDNA specific ΔCq cut off of 10 (represented by solid lines in the figure) would improve BRAF mutation pick up rate in both serum and plasma. By using both cut offs, there was only one discordant case where BRAF mutation was detected in both serum and plasma but not in tumour (represented by an isolated dot below the FFPE tumour cut off line). Extending ΔCq cut off to above 10 would have introduced one extra discordant result in serum (represented by an isolated dot above the cfDNA specific cut off line).*

Results from the second stage-validation data set. Of the 108 matched samples analyzed, 74 were from patients with *BRAF* mutant tumours and 34 were from patients with *BRAF* wild type tumours. Using the mutation calling criteria specific to cfDNA, 38 and 31 mutations were detected in plasma and serum respectively, achieving the sensitivity of 51% (95% CI, 39-63%) for plasma and 42% (95% CI, 31–54%) for serum (Table 8). In 2 patients with *BRAF* wild type tumour, *BRAF* mutation was detected in both serum and plasma giving specificity of 94% (95% CI, 80-99%) for both matrices.

When results from both stages were combined (208 cases, of those 198 were matched), 54 mutations were detected in serum and 65 in plasma using the mutation calling criteria specific to cfDNA giving sensitivity of 44% for serum (95% CI, 35-53%) and 52% for plasma (95% CI, 43-61%) (Table 9). The difference in sensitivities of serum and plasma cfDNA assays was not statistically significant (P = 0.2). Specificity for both serum and plasma was 96% (95% CI, 90-99%). Concordance between tumour mutation status and cfDNA mutation status was 64% (95% CI, 58–71%) and 70% (95% CI, 63-76%) in serum and plasma respectively.

Table 8. Sensitivities and specificities obtained by using different ΔCq cut offs for samples analysed in stage II

ΔCq Cut Offs*	Serum		Plasma	
	Sensitivity	Specificity	Sensitivity	Specificity
≤ 7.5	28%	97%	31%	94%
≤ 8	30%	97%	42%	94%
≤ 9	34%	97%	45%	94%
≤ 10	42%	94%	51%	94%
≤ 11	50%	94%	53%	94%

*To class a sample as "*BRAF* mutant", ΔCq values of both replicates of a sample tested should be ≤ defined cut off

Table 9. Sensitivities and specificities obtained by using different ΔCq cut offs for samples analysed in both stages

ΔCq Cut Offs*	Serum		Plasma	
	Sensitivity	Specificity	Sensitivity	Specificity
≤ 7.5	31%	98%	41%	96%
≤ 8	34%	98%	47%	96%
≤ 9	39%	98%	48%	96%
≤ 10	44%	96%	52%	96%
≤ 11	48%	96%	53%	96%

*To class a sample as "*BRAF* mutant", ΔCq values of both replicates of a sample tested should be ≤ defined cut off

2.3.3. Comparison of mutation fraction in serum and plasma

Of the patients' samples analyzed, 53 cases had *BRAF* mutation in both serum and plasma. In these cases, comparison of ΔCq values, which reflect the proportion of tumour derived mutant DNA present in a sample (the higher the ΔCq, the lower the mutation fraction), between serum and plasma was performed. The ΔCq values were significantly higher in serum compared to plasma (P value < 10^{-6}, Wilcoxon signed rank test) indicating serum has lower mutation fraction. Median ΔCq was 6.2 (range 1.4-9.6, mean 5.9) for serum and 5.1 (range -0.3-9.1, mean 4.9) for plasma. The fact that the median ΔCq of plasma was approximately 1 ΔCq lower than that of serum suggests that proportion of tumour derived mutant DNA is approximately twice higher in plasma. On the other hand, for both plasma and serum, there was no significant difference in distribution of cfDNA concentration between patients with cfDNA mutation and those with no mutation (P=0.2 for plasma, P=0.5 for serum, Mann-Whitney U test).

2.4. Discussion

This study demonstrated that mutation detection in cfDNA using real time PCR needs rigorous analytical validation. The optimum sensitivity for *BRAF* c.1799T>A mutation detection in cfDNA by ARMS was achieved by using mutation calling criteria specific to cfDNA without compromising the specificity. More *BRAF* c.1799T>A mutations were detected in plasma than in serum (65 vs. 54) although differences in assay sensitivity and specificity between them were not statistically significant. Nonetheless, these data are consistent with the contention that plasma contains less wild type genomic DNA than serum and as a result contains a higher mutation fraction.

The assay specificity is robust for both matrices at 96%, which might even be higher considering in three patients where *BRAF* c.1799T>A mutation was detected in cfDNA but not in tumour, mutation was found in both serum and plasma suggesting these may be false negative results in tumour rather than false positive results in cfDNA. In 2 of these cases tumour *BRAF* mutation was tested by ARMS using archival primary tumour excision material with a time gap between tumour sampling and plasma/serum sampling of 16 months in one patient and 33 months in the other. In the remaining case, the tumour mutation was tested by Sanger sequencing using archival tumour material from a metastatic lymph node biopsy and the time gap between the biopsy and serum/plasma sampling was approximately 4 months. There are plausible biological and/or logistical explanations for mutation status discordance between tumour and cfDNA in these cases. The blood samples for cfDNA mutation testing were collected sometime after the tumour biopsy and the tumour may have evolved during that time such that mutant clones now predominate as recently noted in colorectal cancer where the emergence of *KRAS* mutations in cfDNA in patients with *KRAS* wild type tumour was seen after treatment with EGFR targeted therapies[95,96]. Another possibility is that the archival tumour biopsy did not fully represent the tumour heterogeneity and mutant cfDNA might also have originated from a metastatic lesion rather than the site selected for the tumour biopsy. Taken together, it could be argued that specificity of *BRAF* mutation testing for serum or plasma was likely to be 100%.

The sensitivity or 'pick up' of *BRAF* c.1799T>A mutation in cfDNA remained relatively limited at 52% in plasma even by using the optimized mutation calling criteria developed for cfDNA. There are two main reasons for this limitation. The first one is that limit of detection of *BRAF* ARMS assay employed in this study was 2%

(20 mutant copies in 1000 wild type copies) and mutation fraction in a given cfDNA sample could be significantly less than 2%[30,132]. The second reason is the limitation imposed by the amount of input cfDNA. Even if mutation fraction is above 2% in a sample tested, the mutation will still not be detected if the absolute mutant DNA copy number is less than 20. In cases where a *BRAF* mutation was detected in tumour but not in cfDNA by *BRAF* ARMS assay, a more sensitive technology may be needed to detect low level mutations. A more sensitive assay could also establish whether the mutation is not detectable in plasma simply because the tumour is not shedding sufficient DNA which would guarantee the presence of at least one mutant molecule in amount of plasma assayed.

Because of the robust specificity of the *BRAF* ARMS assay, despite a mutation pick up frequency of 50%, when a tumour biopsy is unavailable, mutation testing in cfDNA can be implemented as a surrogate for tumour mutation testing. Furthermore, cfDNA mutation testing could potentially be used as an initial screening step for positive selection of patients with *BRAF* mutant tumours for clinical trials. By adopting this approach, approximately 50% of patients with *BRAF* mutant advanced melanoma could be identified within a very short turnaround time. The requirement to determine mutation status in archival FFPE tumour DNA, however, would still remain in cases where *BRAF* mutation was not detected in cfDNA. Adopting a blood testing approach could significantly save resources and improve efficiency of future clinical trials. However, before this method of patient screening can be utilized, it would be necessary to prove that positive patient selection for clinical trials based on mutation testing from cfDNA is not biased. The biological significance of presence of driver oncogene mutations in patients' circulation is currently unknown. As far as *BRAF* mutation is concerned, it is not known whether advanced melanoma patients

with *BRAF* mutation in cfDNA will respond differently to BRAF or MEK inhibition compared to those with no detectable mutation in cfDNA. An answer to this question is needed before cfDNA based mutation testing could be used as a first screening step for positive patient selection.

In summary, results from this study showed that cfDNA based mutation detection by real time PCR needs rigorous validation to achieve its optimum sensitivity. Plasma was shown to have higher mutation fraction than serum and as such plasma should be the clinical matrix of choice for cfDNA mutation assays. Robust specificity of the *BRAF* ARMS assay was again demonstrated in this large scale study analyzed in a blinded fashion. Based on these results, applications of *BRAF* c.1799T>A mutation testing from cfDNA using ARMS should be considered as a surrogate for tumour testing in cases where tumour materials are not available. However, before this method of mutation detection can be used as a first screening step for positively selecting patients with *BRAF* mutant tumours for inclusion in the current trials of MAPK pathway targeted drugs, it would be still necessary to prove that patients with cfDNA mutation has the same chance of responding to these drugs compared to those without cfDNA mutation. The next chapter seeks to address this question.

3. ASSESSMENT OF BRAF MUTATION IN PLASMA DNA AS A PROGNOSTIC FACTOR AND PREDICTIVE BIOMARKER TO MEK INHIBITION IN BRAF MUTANT MELANOMA

3.1. Introduction

Mitogen-activated protein kinase kinase (MEK) belongs to a group of dual specificity kinases that phosphorylate both tyrosine and threonine residues of a protein[179]. MEK is an integral component of RAS/RAF/MEK/ERK signaling cascade, which is up-regulated in many solid tumours[180]. MEK activates, via phosphorylation, extracellular signal regulated kinase (ERK) that is currently the only known substrate of MEK. ERK, on the other hand, has several known substrates and, via multiple effector pathways, is responsible for cellular proliferation and evasion of apoptosis by tumour cells[181]. RAS/RAF/MEK/ERK pathway is usually activated by three main mechanisms; 1) binding of growth factors to their corresponding receptors on cell membrane, 2) amplification or mutations of upstream growth factor receptors, and 3) mutation of signaling proteins. Genes encoding the key proteins of this pathway, *KRAS* and *BRAF,* are known driver oncogenes and play critical role in tumour initiation and proliferation[182]. Although mutation in *MEK* is a rare biological event, MEK is the key mediator of RAS and RAF activation and as such it is an attractive drug target[183].

Several ATP non-competitive small molecule inhibitors of MEK have been developed [184] and anti-proliferative effect of MEK inhibition on cancer cells has been demonstrated in several in-vitro and in vivo studies[185-196]. Studies, however, also showed that single agent treatment with a MEK inhibitor usually produces cytostatic rather than cytotoxic response on cancer cells[189,196] and this cytostatic effect, moreover, was previously observed to be reversible on drug withdrawal[196]. On the

other hand, cancer cell lines harboring activating mutations in *NRAS*, *KRAS* or *BRAF* were found to be more sensitive to MEK inhibition than the wild type cell lines highlighting that *RAS* and *RAF* mutations could potentially be used as predictive biomarkers for MEK inhibitors response[197-200]. It has been shown that inhibition of MEK potentiates the cytotoxic effect of chemotherapeutic agents[196,201].

In approximately 50% of patients with cutaneous melanoma, RAS/RAF/MEK/ERK pathway is activated by acquiring a mutation in *BRAF*, usually a p.V600E (c.1799T>A)[63,64]. Selumetinib (AZD6244, ARRY-142886) is an orally active, ATP non-competitive, selective MEK1/2 inhibitor that has anti-tumour activity in *BRAF* mutant melanoma[196,202,203]. Selumetinib was found to inhibit *BRAF* mutant cell lines viability and xenograft growth both as monotherapy and in combination with chemotherapy[196,204]. In first-in-human trials, maximum tolerated dose (MTD) of selumetinib was 75mg twice daily[205,206]. However, single agent activity of selumetinib in pretreated melanoma population was very modest in a single arm phase II study and responses were seen exclusively in patients with *BRAF* mutant tumours[207]. Based on these findings, a double blind placebo controlled randomized phase II study (NCT00936221) was initiated in 2009 to test the therapeutic efficacy of selumetinib combined with dacarbazine in *BRAF* mutant advanced cutaneous or unknown primary melanoma in the first line setting.

Patient selection for participation in the study NCT00936221 was based on the presence of a *BRAF* c.1799T>A mutation in tumour. However, as discussed earlier in this thesis, patients with *BRAF* mutant cancer can further be stratified according to cfDNA mutation status, those with *BRAF* mutation in cfDNA and those without mutation. Of patients with *BRA*F mutant melanoma measured in tumour, approximately 50% of patients will have a *BRAF* mutation in cfDNA detected by real

time PCR as reported in the previous chapter and in a previous study[136]. Currently, it is not clear whether this stratification will have any predictive value with respect to clinical outcomes achieved with MEK inhibition or provide additional prognostic information in patients with *BRAF* mutant advanced melanoma.

Theoretically, biology and clinical behavior of patients with cfDNA mutations could be different from that of those without mutation in cfDNA and they could respond differently to MEK inhibition with selumetinib. It is possible that presence of oncogenic driver mutation in cfDNA is not just the reflection of tumour burden. A recent study reported that amplification of mutant *BRAF* gene was present in some clones of parental cancer cell lines and one out of 11 *BRAF* mutant colorectal tumours[208]. Moreover, amplification of mutant *BRAF* gene was found to confer acquired resistance to selumetinib in colorectal cancer cell lines harbouring a p.V600E mutation in a separate study[209]. So far no published study has reported that there is a linkage between mutant driver gene amplification and presence of cfDNA mutation and it still remains unclear how common *BRAF* mutant gene amplification as a biological phenomenon is in malignant melanoma. However, if there is any linkage between mutant gene amplification and presence of mutation in cfDNA, presence of a *BRAF* mutation in cfDNA could potentially be a negative predictor of response to MEK inhibition by selumetinib. On the other hand, absence of cfDNA mutation might also be the positive predictor of response. If there is a linkage it can be hypothesized that ORR and clinical outcomes (PFS and OS) will be better in patients who have *BRAF* mutations in tumour but not in plasma compared to those who have mutation in both tumour and plasma. It will be important to prove or refute this hypothesis as this could have an impact on further development of mutation testing from cfDNA as a companion diagnostic. To address this issue, clinical samples

and clinical outcome data from study NCT00936221 were used. The relationship between cfDNA *BRAF* mutation status and clinical outcomes (PFS and OS) regardless of treatment were compared testing cfDNA as a poor prognostic factor. The interaction between cfDNA *BRAF* mutation status and treatment specific efficacy outcomes (i.e. testing cfDNA mutation status as a predictive marker of selumetinib treatment related outcomes) were also examined. This study was submitted to Molecular Cancer Therapeutics for publication as an original research article in February 2014.

3.2. Materials and Methods

3.2.1. Patients and samples

Clinical samples were available from AstraZeneca sponsored study NCT0093622. This was a double blind placebo controlled randomised phase II trial that set out to test the therapeutic efficacy of an ATP non-competitive specific MEK 1/2 inhibitor, selumetinib, in combination with dacarbazine (DTIC), in treatment naive *BRAF* mutant advanced (inoperable Stage III or Stage IV) cutaneous or unknown primary melanoma. The full efficacy results of NCT0093622 have been reported elsewhere[210]. Patients were randomised on a 1:1 basis to receive either selumetinib 75 mg twice daily, or matching placebo, in combination with dacarbazine. Of the 385 screened, 91 patients with *BRAF* mutant tumours were randomised to NCT00936221. The first patient was randomised on 20th July 2009 and the last patient was randomised on 8th April 2010. The data cut off date for the study was 20th November 2011. Forty six patients were assigned to DTIC/placebo arm and 45 patients to DTIC/selumetinib arm. Two patients, one in each arm of the study, died before the first dose of allocated treatment. Forty one out of 44 patients in DTIC/selumetinib arm

and 42 out of 45 patients in DTIC/placebo arm had valid cfDNA *BRAF* mutation results (summarised in Figure 11). Patients with no plasma cfDNA mutation result were removed from further analysis resulting in an exploratory study population of 83 patients. The reasons for removal are 1) no plasma sample (n= 2), DNA extraction failed (n= 3) and unknown mutation result (n= 1).

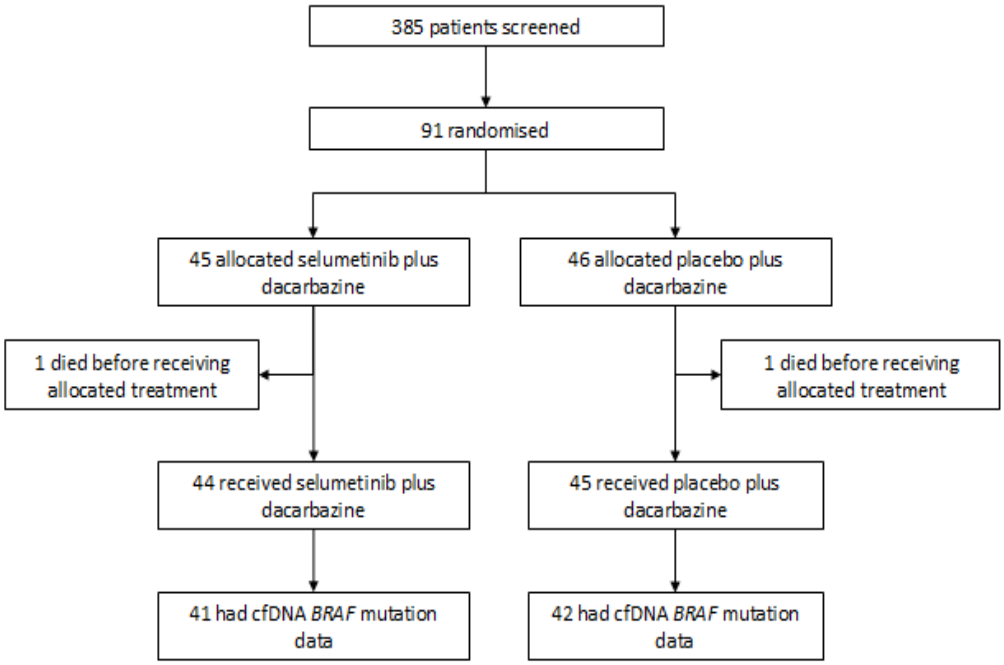

Figure 11. CONSORT diagram showing exploratory study patient population. *cfDNA BRAF mutation results were not available for 6 patients, 3 in each arm of the study.*

3.2.2. Study procedures

DTIC (1000 mg/m^2) was given as intravenous infusion on day 1 of each 21 day cycle up to 8 cycles in the absence of significant toxicity. Selumetinib hydrogen sulphate capsule 75mg twice a day or matched placebo was continued until disease progression or unacceptable toxicities occurred. Tumour assessment was performed every 12 weeks after initiation of the study treatment using a computed tomography (CT). Radiological responses

were reported according to the response evaluation criteria in solid tumour (RECIST) version 1.0. Once patient had been shown to have objective disease progression and withdrawn from study treatment, survival contacts occurred every 8 weeks until the patient's death. After disease progression, patients were allowed to have subsequent therapies according to the treating physicians' discretion but no crossover was allowed.

3.2.3. Tumour *BRAF* mutation testing

As described in the section 2.2.7, after extracting DNA form 8 x 5µm sections of FFPE tumour tissue by QIAamp FFPE Tissue kit (Qiagen, Hilden, Germany), *BRAF* c.1799T>A mutation status in tumour was established by an AstraZeneca appointed central laboratory (Cranford, New Jersey, USA) using Amplification Refractory Mutation Testing System allele specific real time PCR or Sanger sequencing or by AstraZeneca appointed local laboratories using AstraZeneca agreed methods (Sanger sequencing, allele specific PCR, pyrosequencing or TaqMan PCR). This data was made available to me for comparison with cfDNA data.

3.2.4. *BRAF* mutation testing in plasma derived cfDNA

BRAF c.1799T>A mutation was tested using 2ml of plasma collected at baseline i.e. before initiating study treatment. The process of blood collection, preparation of plasma from whole blood and extraction of cfDNA from plasma using QIAamp circulating nucleic acids kit (Qiagen, Hilden, Germany) were described in details in the previous chapter. *BRAF* mutation status in cfDNA was established by using ARMS allelic specific real time PCR (AstraZeneca Pharmaceuticals, Alderley Park,

Cheshire, UK) and validated optimised mutation calling criteria for cfDNA was used for mutation calling as described earlier.

3.2.5. Statistical Analysis

Comparisons of PFS and OS between the patient groups were performed using Cox proportional hazards modelling. PFS is defined as the time from randomization to the first documented radiological progression or death. OS is defined as the time from randomization to death from any cause. Cox models included terms for treatment, factor (cfDNA mutation status) and treatment-by-factor interaction terms as appropriate. All models also include adjustment for baseline covariate terms of WHO performance status, level of LDH and disease stage. Median survival times and associated confidence limits were estimated from the Survival Density Function which was calculated as the Kaplan-Meier product limit estimates for survival probability at each recorded time. Further analyses employing stratified log-rank tests were also undertaken as a sensitivity analysis. An assessment was also made to examine that differences in survivals between groups were not biased by differences in CT scanning frequency using a grouped survival method. The results of the different statistical analyses were found to be consistent. The correlation between ORR (defined as the number (%) of subjects with at either Complete or Partial Response) and cfDNA mutation status was examined by using a logistic regression model that included treatment, factor and treatment-by-factor interaction term in addition to the baseline covariate terms of WHO performance status, level of LDH and disease stage. All statistical analyses including the 95% confidence intervals (CI) were performed using R software (version 3.0.1., Vienna, Austria) and all the P values were two sided and considered significant if <0.05.

3.3. Results

3.3.1. Patient characteristics

Characteristics of the patients included in this exploratory analysis (n=83) are summarised in Table 10. Of 83 patients, 55% were male and 45% female. All were white Caucasian. Median age was 55 years (range, 18-84 years). Majority (96%) of the patients had metastatic disease and 64% had M1c disease. Fifty two percent and 46% of the patients had cfDNA mutation in DTIC/placebo arm and DTIC/selumetinib arm respectively. Patients' characteristics were similar between the two study arms.

Table 10. Characteristics of exploratory study patients

Characteristics	Study Arms		Total
	Placebo + DTIC N (%)	Selumetinib + DTIC N (%)	N (%)
Male	25 (59)	21 (51)	46 (55)
Female	17 (41)	20 (49)	37 (45)
White	42 (100)	41 (100)	83
Age			
Median	52	57	55
Range	24-84	18-79	18-84
Performance Status			
Normal (PS 0)	31 (73)	33 (80)	64 (77)
Restricted (PS 1)	11 (26)	8 (20)	19 (23)
Stage			
Stage III (inoperable)	1 (2)	2 (5)	3 (4)
M1a	6 (14)	5 (12)	11(13)
M1b	9 (21)	7 (17)	16 (19)
M1c	26 (62)	27 (66)	53 (64)
cfDNA BRAF Mutation			
Mutation detected	22 (52)	19 (46)	41 (49)
Mutation not detected	20 (48)	22 (54)	42 (51)

Total	42	41	83

3.3.2. Clinical characteristics of patients with cfDNA *BRAF* mutation

Of 83 patients, 41 (49%) had cfDNA mutation in plasma. cfDNA *BRAF* mutation was detectable in 33%, 45%, 19% and 60% of patients with stage III disease, M1a disease, M1b disease and M1c disease respectively (Table 11). No clear correlation was observed between number of metastatic sites and cfDNA mutation status. However, notably, 65% of patients with hepatic metastases had cfDNA mutation and 72% of patients with high LDH level (>550) had a detectable cfDNA mutation (Table 11). A non-statistically significant gender imbalance that was not marked enough to impact downstream analyses was observed.

Table 11. Clinical characteristics of patients with cfDNA mutation

Characteristics	cfDNA BRAF Mutation Status		Total
	Mutant (%)	Wild Type (%)	
Sex			
Female	16 (43)	21 (57)	37
Male	25 (54)	21 (46)	46
Age			
> median	18	21	
< median	20	21	
Performance status (PS)			
Normal (PS 0)	30 (47)	34 (53)	64
Restricted (PS 1)	11 (58)	8 (42)	19
Stage			
Stage III (inoperable)	1 (33)	2 (67)	3
M1a	5 (45)	6 (55)	11
M1b	3 (19)	13 (81)	16
M1c	32 (60)	21 (40)	53
Metastatic sites			
Lymph node	26 (53)	23 (47)	49
Hepatic	13 (65)	7 (35)	20
Respiratory	19 (46)	22 (54)	41
Skin soft tissue	14 (54)	12 (46)	26
Number of metastatic sites			
0	1 (33)	2 (67)	3
1	9 (36)	16 (64)	25
2	15 (54)	13 (46)	28
≥ 3	12 (52)	11 (48)	23
LDH			
<550	23 (40)	35 (60)	58
>550	18 (72)	7 (28)	25

3.3.3. Correlation between cfDNA *BRAF* mutation status and clinical outcomes

3.3.3.1. Objective response

The objective response rates (ORR= complete responses + partial responses) were 29% in the DTIC/placebo arm and 44% in the DTIC/selumetinib arm. There were two complete responses (CR), one in each arm of the study. Eleven patients (26%) had partial response (PR) in DTIC/placebo arm and 17 (42%) in DTIC/selumetinib arm. Twenty one patients (50%) in DTIC/placebo arm and 13 (32%) in DTIC/selumetinib arm had progressive disease (PD). The stable disease (SD) rates were 21% and 24% in DTIC/placebo arm and DTIC/selumetinib arm respectively. When the patients in the two study arms were stratified further by cfDNA *BRAF* mutation status, for those with cfDNA mutation, the objective response rates (ORR) were remarkably similar between those treated with DTIC/placebo or those treated with DTIC/selumetinib (Table 12, Figure 12). In contrast, for patients with no cfDNA mutation detected, patients treated with DTIC/selumetinib had higher PR rate than patients in DTIC/placebo arm (Table 12). These results showed that higher PR rate observed in selumetinib arm in NCT00936221 was mainly derived from cfDNA wild type subgroup of the selumetinib arm. Moreover, only 9% of patients who had no cfDNA mutation and treated with selumetinib had PD whilst 35% of patients who had no cfDNA mutation and treated in the placebo arm had PD. However, a logistic regression analysis of ORR (CR+PR) did not conclusively show a significant correlation between treatment effect and cfDNA *BRAF* mutation status (P=0.45) and as such data should be interpreted with caution.

Table 12. Objective responses in the two arms of the study with respect to cfDNA mutation status

Type of Response	DTIC + Placebo		DTIC + Selumetinib	
	cfDNA Mutant	cfDNA Wild Type	cfDNA Mutant	cfDNA Wild Type
CR	0 (0%)	1 (5%)	0 (0%)	1 (4%)
PR	5 (23%)	6 (30%)	5 (26%)	12 (54%)
SD	3 (14%)	6 (30%)	3 (16%)	7 (32%)
PD	14 (64%)	7 (35%)	11 (58%)	2 (9%)
Total	22	20	19	22

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; DTIC, dacarbazine.

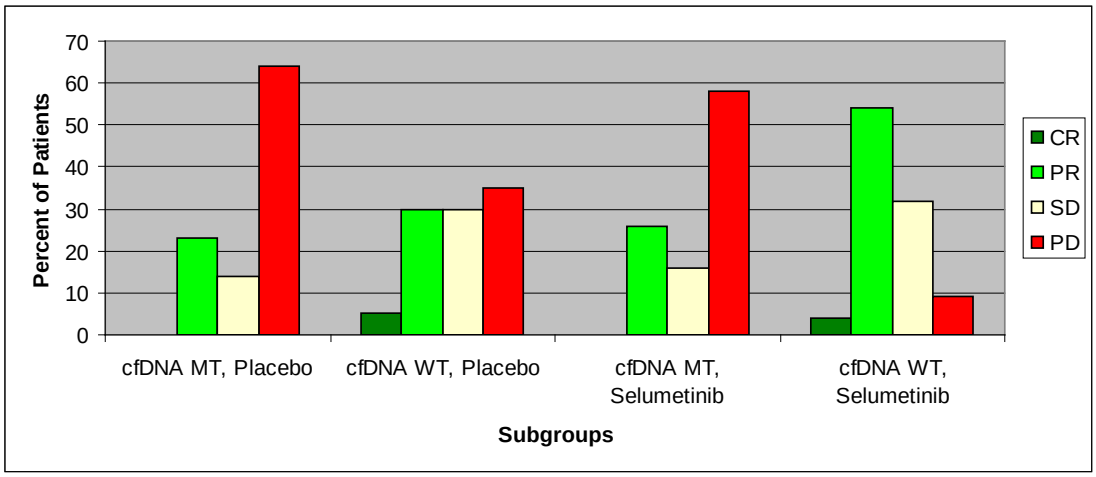

Figure 12. Comparison of objective response rates in subgroups stratified by cfDNA mutation status and treatment received. *Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; MT, mutant; WT, wild type.*

3.3.3.2. Progression free survival

At the time of data analysis, 79 events, 41 in the placebo arm and 38 in the selumetinib arm, had occurred. Median PFS of patients in DTIC/placebo arm was 103 days whilst median PFS of patients who had DTIC/selumetinib was 175 days (Figure 13A). A Cox proportional analysis, adjusting for baseline covariates, suggested that

survival may be prolonged in the DTIC/selumetinib arm (adjusted HR 0.6 (95% CI 0.29-1.08), P=0.09) however the result was not significant at the level used in this exploratory analysis. Median PFS of patients with cfDNA *BRAF* mutation was significantly shorter (87 days) compared to that of those without the mutation (230 days) (adjusted HR 2.05 (95% CI 1.27-3.33), $P<0.01$) (Figure 13B).

When patients in the two study arms were further stratified by the cfDNA mutation status, of patients with no cfDNA mutation, median PFS was longer in patients treated with selumetinib when compared to those treated with placebo (median PFS 246 days (95%CI 180-336) vs. 164 days (95%CI 99-259)). However, this difference of 82 days was not statistically significant by a stratified log rank test (HR 0.76 (95%CI 0.36-1.59), P=0.46). On the other hand, of patients with cfDNA mutation, median PFS of those treated with selumetinib and that of those treated with placebo were approximately the same (88 days (95%CI 81-162) vs. 86 days (95%CI 84-237)) with HR of 0.70 (95%CI 0.33-1.5, $P=0.36$). A Cox proportional hazard model that included treatment-by-factor interaction terms and baseline covariate terms suggested that patients with cfDNA mutation might derive lesser PFS benefit from MEK inhibition with selumetinib than those with no cfDNA mutation (HR 1.6, 95% CI 0.62-4.11; P=0.33) (Figure 14).

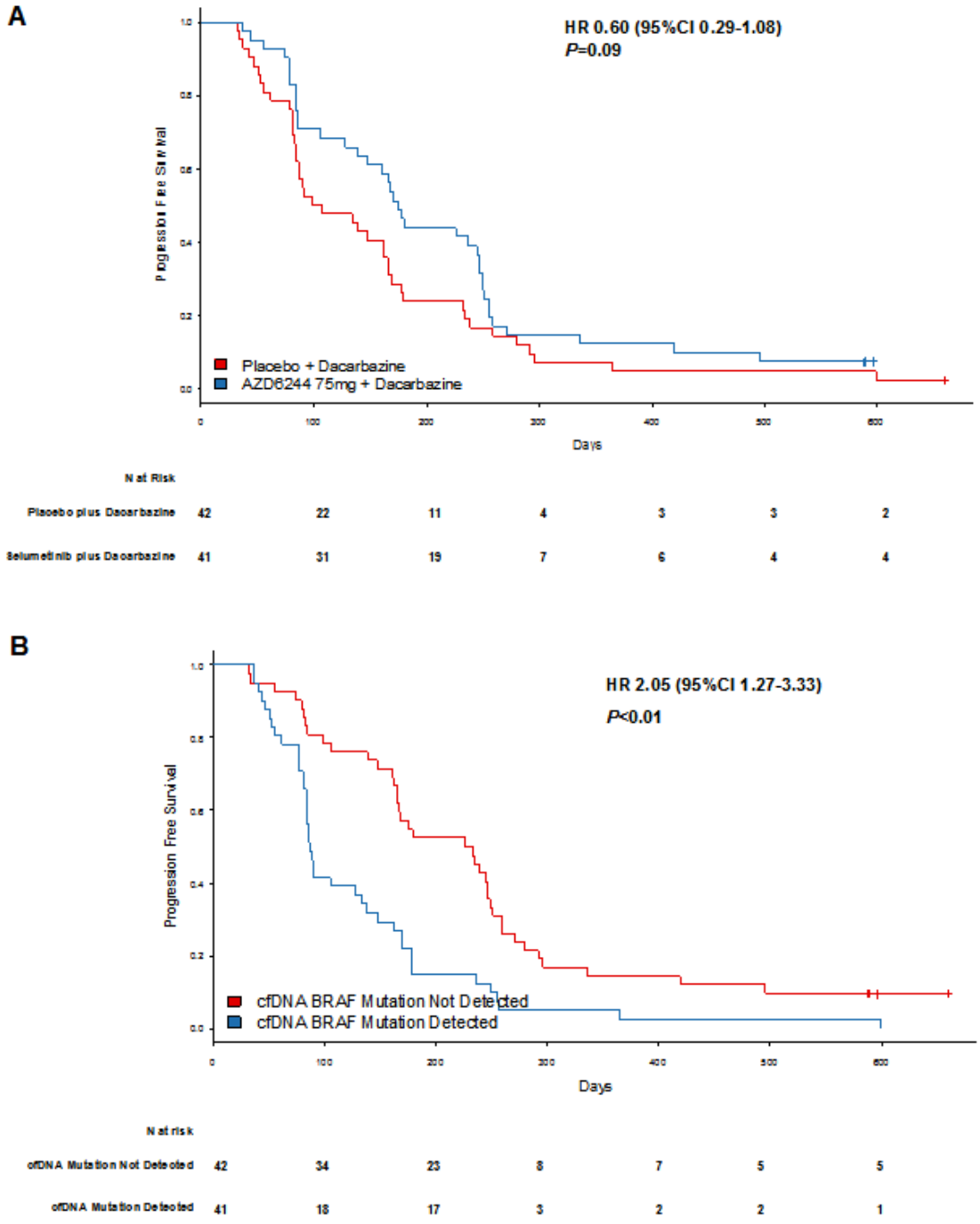

Figure 13. Kaplan-Meier plots of progression free survival: A) comparison of PFS between the two treatment arms; B) comparison of PFS between patients with cfDNA mutation and those without cfDNA mutation. *Hazard ratios, 95%CIs and P-values are derived from an adjusted Cox proportional hazards regression model which includes a cfDNA factor term and baseline covariate terms of WHO performance status, LDH and disease stage.*

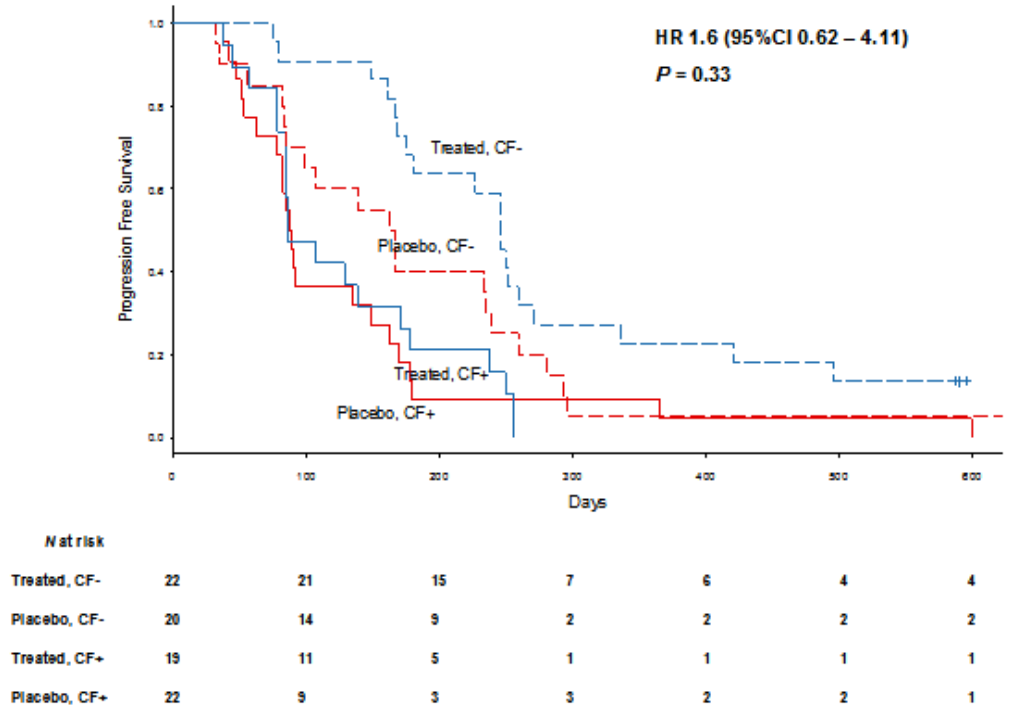

Figure 14. Kaplan-Meier estimates of PFS in subgroups stratified by cfDNA BRAF mutation status and treatments they received. *Abbreviations: Treated CF-, patients with no cfDNA BRAF mutation treated with selumetinib and DTIC; Treated CF+, patients with cfDNA BRAF mutation treated with selumetinib and DTIC; Placebo CF-, patients with no cfDNA BRAF mutation treated with placebo and DTIC; Placebo CF-, patients with cfDNA BRAF mutation treated with placebo and DTIC. *Hazard ratio (HR) is shown here with its 95% confidence interval (CI). The analysis was performed using a Cox proportional hazard model with treatment-by-factor interaction term and baseline covariate terms of WHO performance status, LDH and disease stage.*

3.3.3.3. Overall survival

Of 83 patients included in this exploratory analysis, 61 deaths, 32 in the DTIC/placebo arm and 29 in the DTIC/selumetinib arm, had occurred at the time of data analysis (November 2011). Median follow up time was 12.3 months. Median OS of patients who were treated in the DTIC/placebo arm was 302 days and that of the patients in the DTIC/selumetinib arm was 414 days (Figure 15A). However, when the whole hazard curve was analysed by a Cox proportional hazard model analysis, with adjustment for baseline covariates, no significant treatment effect was present (adjusted HR 0.96 (95%CI 0.57-1.6), P=0.9). Median OS of patients with cfDNA *BRAF* mutations was 282 days and that of those without mutations was 451 days (Figure 15B). Estimates of the HR from a Cox proportional hazard model suggested that presence of a cfDNA mutation might be an independent poor prognostic indicator (adjusted HR 1.5 (95%CI 0.88-2.6), P=0.1), although the P-value was below that considered to be significant in this exploratory analysis. Assessment of the interaction between treatment and cfDNA mutation status was not performed for OS as it was considered that there were not enough events to give robust results.

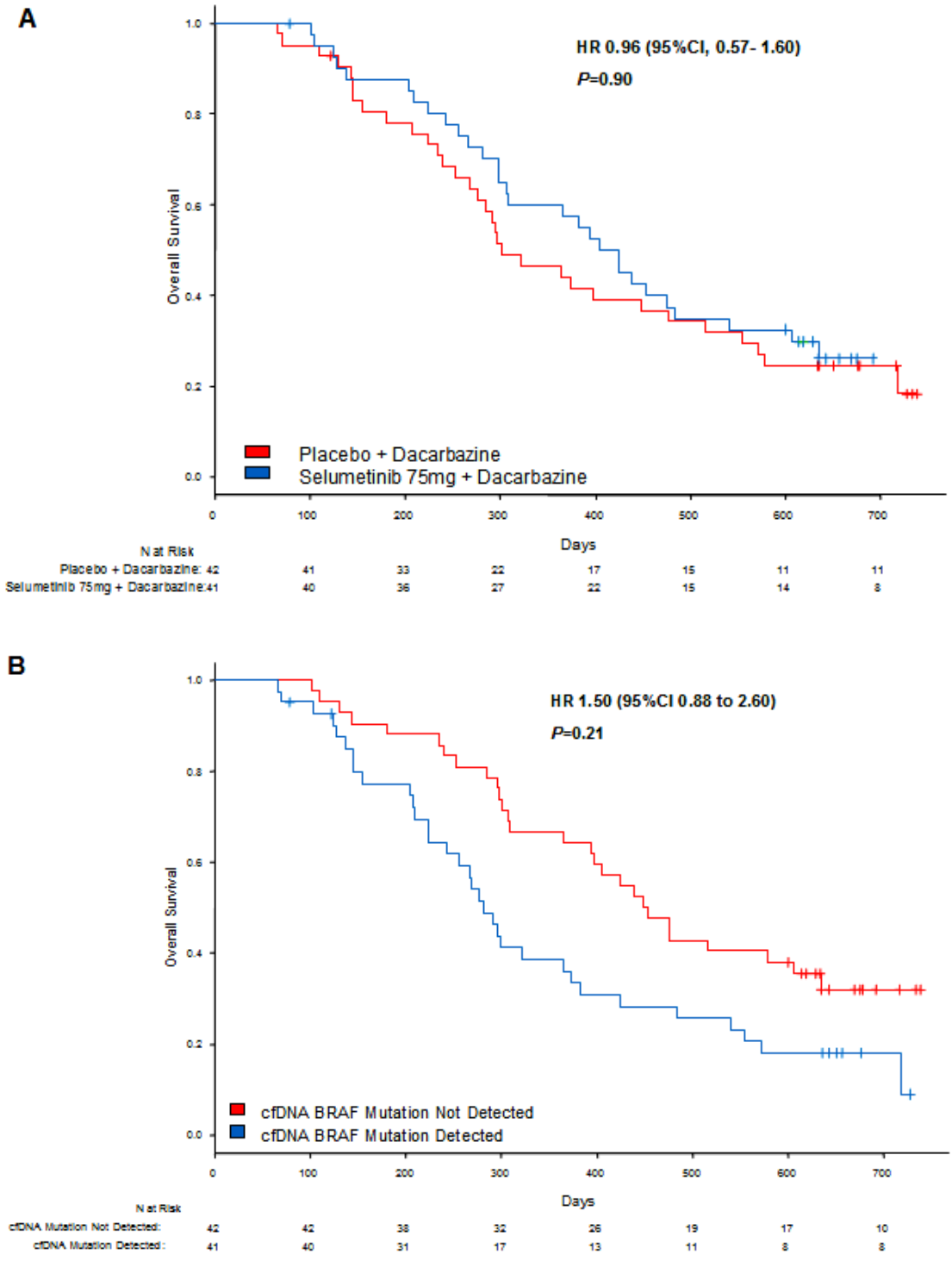

Figure 15. Kaplan-Meier estimates of OS: A) comparison of OS between the two treatment arms; B) comparison of OS between patients with cfDNA mutation and those without cfDNA mutation. *Hazard ratios, 95%CIs and P-values are derived from an adjusted Cox proportional hazards regression model which includes a treatment term and baseline covariate terms of WHO performance status, LDH and disease stage.*

3.4. Discussion

In patients with advanced (inoperable stage III and stage IV) melanoma harbouring a *BRAF* mutation, the biological significance of detectable *BRAF* mutation in plasma with respect to patients' responses to MEK inhibition is currently unclear. Here, *BRAF* mutation status in cfDNA was explored as a prognostic biomarker and predictive biomarker of response to the MEK inhibitor selumetinib in treatment naive advanced *BRAF* mutant cutaneous or unknown primary melanoma using clinical samples from the NCT00936221 clinical trial.

An acknowledged limitation is the exploratory nature of the retrospective subgroup analysis (n=83) within the randomised phase II trial where not all the patients randomised had cfDNA *BRAF* mutation data. The original study was not designed to conduct subgroup analyses and as a consequence has very limited power to detect treatment subgroup interactions. It is also acknowledged that the limit of detection of the real time allelic specific PCR method employed in this study for *BRAF* mutation detection in cfDNA was 2% and this might have confounded the results by misclassifying patients with <2% mutation level in cfDNA as cfDNA mutation not detected. Nevertheless, results and the trends within the data are intriguing and worthy of follow up study.

Estimates of median OS, calculated as described for PFS above, were shorter in patients with cfDNA mutation (282 days) when compared to patients without the mutation (451 days) although differences in OS between these two groups failed to reach statistical significance (adjusted HR=1.5, 95% CI 0.88-2.6: P=0.1). There was statistical evidence, from the analysis of PFS of patients with cfDNA *BRAF* mutation to indicate that survival may also be reduced in this patient population compared to

those without the mutation (adjusted HR 2.05 (95% CI 1.27-3.33), P<0.01) (Figure 13B) suggesting a prognostic relevance of the *BRAF* mutation detection in cfDNA.

In subgroup analysis of PFS where patients in the two study arms were further stratified by cfDNA *BRAF* mutation status, patients with no cfDNA mutation who were treated with DTIC/selumetinib had the best PFS (Figure 14) and highest ORR (Table 12). Even though there were no statistically significant differences in PFS between the subgroups and a logistic regression analysis of ORR failed to show a significant association between treatment effect and cfDNA mutation status, these results from multiple endpoints still suggest that, of patients with *BRAF* mutant tumours, those with no cfDNA mutation might derive improved clinical benefit from MEK inhibition with selumetinib and this hypothesis is now needed to be tested prospectively in a larger study.

It was found that cfDNA *BRAF* mutation was most prevalent in patients with liver metastases (65%) and a high (>550) LDH level (72%). These novel findings suggest that patients with highly proliferative or aggressive tumour shed more DNA into peripheral circulation and might explain why patients with cfDNA mutation are likely to have poorer prognosis than those without the mutation. As the study population was relatively small no formal statistical analyses was performed to test the correlation between patients' clinical characteristics and cfDNA mutation status and larger studies are now warranted.

Overall, the results suggest that the biological behaviour of patients with and without *BRAF* mutation detectable in cfDNA may be distinct with differing prognosis and might derive different magnitude of clinical benefits from MEK inhibition with selumetinib. It is plausible that those patients with plasma detected *BRAF* mutation may have a higher disease burden and/or aggressive biology and thus although all

have *BRAF* mutant tumours, those without plasma detected *BRAF* mutations tend to have a better prognosis and in this study a more durable response to MEK inhibition. Larger prospective studies are needed to formally test this hypothesis and to examine the predictive value of cfDNA *BRAF* mutation status to other MAPK pathway therapeutic agents, so that the biological significance of cfDNA *BRAF* mutation detection can be better understood and exploited within specific clinical contexts. It can be postulated that for patients with *BRAF* mutant tumours, a decrease in *BRAF* mutation load in plasma will indicate benefit, whilst if absent at baseline, appearance of plasma *BRAF* mutation over time on treatment may serve an 'early warning' that salvage therapy will be required. This may inform the decision on when to switch treatment. Given the slow rate of response to immunotherapy in patients with advanced melanoma, and the requirement for patients to be exposed to treatment for 9-12 weeks minimum to have a reasonable chance of benefit, this lead time advantage may be very relevant clinically.

4. TARGETED NEXT GENERATION SEQUENCING OF PLASMA DERIVED CIRCULATING FREE DNA IN PATIENTS WITH ADVANCED COLORECTAL CANCER: A PROOF OF CONCEPT STUDY

4.1. Introduction

In patients with advanced CRC, using combination chemotherapy in a serial fashion improves median survival from approximately 12 months with 5-fluouracil (5-FU)/folinic acid (FA) chemotherapy to approximately 21 months with combination regimens 5-FU/FA/Irinotecan (FOLFIRI) and 5-FU/FA/Oxaliplatin (FOLFOX)[211-214]. Addition of biological agents to 5-FU based combination chemotherapy (bevacizumab in non-selected patient populations and cetuximab and panitumumab in *KRAS* wild type patients), has also been shown to improve clinical outcomes including survival[75,215,216]. However, despite significant improvements in median survival, 5 year survival of patients with advanced (unresectable) colorectal cancer remains poor at approximately 12%[217]. A major stumbling block for achieving long term survival in this group of patients remains primary and secondary drug resistance.

In colorectal cancer, it has been shown that tumour-derived plasma DNA can be used to study mechanisms of resistance to EGFR monoclonal antibodies[95,96,117]. Moreover, in a proof-of-concept study, it has been demonstrated that next generation sequencing (NGS) of plasma DNA can reveal clonal evolution of cancer cells that acquired resistance to anti-cancer therapy in patients with ovarian and breast cancers[173]. However, there are several technical challenges for sequencing cfDNA with NGS. First and foremost, the quantity of cfDNA per ml of plasma is within nano-gram range and this imposes limitation on input DNA for NGS. Moreover, cfDNA is highly fragmented and some DNA molecules in a given sample might not be amplifiable to produce good quality libraries. Initial multiplex amplification of cfDNA needed for

producing sequencing libraries could also result in significant loss of tumour derived mutant alleles in cfDNA samples where mutation fractions are very low. Furthermore, sequencing error rate of NGS platforms limit the sensitivity of mutation detection from cfDNA. For example, if the sequencing error rate of a platform is 5%, it is not technically possible to detect mutation with <5% allelic frequency with high accuracy even if sequencing was performed with enough depth to detect mutant alleles with allelic frequencies significantly less than 5%. At present sequencing costs also remain quite high prohibiting large scale cfDNA sequencing studies.

In my project, collaboration with Professor Marilyn Li at Baylor College of Medicine was formed to conduct a proof of concept study for targeted NGS of plasma derived cfDNA. Ion-Torrent PGM™ platform was chosen to perform targeted NGS of cfDNA and FFPE tumour DNA in 8 patients with advanced colorectal cancer (how these samples were selected for sequencing is described in detail below). This method had been validated in the Cancer Genetics Laboratory (Baylor College of Medicine) to detect mutations with allelic frequency of 5% or above using FFPE tumour derived DNA[218]. As FFPE tumour DNA is highly fragmented, it was reasoned that this technique could also assess cfDNA successfully. One of the main advantages of this technique is that only 10ng of input DNA is needed for library preparation. This amount of DNA was feasible to obtain from almost all cases of patients with advanced cancer provided that sufficient blood volume is collected for plasma preparation. Moreover, multiplex amplification of template DNA for detecting all the mutations could be performed in a single tube PCR reaction. This means that it is not necessary to split the samples repeatedly to perform several multiplex amplifications to produce sequencing libraries and importantly also reduces the risk of contamination. The main limitations, however, were that only 739 targeted mutations

within 46 selected cancer candidate genes with >5% allelic frequency will be detected by this method limiting the assay sensitivity. As this is also amplicon based sequencing, novel somatic mutations acquired outside the amplicons of genes tested will not be detected by this technique. The major advantage, however, is the capacity to analyse 739 mutations from 10ng of input DNA within a single sequencing run. This collaboration between CRUK Manchester Institute and Baylor College of Medicine was submitted to Cancer Discovery as a research brief in February 2014.

4.2. Patients and samples

Patients from The Christie, Manchester, UK were recruited to a translational research study (protocol reference 10_CLPHA_62) that was approved by a local research ethics committee (reference no. 10/H1003/88). The study was conducted according to the GCP guidelines and the declaration of Helsinki. Between February 2011 and April 2012, archival tumour samples and serial plasma samples were collected from 51 patients who were undergoing a course of palliative 1st line, 2nd line or 3rd line chemotherapy for advanced colorectal cancer. The plasma samples were collected at baseline (before commencing chemotherapy), 6 weeks and 12 weeks after starting treatment and at disease progression. Of those 51 patients, matched tumour and baseline plasma samples were available in 34 patients. *KRAS* mutations in tumour and baseline plasma samples were examined initially in those 34 patients by using ARMS-Scorpion real time PCR. Depending on the *KRAS* mutation status of the tumour and plasma and the availability of serial plasma samples, 8 cases were selected for targeted next generation sequencing. Eight tumour and 16 plasma DNA samples from the selected patients were sequenced using Ion Torrent PGM^{TM} platform. Germ line DNA was also sequenced in all 8 patients. Sequencing was successful in all tumour

and germ line DNA samples but in 4 patients, plasma DNA sequencing failed at the library preparation step. As such, complete data from 4 patients, whose case histories and timing of tumour and plasma samplings were summarised in Figure 16, are described here.

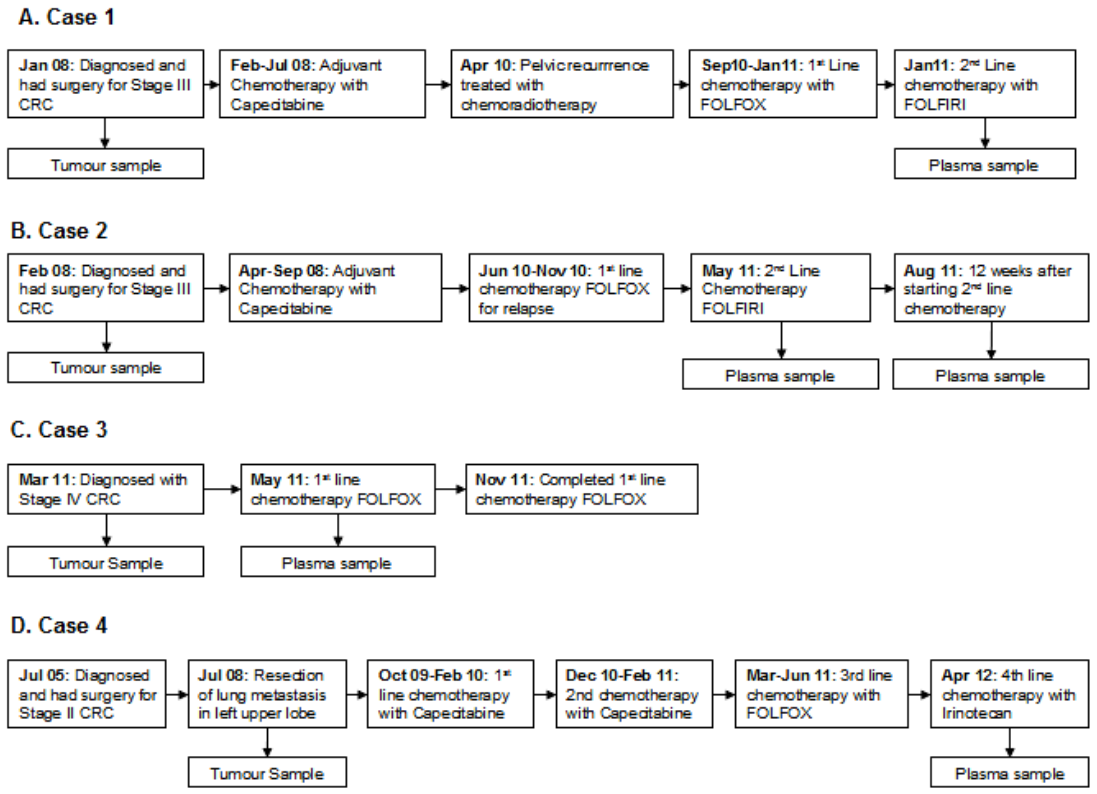

Figure 16 (A-D). Treatment history and timing of tumour and plasma samplings in the 4 cases studied

4.3. Methods

4.3.1. Blood collection and preparation of plasma

For each patient 20ml of blood was collected at each time point for plasma preparation using 2 x 10ml Becton-Dickinson Vacutainer Collection Tube containing EDTA. Within 30 minutes of collection, the tubes were centrifuged at 2000g x 10 minutes and the supernatant plasma from both tubes was transferred to a 15ml falcon

tube using a pipette. The falcon tube was subsequently centrifuged at 2000g x 10 minutes to remove cell debris. The resultant supernatant plasma was transferred into 4ml cryovials and stored at -80°C immediately afterwards until further use. Separately 4ml of whole blood was collected into a Becton-Dickinson Vacutainer Collection Tube containing EDTA for preparation of genomic DNA from peripheral blood mononuclear cells (PBMCs).

4.3.2. DNA extraction and quantification

DNA was extracted from 4mls of plasma using QIAamp circulating nucleic acids (Cat. no.551144, Qiagen, Hilden, Germany) as described in details in section 2.2.4 of this thesis. Reaction volumes were adjusted accordingly to the manufacturer's instructions to extract cfDNA from 4mls of plasma. After tumour cell content was scored by a qualified pathologist (Dr Richard Byers, University of Manchester) using a H&E slide, genomic DNA was extracted from 8 x 5 µm sections of FFPE tumour tissues using Qiagen FFPE Tissue Kit (Cat. no. 6404, Qiagen, Hilden, Germany). Genomic DNA from PBMCs was extracted by using Wizard® genomic DNA purification kit (Cat. no. A1120, Promega, Madison, WI, USA). All the experiments were performed according to the manufacturers' instructions. DNA was quantified by using RNase P PCR (Cat. no. 4316831, Life technologies, Foster City, CA, USA) as described in detail in section 2.2.5.

4.3.3. Next generation sequencing of plasma, tumour and germ line DNA

DNA sequencing was performed using IonTorrent PGM™ platform and Ampliseq™ cancer panel (Life Technologies, Calsabad, CA, USA) that can detect 739 targeted mutations in 604 loci of 46 cancer candidate genes (the details of the panel can be

found in Appendix 1 or on https://www.ampliseq.com/browse.action with free registration) according to manufacturer's instructions by Cancer Genetics Laboratory, Baylor College of Medicine, Houston, Texas, USA. Detailed methods are described as follows.

Library construction The library construction was performed using the Ion AmpliSeq™ Kit 2.0 and the Ion Ampliseq™ Cancer Panel (Life Technologies, Carlsbad, CA) which contains 190 primer pairs and covers 739 targeted mutations. 10ng of plasma DNA was used as template to perform multiplex PCR for preparation of amplicon library from targeted regions of 46 genes, including *ABL1, AKT1, ALK , APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNAS, HNF1A, HRAS, IDH1, JAK2, JAK3, KDR, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC,STK11, TP53, VHL*. The partial digestion of the primer sequences with FuPa reagent, the ligation of Ion AmpliSeq™ Adapters or Ion Xpress™ Barcode Adapters onto the amplicons, the library purification with Agencourt® AMPure® XP Reagent (Beckman Coulter, Brea, CA) and re-amplification were all performed according to the manufacturer's user guide (Life Technologies, Carlsbad, CA).The amplified library was quantified using Agilent® 2100 Bioanalyzer instrument with the Agilent®High Sensitivity DNA Kit (Agilent Technologies Inc, Santa Clara, CA).

Template preparation and sequencing Sequencing template preparation was performed using the Ion One Touch™ 200 Template Kit and Ion One Touch ™ System (Life Technologies) following the manufacturer's guidelines. The library stocks were pooled (if barcoded) and diluted to further generate a working library concentration of 24-32 pM with low TE solution. The template Ion Spheres™

Particles (ISPs) were enriched with the automated Ion One Touch™ ES instrument. Quality and quantity of the enriched ISPs were assessed using the Guava® easyCyte™ 5 Flow Cytometer (Millipore, Billerica, Massachusetts). Sequencing of the templated ISPs was carried out on the Ion PGM™ system using the Ion PGM™ 200 Sequencing Kit (Life Technologies, Carlsbad, CA) following the manufacturer's manual.

Data Analysis NGS Sequencing results were analyzed using VariantCaller and CoverageAnalysis plugins in the Torrent Suite software V2.0.1 or later versions (Life Technologies, Carlsbad, CA) with hg19 reference genome compared to targeted region BED files and "Hotspot" BED files. All variant calls provided by VariantCaller were reviewed using Integrative Genomics Viewer (IGV) 2.2 to avoid strand biases and sequencing errors. Only variants with allelic frequency of >5% were reported to avoid false positives.

4.3.4. *KRAS* mutation testing in FFPE tumour DNA and plasma DNA

Six codon 12 (c.35G>C, c.35G>A, c.34G>C, c.34G>T, c.34G>A, c.35G>T) and one codon 13 (c.38G>A) *KRAS* mutations were detected by Therascreen® KRAS RGQ PCR Kit (Cat. no. 870011, Qiagen, Manchester, UK) in both tumour and plasma DNA using Rotor-Gene Q 5plex HRM System (Cat. no. 9001650, Qiagen, Hilden, Germany) according to manufacturer's instructions.

4.4. Results

4.4.1. Detection of mutations in plasma is platform dependent

For case 1, archival tumour was collected in January 2008 when the patient was diagnosed with stage III rectal cancer. Tumour cell content of the biopsy estimated by

a qualified pathologist was 50%. Tumour sequencing by IonTorrent PGM™ revealed a *KRAS* c.35G>T mutation and a *TP53* c.524G>A mutation with allelic frequencies of 22% and 32% respectively (Table 13). Using IonTorrent PGM™ neither mutations were detected in plasma collected approximately 41 months after tumour biopsy although the *KRAS* mutation was detected in the same plasma sample using real time PCR (Table 14) indicating that the level of tumour derived *KRAS* mutation in the plasma sample dropped below the detection limit of the IonTorrent PGM™ (set at 5%). In contrast, in case 4 where *KRAS* and *TP53* mutations were detected in tumours, both were detected in plasma by IonTorrent PGM™ sequencing as allelic frequencies were higher in plasma (32% and 16% for *KRAS* and *TP53* mutation respectively) (Table 13).

Table 13. Mutations detected in tumour, plasma and germ line DNA samples sequenced by IonTorrent PGM™*

Case No.	Mutations Detected (AF)				Cosmic ID
	Tumour	**1st Plasma**	**2nd Plasma**	**Germ Line**	
1	*KRAS* c.35G>T (22%)	ND	NA	ND	520
	TP53 c.524G>A (32%)	ND	NA	ND	10648
2	*KRAS* c.35G>A (33%)	ND	NA	ND	521
	TP53 c.488A>G (26%)	ND	NA	ND	10808
	SMAD4 c.1067C>G (33%)	ND	NA	ND	De novo
	ND	*FBXW7* c.1394G>A (69%)	*FBXW7* c.1394G>A (52%)	ND	22965
	ND	*TP53* c.524G>A (55%)	*TP53* c.524G>A (52%)	ND	10648
	ND	ND	*ATM* c.9137G>C (6%)	ND	De novo
3	*MET* c.3029C>T (50%)	*MET* c.3029C>T (41%)	NA	*MET* c.3029C>T (48%)	707
	BRAF c.1799T>A (11%)	ND	NA	ND	476
	ND	*TP53* c.742C>T (68%)	NA	ND	10656
	KRAS c.34G>T (56%)	*KRAS* c.34G>T (32%)	NA	ND	516
4	*TP53* c.404G>A (63%)	*TP53* c.404G>A (16%)	NA	ND	10801

Abbreviations: AF, allelic frequency; ND, not detected; NA, not available. * Primary sequence data of the tumour and plasma DNA samples analysed can be found in Appendix 2

Table 14. Tumour and plasma mutations detected by Therascreen® KRAS RGQ PCR

Case No.	*KRAS* Mutations		Cosmic ID
	Tumour	1st Plasma	
1	c.35G>T	c.35G>T	520
2	c.35G>A	ND	521
3	ND	ND	-
4	c.34G>T	c.34G>T	516

Abbreviation: ND, not detected

4.4.2. Matched tumour and plasma DNA sequencing provides a more representative picture of total tumour mutational burden and heterogeneity

In Case 3 a plasma sample was collected within 2 months of colonic biopsy with a pathological tumour cell content of 20%. IonTorrent PGM™ analysis of the tumour revealed a somatic *BRAF* (c.1799T>A) mutation and a germline *MET* (c.3029C>T) mutation (Table 13). Maldi-Tof mass spectrometry (Sequenom®) mutation profiling also detected the same two mutations in the same tumour sample (courtesy of Professor Bill Newman, The Manchester Centre for Genomic Medicine). By plasma IonTorrent PGM™ sequencing the *BRAF* mutation was not detected but a novel *TP53* mutation with an allelic frequency of 68% (Table 13) was detected. This indicates that the *TP53* mutant clone present in the majority of cfDNA was missed during biopsy and that matched tumour and plasma sequencing provides a more representative profile of the entire tumour mutational burden.

4.4.3. Emergence of a *FBXW7* mutation in plasma following chemotherapy

For Case 2 the tumour sample was collected when the patient underwent surgery for stage III colorectal cancer in February 2008. The patient was diagnosed with a disease relapse in June 2010 and was treated with 1st line chemotherapy FOLFOX. At

progression in May 2011, a plasma sample was collected before commencing second line chemotherapy with FOLFIRI and a further plasma sample was collected 12 weeks after commencement of second line chemotherapy. IonTorrent PGM™ Sequencing of the primary archival tumour (pathological tumour cell content 40%) revealed a *KRAS* c.35G>A, mutation, a *TP53* c.488A>G mutation and a *SMAD4* c.1067C>G mutation with AFs of 33%, 26% and 33% respectively (*KRAS* mutation was also confirmed by Therascreen® KRAS RGQ PCR (Table 14) and Sequenom® analysis) (Table 13). IonTorrent PGM™ sequencing of the 1st plasma sample (collected approximately 39 months after tumour sampling) failed to detect any of the tumour mutations but identified two new mutations one in *FBXW7* (c.1394G>A) and one in *TP53* (c.524G>A) with AFs of 69% and 55% respectively. IonTorrent PGM™ sequencing of second plasma samples (collected 12 weeks after 1st plasma sample and starting 2nd line chemotherapy FOLFIRI) again showed the continued presence of these two new mutations in plasma at high allelic frequency (52% for both mutations) in the absence of other detectable mutations raising the possibility that the cells harbouring these mutations were selected due to conferred chemotherapy resistance. Furthermore, sequencing of the latter plasma sample also revealed a de novo *ATM* mutation (c.9137G>C) at low AFs of 6% (Table 13) indicating the potential emergence of a further subclone. These results were summarised in the Figure 17.

Primary sequence data of the tumour and plasma DNA samples analysed can be found in Appendix 2.

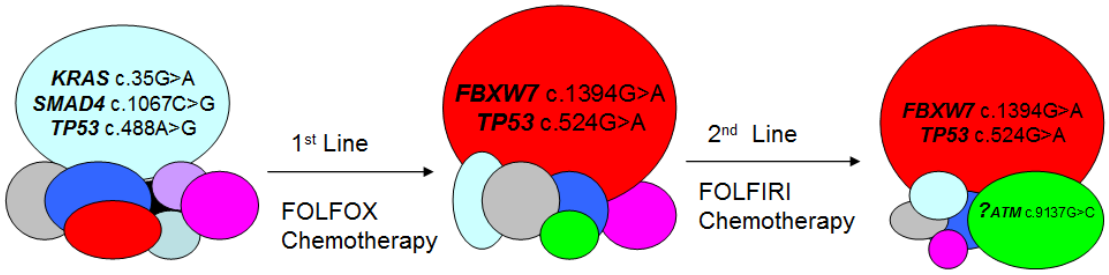

Figure 17. Clonal evolution of cancer cells after exposure to chemotherapy in Case 2. *It is plausible that the cancer subclone harbouring FBXW7 c.1394G>A and TP53 c.524G>A (red bubble) exited as a minor clone in the primary tumour and was selected for chemotherapy resistance after exposure to 1st line chemotherapy FOLFOX. Persistence of both mutations in patient's plasma 12 weeks after second line chemotherapy FOLFIRI suggests that this clone is resistant to 5-Fu based combination chemotherapy. It is possible that a separate minor clone harbouring ATM c.9137G>C (green bubble) is also selected for chemotherapy resistance although data is very preliminary.*

4.5. Discussion

This pilot study reports for first time the emergence of *FBXW7* mutant cells following 5-Fluouracil based chemotherapy in advanced CRC. F-Box and WD repeat domain containing 7 gene (*FBXW7*) encodes a member of the F-Box protein family that constitutes one of the four subunits of an ubiquitin protein ligase complex SCF (SKP1-cullin-F-box). SCF mediates proteosomal degradation of target proteins by ubiquitination[219]. FBXW7 functions as the substrate recognition subunit of a SCF and was found to be a TP53-dependent haplosinufficient tumour suppressor that exerts its function by promoting the degradation of several oncoproteins such as MYC, cyclin E, Notch and JUN [220,221]. FBXW7 was also shown to play critical role, upstream of MCL1, in the regulation of apoptosis[222]. Loss of FBXW7 function through mutation was found in 8% of patients with colorectal cancer and at variable frequencies in multiple other cancer types. *FBXW7* mutations are relatively common in cancers of endometrium (13%), cervix (7%), bladder (7%), stomach (5%), ovary (3%) and lung

(3%)[223]. However, unlike other tumour supressors, loss of FBXW7 function is mainly mediated by acquiring somatic heterozygous missense mutations rather than following the classical pattern of loss of function through mutations in both alleles[221,224,225]. Although a wide spectrum of mutations, 94% of them single nucleotide changes and 6% indels, are seen involving various regions of coding exons of *FBXW7,* hot spot missense mutations that account for approximately 43% of the mutations are found in codons 465 and 479 encoding amino acid arginine.

Arginine[465 and 479] resides in β propeller structure formed by FBXW7's WD40 repeat domain that plays critical role in substrate recognition by the protein[221]. Results from previous in-vitro and in-vivo studies have shown that *FBXW7* heterozygous mutations have 'dominant negative' effect on normal allele by forming heterodimers and disrupt the function of the normal counterpart[221,225,226]. The *FBXW7* mutation observed in this study in plasma of a patient with advanced CRC after exposure to FOLFOX combination chemotherapy was a non-synonymous c.1394G>A mutation replacing codon 465 arginine with histidine (p.R465H). As mentioned earlier, this is one of the codon 465 arginine hot spot mutations that disrupt the substrate recognition of FBXW7. The functional consequence of this failure of substrate recognition will be non-degradation of substrate oncoproteins such as cyclin E1, MYC and Notch resulting in increased intracellular concentration and deregulation of these oncoproteins[227,228]. However, although evidence is emerging that *FBXW7* mutant cells are resistant to various chemotherapeutic and targeted anticancer agents such as paclitaxel, vincristine, Bcl-2 inhibitor ABT737, gamma secretase inhibitors and sunitinib[229-231], further mechanistic studies are still needed to elucidate drug resistance mechanism mediated by *FBXW7* mutant cells within specific contexts to link

functional consequences of loss of FBXW7 with specific anticancer treatment resistance including resistance to 5-Fu based combination chemotherapy.

Our early findings indicate that *FBXW7* may be an important regulator of resistance to 5-FU. If true, this finding has important consequences for both palliative and adjuvant treatment decision making in patients with colorectal cancer. Preclinical studies to test this hypothesis were not initiated as the PhD study time had completed. It is plausible that patients with *FBXW7* mutant tumour are unlikely to respond to combination chemotherapy in advanced settings and similarly, in adjuvant setting, presence of this mutation in a tumour might predict high risk of tumour recurrence as adjuvant chemotherapy might not be effective in these patients. On the contrary, combining FOLFOX or FOLFIRI chemotherapy regimens with agents that *FBXW7* mutant cells are sensitive to such as MS-275, a histone deacetylase inhibitor and CGP-60474, a cyclin dependent kinase inhibitor[231], might improve survival outcomes in patients with *FBXW7* mutant colorectal cancer. These hypotheses should be tested preclinically, and our mutation findings tested prospectively in a larger cohort of colorectal cancer patients. Moreover, these might also be applicable to other tumour types such as endometrial and cervix cancers where *FBXW7* mutations can be found relatively commonly[223].

Results from this study also confirmed the previous finding that clonal evolution of a tumour can be monitored using sequencing of plasma DNA and also showed that more comprehensive profile of a heterogeneous tumour genetic landscape can be obtained by sequencing matched tumour and plasma DNA. On the other hand, it has to be noted that failure rate of non-optical semiconductor based sequencing of plasma DNA using IonTorrent PGM™ platform, in our hands, was high and more stringent sample selection criteria or further method optimization will be necessary to minimize

the failure rate. It was possible that in some plasma samples, cfDNA were so fragmented that they were not amplifiable by PCR resulting in failures to produce good quality AmpliSeq libraries. It is not just that total quantity of DNA in a sample determines the success and failure of library preparation but also the integrity of DNA. Furthermore, variability of coverage depth of reference and variant sequences interrogated in both tumour and plasma DNA samples was very high (details of the primary sequence data of the mutations identified in the tumour and plasma samples analysed are shown in Appendix 2). This highlights the fact that the allelic frequencies of the mutations detected were unlikely to be accurate and mutations with low level of allelic frequencies and those detected with low variance coverage should be interpreted with caution and need confirmatory validation using separate methodologies. Nevertheless, results from this preliminary pilot study showed that profiling of mutations in tumour and plasma DNA by a non-optical semiconductor based sequencing can be used to study tumour biology in cancer patients and this could advance our understanding of drug resistance mechanisms in individual patients.

5. SUMMARY DISCUSSION AND FUTURE DIRECTIONS

Undoubtedly, significant progress has been made in cfDNA research field since the discovery of CNAs in human by Mandel and Metais in 1948. With rapid advances in PCR based technologies and next generation DNA sequencing, it is quite clear that cfDNA mutation testing has enormous potential to be used as surrogate for tumour mutation testing. As a research tool, genomic analysis from cfDNA provides exciting opportunities for monitoring tumour genetic evolution in real time to study novel drug resistance mechanisms. However, cfDNA research suffered a great deal in the past from lack of standardization and robust validation[156] and currently whether cfDNA based assays are fit for diagnostic applications in oncology clinics remains uncertain. Moreover, several questions related to the biological significance of detection of driver oncogene mutations in cfDNA remain unanswered.

My PhD project, at its conception in February 2010, was designed to bring cfDNA mutation analysis one step closer to the clinic. At the time, real time PCR was the most widely used technique for mutation testing from cfDNA (Table 2). However, sensitivity of mutation testing from cfDNA using conventional PCR techniques such as allelic specific PCR is relatively limited because mutant fraction in cfDNA samples could be far below the limit of detection by these techniques[30,132]. Although modified PCR techniques such as mutant enriched PCR, LNA/PNA mediated PCR and COLD PCR were reported to be exquisitely sensitive in the literature, most of the techniques fell short of widespread clinical adoption because of problems in reproducibility [145,146,232]. More recently, two emerging novel technologies, digital PCR and BEAMing, have been successfully used to detect very low level mutations in cfDNA in patients with advanced cancers. However, while both techniques are suited

perfectly well for research use in expert hands, their cost, complexity and accessibility currently limit the routine use of these techniques routinely in clinics.

In contrast to digital PCR and BEAMing, conventional real time PCR techniques such as ARMS, which was employed in this study to detect *BRAF* mutation in cfDNA, are relatively easy to use and cost effective. Furthermore, even though sensitivity is limited when compared to digital PCR and BEAMing, ARMS has very high specificity that can be exploited within a specific clinical context to make cfDNA mutation testing more applicable to the clinic. As argued earlier in this thesis, in a particular tumour type where a mutation is highly prevalent, mutation testing in cfDNA could potentially be used as a surrogate for tumour mutation testing for positive patient selection. A classic example of this is *BRAF* mutation testing in cutaneous melanoma. *BRAF* is mutated in approximately 50% of patients with cutaneous melanoma[63,64] and *BRAF* c.1799T>A transversion can be detected in cfDNA by using ARMS allelic specific real time PCR in approximately 50% of patients with tissue defined *BRAF* mutant tumours[136]. By using cfDNA mutation testing approach, 50% of patients who might be suitable for treatment with BRAF inhibitors or other MEK pathway targeted drugs within or outside clinical trials could be identified by a blood test. One could argue that only in patients with cfDNA mutation negative results, would tumour mutation testing be necessary. Adopting this approach could potentially facilitate patient recruitment into clinical trials as there are inherent problems with mutation testing from archival FFPE tumour DNA as discussed earlier. However, robust assay validation would be necessary before any cfDNA based mutation assay could be used in routine clinical practice. This formed the scientific rationale of the work described in the chapter 2 of this thesis.

By using clinical samples from a randomised phase II study (NCT00936221), analytical validation of *BRAF* c.1799T>A mutation testing by ARMS was performed during the first year of the project to establish the optimum clinical matrix for cfDNA mutation testing and to derive mutation calling criteria specific for cfDNA *BRAF* mutation detection. The results showed that plasma had significantly higher mutation fraction when compared to serum ($P < 10^{-6}$) and more *BRAF* mutations were detected in plasma although the difference between the sensitivity of plasma *BRAF* mutation detection and that of serum *BRAF* mutation detection did not reach statistical significance ($P = 0.2$). Total cfDNA concentration in serum was found to be approximately two times higher than for plasma ($P < 10^{-6}$) whilst mutation fraction was found to be significantly higher in plasma (($P < 10^{-6}$), supporting the hypothesis that serum contains higher background wild type DNA. This is the reason why more mutations were detected in plasma than in serum at all levels of exploratory cut off criteria for mutation calling. As such, it is argued here that plasma should be the clinical matrix of choice for cfDNA mutation testing.

The results also clearly indicated that robust assay validation is necessary to achieve optimum sensitivity of cfDNA mutation testing. Applying the same mutation calling criteria validated for FFPE tumour mutation testing to cfDNA testing would limit the assay sensitivity for cfDNA mutation detection. In this study using validated *BRAF* mutation calling criteria specific for cfDNA was shown to improve the sensitivity of the assay without compromising its specificity. And this method now needs to be validated on an independent sample set for full qualification of the assay for routine clinical use.

On the other hand, having a validated assay should not be the only prerequisite for using a particular assay in a clinical setting. It could be argued that assays also need

clinical qualification to prove they are fit for purpose within the specific clinical contexts for which their utility is proposed. For *BRAF* mutation testing from cfDNA for selecting patients for treatment with BRAF inhibitors or MEK inhibitors, it should be considered that biological and clinical behaviour of patients with mutations in plasma and that of those without could be different and might respond differently to targeted anticancer therapeutics. One intriguing question is why cfDNA mutation is detectable relatively easily in one group of patients but not in others? Is it just simply determined by the anatomy and physiology of tumour vasculature and/or level of intrinsic tumour apoptosis or is it related to other aspects of tumour biology? If cfDNA prevalence is related to biophysical properties, selecting patients for treatment with a particular drug on the basis of cfDNA mutation status could potentially introduce a selection bias that is not fully understood. In other words, there is the danger of selecting patients who are less likely to benefit from treatment. Thus, it could be argued that this challenging question should be answered within specific clinical contexts before cfDNA assays are used for positive patient selection for treatment with mechanism based cancer therapeutics.

In chapter 3 of this thesis, attempts were made to answer this question within the context of *BRAF* mutation and MEK inhibition with selumetinib in advanced melanoma. By using clinical samples and outcome data from study NCT00936221, the prognostic significance of cfDNA *BRAF* mutation detection and its value for predicting response to the MEK inhibitor, selumetinib, was explored in patients with *BRAF* mutant advanced cutaneous or unknown primary melanoma. The limitations of this exploratory analysis were that it was a retrospective subgroup analysis of a randomised phase II study and that only 83 patients of 91 randomised patients had cfDNA mutation data. Nevertheless, the baseline characteristics of two patient groups

were similar. In a Cox proportional hazard model analysis of patients with cfDNA *BRAF* mutation, PFS was found to be significantly shorter than that of those without the cfDNA mutation (median PFS 87 vs. 230 days, adjusted HR 2.05 (95% CI 1.27-3.33), P<0.01). Furthermore, OS analysis using the same Cox model showed a non-significant trend that patients with cfDNA mutation had shorter OS (adjusted HR 1.5 (95%CI 0.88-2.6), P=0.1). The prognostic significance of cfDNA *BRAF* mutation detection was further supported by the fact that it was found to be more prevalent in patients with liver metastases and high LDH level. On the other hand, there was no clear association between the cfDNA mutation status and number of metastatic sites consistent with the findings recently reported in a similar study[233].

When interaction between cfDNA mutation status and PFS was analysed by a Cox proportional hazard model, no statistically significant interaction was found although there was a trend suggesting that patients with no cfDNA *BRAF* mutation derive better PFS benefit from MEK inhibition with selumetinib (adjusted HR for increased treatment benefit derived by patients without cfDNA mutations =1.6 (95% CI 0.62-4.11), P=0.33). Patients with *BRAF* mutant tumours with no cfDNA mutation who were treated in DTIC/selumetinib arm had a better median PFS than that of those who had DTIC/placebo. In contrast, in patients with cfDNA mutation, the median PFS did not differ between those who received DTIC/selumetinib and those who had DTIC/placebo. Of all the subgroups analysed, those who had no cfDNA mutation and who were treated with DTIC/selumetinib had the best median PFS. Moreover, highest ORR was also seen in this sub-group. Taken together, these results suggest that of patients with *BRAF* mutant tumours those without the mutation in cfDNA derive greater clinical benefits from MEK inhibition with selumetinib.

The results from this study, even though it is preliminary, were indeed intriguing. cfDNA *BRAF* mutation status was an independent prognostic indicator of PFS regardless of the treatment patients received. Although it was not found to be an independent predictor of OS, this was most likely to be due to the fact that this exploratory analysis was underpowered and there were not enough events to detect the difference as a non-significant trend was seen that patients with cfDNA *BRAF* mutation had shorter overall survival when compared to those without. Considering findings from other studies that reported prognostic significance of cfDNA *BRAF* mutation detection in melanoma[233,234], it is likely that cfDNA *BRAF* mutation status is prognostic in this disease group. Similar findings have been recently reported in lung cancer for *KRAS* mutation[29]. The main question, however, is whether there is any plausible biological reason to explain this prognostic significance.

The fact that cfDNA mutation is more prevalent in patients with liver metastases and high LDH level and that there is no clear association between cfDNA mutation status and number of metastatic sites indicates that patients with cfDNA mutation may have clinically more aggressive disease and this difference is not purely a reflection of disease burden. However, it is not clear why more aggressive tumours would shed more mutant DNA into the peripheral circulation. This could, perhaps, just reflect high cell turn over within the highly proliferative and aggressive tumour. Highly proliferative tumours are more likely to develop central tumour necrosis and this might be one of the reasons why more mutant DNA molecules were detected in circulation. Possibly, patients with metastatic disease in highly vascularised organs such as liver might also have higher mutation burden in the peripheral circulation.

It could be argued that stratification of *BRAF* tumour mutant patients by cfDNA mutation status in this study was somewhat arbitrary. The *BRAF* ARMS assay

employed in the study to detect cfDNA *BRAF* c.1799T>A mutation has LOD of 2% and as such, of patients who were classed as those with no detectable *BRAF* mutation in plasma, some might have *BRAF* mutation with mutation fraction less than 2%. It could be argued that this is very much akin to comparing the prognosis of patients with *BRAF* mutation fraction >2% in plasma to that of those with mutant fraction <2%. If more cutting edge technologies such as BEAMing had been used it would have been able to detect cfDNA mutation with lower than 2% and stratification would have been more stringent and accurate. Moreover, it could have been possible to calculate mutation fraction more accurately to determine whether there is positive correlation between *BRAF* mutation load in plasma and patients' prognosis. That correlation analysis could significantly strengthen prognostic implications of cfDNA mutation testing.

Another unanswered question is why is it that it is not possible to detect mutations in cfDNA in a proportion of patients with advanced cancers even by exquisitely sensitive methods like BEAMing or droplet PCR or CAST PCR[233,235,236]. Some would argue that the answer might lie in tumour heterogeneity. In advanced melanoma, not all *BRAF* mutant tumours are identical. For example not all patients with *BRAF* mutant tumours respond to BRAF inhibitor vemurafenib as approximately 20% of patients have primary resistance and are non-responders to this drug. Although *BRAF* mutation is an early event, it is still possible that not all tumour clones in a melanoma harbour this mutation and *BRAF* mutant clone is not the dominant clone in each and every *BRAF* mutant melanoma. The polyclonal nature of *BRAF* mutant tumours is a recognized biological phenomenon[237,238]. It could then be hypothesized that *BRAF* mutation in cfDNA can be detected relatively easily in patients with tumours where the *BRAF* mutant clone is the dominant clone and it is difficult to detect when it is not

the dominant one. However, further studies are still needed to prove or refute the above hypothesis.

Another plausible biological factor that could potentially determine the level of mutation present in the circulation is mutant gene amplifications. Mutant gene amplification has been recognised recently as a biological phenomenon that plays an important role in acquired drug resistance. Both *BRAF* and *KRAS* mutant gene amplifications have been shown to mediate resistance to MEK inhibition with selumetinib in-vitro[209,239]. Although it is currently not known how common amplification of *BRAF* mutant gene as a biological event is in cutaneous melanoma, allelic imbalance of *EGFR*, *KRAS* and *BRAF* mutant genes has been reported[208,240,241]. As discussed in earlier sections of this thesis, if there is any linkage between *BRAF* mutant gene amplification and presence of *BRAF* mutation in plasma, it can be hypothesised that patients with cfDNA *BRAF* mutation will derive less clinical benefits from MEK inhibition. The results from this project, however, did not conclusively confirm or refute the above hypothesis. Although patients without *BRAF* mutation in plasma had better prognosis and seemed to have derived better clinical benefits from MEK inhibition with selumetinib, the results were not robust enough to conclude that cfDNA *BRAF* mutation status is a predictive biomarker for response to the MEK inhibitor selumetinib. These results could still be the reflection of the fact that patients with cfDNA mutation have generally aggressive disease that respond poorly to anti-cancer treatment in general, not just specifically to MEK inhibition as reported in a separate study within the same context[233].

When analysis of clinical samples from study NCT00936221 was completed in 2011, considering rapid progress in next generation sequencing technologies during the course of the project, a proof of concept study was conducted to study the clinical

utility of mutation profiling of plasma derived cfDNA in patients with advanced colorectal cancer. NGS approach has some advantages over a real time PCR approach. While real time PCR can only be used to detect known mutations, NGS allows detection of both known and novel mutations. Secondly, gene copy number changes can also be studied from next generation sequencing data. Thirdly, multiplex potential is far greater with NGS than real time PCR. Lastly, although NGS is very costly at present, in the long term with further decrease in the cost of NGS, the cost of analysis per mutation will be a lot cheaper than real time PCR. Considering these advantages, the clinical utility of targeted next generation sequencing of cfDNA in patients with advanced colorectal cancer was explored in the final year of this PhD project using Ion-Torrent PGM™ platform, the benefits and pitfalls of this platform were discussed previously in the introduction of chapter 4.

From a technical point of view, the failure rate of the Ion-Torrent PGM platform for cfDNA sequencing was disappointingly high as library preparation was successful in only 50% of cfDNA samples tested. However, from biological and clinical points of view, even though complete data was available from only 4 of the 8 patients tested, this pilot study was a success. It was shown that by performing mutation profiling of matched tumour and cfDNA at the same time, more comprehensive and representative picture of tumour mutation burden can be obtained. Some mutations that could not be detectable in tumour sequencing were detected in cfDNA highlighting that cfDNA represents mutations from multiple sub clones of a tumour. Moreover, the emergence of a *FBXW7* mutation in cfDNA after treatment with FOLFOX chemotherapy and the persistence of this mutation in the circulation 6 weeks after exposure to second line chemotherapy FOLFIRI was demonstrated in a patient with metastatic CRC. The results indicate that *FBXW7* mutant cells may be selected for resistance to 5-FU based

combination chemotherapies and this is the first time that this association has been demonstrated. Moreover emergence of another chemotherapy resistant clone harbouring *SMAD4* and *ATM* mutation was observed in the same patient after exposure to second line chemotherapy FOLFIRI. These preliminary data suggest that targeted next generation sequencing of cfDNA could be used to advance our understanding of tumour biology in individual patients with advanced colorectal cancer.

The results reported and discussed here, together with results from other studies recently reported in the literature, are shaping the future direction of cfDNA research. From a technological perspective, pushing boundaries for improving sensitivity for detection of somatic mutations from cfDNA will continue to be the dominant theme in this research field. Currently, it is not known why cfDNA mutations cannot be detected in some patients with advanced cancers. Even by most exquisitely sensitive technique break point digital PCR, mutation cannot be detected in cfDNA in all patients with advanced cancers and can be detected in approximately 50% of patients with early stage cancer[120]. Whether this is because no mutant DNA molecule is present in the circulation to detect or whether currently available technologies are not sensitive enough to detect very low level mutations remains open to question. Most would like to argue, however, that it is mainly due to technology limitations and with further improvement in exiting technologies and emergence of new innovative ones, it would be possible to detect tumour specific mutations in cfDNA in almost all, even if not all, patients with advanced cancers.

Ultimately, with further advances in technologies, cfDNA research will expand into the arena of screening for early detection of cancer. However, to be used as a screening test; a technique has to have a very high sensitivity and specificity to avoid

harmful consequences of a false positive or false negative result. At present, one has to be sceptical about readiness for cfDNA based assays to be used in early detection of cancer considering only approximately 50% of patients with early stage cancer have detectable ctDNA[120]. Furthermore, most of the cfDNA based assays have not been subjected to rigorous validation/qualification and this currently limits their use in diagnostic settings. On the other hand, it could be envisaged that future cfDNA research will see extensive attempts to use cfDNA mutation testing as surrogate for tumour mutation testing in patients with advanced cancers especially in tumour types where good quality tumour materials are scarce such as pancreatic cancer and lung cancer mainly by using innovative PCR based technologies such as BEAMing, digital PCR, droplet PCR, CAST PCR, COLD-PCR and pyrophosporolyis-activated polymerization. Just before submission of this thesis Thierry *et al.* has reported that in 106 patients with metastatic CRC, 100% sensitivity and specificity of *BRAF* mutation detection was achieved by a cfDNA based assay using a modified allele-specific PCR called Intplex PCR. In the same study, sensitivity and specificity of cfDNA Intplex assay for testing 7 exon 2 *KRAS* mutations (p.G12V, p.G12A, p.G12D, p.G12S, p.G12C, p.G12R and p.G13D) was found to be 92% and 98% respectively with 96% concordance with tumour mutation results[242].

With rapid progress seen in NGS research it is quite clear that next generation sequencing of cfDNA can only improve and will achieve better precision. Further advances in NGS will eventually allow enumerating all cfDNA molecules in a given volume of blood and determining their sequence informing molecular composition of a tumour and its heterogeneity. I predict that in the near future it would be possible to perform targeted NGS from as low as 10ng of input DNA as demonstrated in this project. Although failure rate was high in my pilot study, it was still possible to get

meaningful data in 50% of the cfDNA samples tested. With further optimization, it will be possible to improve the success rate. Moreover, it has now been demonstrated that whole genome sequencing of cfDNA was feasible in selected patients with advanced breast cancer and colorectal cancer[153]. With rapid advances in this technology, it would be realistic to perform whole exome or genome sequencing of cfDNA using 50-100ng of input DNA range at a reasonable cost. This will eventually allow performing integrated genomic analysis of cfDNA to study tumour biology from a blood based test. This now seems a realistic and achievable goal.

However, it has to be noted here that sensitivity of NGS for detection of somatic mutations from cfDNA remains low. Using the Ion-Torrent PGM platform, only mutations with very high allelic frequencies were detected in cfDNA. As such, tumour mutation testing remains the gold standard and cfDNA sequencing remains an exploratory research tool or a complementary test to tumour mutation testing. One feasible approach will be to perform ultra deep sequencing of small number of genes to achieve a very high sensitivity. To be successful, this approach needs a very efficient target enrichment system, without which ultra deep targeted NGS will not necessarily increase the sensitivity of cfDNA mutation detection. As discussed earlier, during library preparation, there could be significant loss of minor alleles and this will limit sensitivity of the technique regardless of the sequencing depths. However, results from a recent exploratory study that employed massively parallel sequencing platform Safe-SeqS for detection of hot spot mutations in *TP53, APC, KRAS, BRAF, PIK3CA* and *FBXW7* from cfDNA in 19 patients with metastatic CRC were encouraging[243].

Another promising technology platform for sequencing cfDNA is third generation DNA sequencing or single molecule DNA sequencing platform. These approaches do

not need target enrichment or PCR amplification for library preparation before performing the sequencing runs. This could be advantageous for cfDNA sequencing as loss of minor alleles during library preparation is no longer an issue with these platforms. The mutant DNA templates present in the samples should be detectable regardless of the quantity of wild type templates at least in theory. At present, the rate limiting factor for improving sensitivity for mutation testing remains sequencing error rate and these platforms are not yet ready for widespread research applications. There are also limited literature describing these platforms and as such the full potential of these cannot be assessed.

While technological advances are crucial for progress, the main challenge is how genetic information obtained from cfDNA analysis could be translated into clinical settings to aid better patient management decisions. Most experts would perhaps argue that translation lags far behind technological advances in the field of cancer genomics. This is also true for cfDNA research. As demonstrated by recent studies, cfDNA analysis offers an exciting opportunity to study the emergence of novel drug resistance mutations in real time at longitudinal time points. This could be clinically and biologically useful. Firstly, emergence of known resistance mutations can be monitored and it might be possible to detect development of drug resistance before radiological progression with more time to implement treatment change that could impact outcomes. For example, in patients with advanced non-small cell lung cancer who are undergoing treatment with EGFR TKIs, erlotinib or gefitinib, it would be possible to monitor early emergence of EGFR p.T790M mutation by using cfDNA analysis by real time PCR or other PCR based techniques such as BEAMing. This might provide a window of opportunity to change or augment therapy prior to significant increase in disease burden. Obviously, this theoretical advantage needs to

be translated into clinical reality within specific clinical contexts. Recently it was demonstrated that emergence of *KRAS* mutations can be detected in cfDNA in advanced colorectal cancer patients undergoing EGFR targeted therapies with cetuximab or panitumumab before radiological progression validating cfDNA based monitoring approach[95,96,244].

Another challenge from biological point of view is that, although it has been demonstrated that emergence of novel drug resistance mutations can be detected in cfDNA using next generation DNA sequencing, how can we differentiate between driver and passenger mutations? Not all novel mutations emerging and detectable in the cfDNA after exposure to anti-cancer therapeutics are likely to be driver mutations. A majority of them will probably be passengers and this will impose difficulties in interpreting cfDNA mutation profiling data. Although allelic frequency changes in plasma over time might inform how patients are responding to therapy, what do these changes mean biologically? It could be argued that monitoring changes in the level of known driver mutations in the plasma during the course of treatment might give more meaningful information for clinical decision making. For example, serial monitoring of the *BRAF* mutation load in plasma could potentially inform the level of response and development of drug resistance in advanced melanoma patients with detectable *BRAF* mutation in plasma. More translational research studies are now required to study the predictive value of changes in the level of cfDNA mutation on treatment response and resistance.

In the near future cfDNA research protocols will be incorporated into various clinical trial protocols so that the clinical or biological merits of studying cfDNA mutations by PCR based techniques or NGS could be studied and understood within specific clinical contexts. This will help identify the value of cfDNA mutation testing and

provide an opportunity to validate cfDNA based assays as companion diagnostics from the very early stage of drug development. However, for this approach to be successful, one has to start development and validation (both technically and biologically) of cfDNA assays at a preclinical stage. This would give ample opportunity for studying pre-analytical and analytical characteristics of cfDNA based assays and their biological and potential future clinical role within specific contexts from a very early stage and will maximise the success of cfDNA translational research and its impact upon the progress of anti-cancer therapeutics.

With recent progress in cfDNA research, potential clinical utility of cfDNA based mutation testing as 'liquid biopsies' in patients with cancer is now increasingly being recognised (recently reviewed by Luis A. Diaz Jr and Alberto Bardelli)[245]. The authors argued that 'to overcome the limitations of tissue biopsies, less invasive techniques capable of capturing tumour heterogeneity and the molecular changes cancer cell undergo when they are exposed to therapy are needed' and genotyping from ctDNA could serve this purpose in the near future in patients with metastatic cancer. Authors also foresee that, in addition to already recognised role of monitoring resistance and heterogeneity, liquid biopsies may also be useful to monitor tumour burden and detection of minimal residual disease after tumour resection with surgery or therapy with curative intent. The results presented and discussed in chapter 4 of this thesis also suggest that mutation profiling of plasma derived cfDNA compliments tumour mutation profiling in patients with advanced CRC. One intriguing question is whether genomic analysis of CTCs would add additional value to tumour and ctDNA analyses. In my opinion, ctDNA analysis and CTC analysis serve different purposes. Genotyping of ctDNA provides a way to overcome the limitations imposed on tissues biopsies by tumour heterogeneity and present an ideal opportunity to study genetic

evolution of heterogeneous tumours in real time using a non-invasive test. CTC molecular analysis on the contrary provides an exciting opportunity to study the molecular mechanisms underpinning the metastatic tumour progression. The major caveat in analysing CTC for understanding the biology behind metastasis process is that it is not clearly known at what stage cancer metastasis occurs[246] and whether captured CTCs in patients with advanced cancer are the ones on the transit to seed and soil. One could argue that they could just still be cells shedding from different metastatic sites without biological intention to seed and soil at a distant site. If this is the case, CTC analysis might be better suited to study tumour heterogeneity and complimentary to ctDNA analysis in patients with advanced cancer. However, currently, it is not known how many CTCs are needed to be analysed to closely reflect tumour heterogeneity and technology hurdles are still needed to be overcome to answer this question (recently reviewed by Krebs *et al.*)[247]. From a clinical perspective CTCs analysis will be ideal for studying biology of cancer metastasis if they could be detectable in patients with early stage cancers to provide new therapeutic targets and scientific rationale for adjuvant treatment strategies. To achieve this, one has to characterise CTCs in transit in patients with early stage cancers before tumour resection or during follow up after tumour resection ideally with parallel characterisation of disseminated cancer cells and primary tumours. In patients with advanced cancer, at present, ctDNA analysis seems to have more advantages over CTC analysis as its clinical utility as surrogate for tumour mutation testing and as liquid biopsy for disease monitoring and elucidating treatment resistance mechanisms are better defined. Moreover, tumour somatic mutations are specific and with better improvement in sensitivity of ctDNA based assays, they could potentially be utilised as a cancer screening tool in the future.

In conclusion, with rapid advances in the field of cancer genomic and in DNA sequencing technologies, it is likely that cfDNA research field will expand rapidly in the near future. However, smarter translational approaches from the oncology community will be necessary to ensure that progress made in this research field is not all lost in translation. One could, however, be cautiously optimistic that the future of cfDNA research is bright and further advances in cfDNA research will bring future benefits for patients with cancer.

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APPENDIX 1: Ion AmpliSeq Cancer Panel Version 1

Gene	accession number	COSMIC id	cds_mut_syntax	aa_mut_syntax	Strand	hg19 coordinates
ABL1	X16416	12560	c.944C>T	p.T315I	+	9:133748283-133748283
ABL1	X16416	12573	c.763G>A	p.E255K	+	9:133738363-133738363
ABL1	X16416	12574	c.764A>T	p.E255V	+	9:133738364-133738364
ABL1	X16416	12575	c.951C>G	p.F317L	+	9:133748290-133748290
ABL1	X16416	12576	c.757T>C	p.Y253H	+	9:133738357-133738357
ABL1	X16416	12577	c.749G>A	p.G250E	+	9:133738349-133738349
ABL1	X16416	12578	c.1052T>C	p.M351T	+	9:133748391-133748391
ABL1	X16416	12602	c.827A>G	p.D276G	+	9:133747520-133747520
ABL1	X16416	12604	c.1187A>G	p.H396R	+	9:133750356-133750356
ABL1	X16416	12605	c.1075T>G	p.F359V	+	9:133748414-133748414
ABL1	X16416	12608	c.730A>G	p.M244V	+	9:133738330-133738330
ABL1	X16416	12609	c.756G>C	p.Q252H	+	9:133738356-133738356
ABL1	X16416	12610	c.758A>T	p.Y253F	+	9:133738358-133738358
ABL1	X16416	12611	c.1064A>G	p.E355G	+	9:133748403-133748403
ABL1	X16416	12631	c.742C>G	p.L248V	+	9:133738342-133738342
ABL1	X16416	12632	c.756G>T	p.Q252H	+	9:133738356-133738356
ABL1	X16416	49074	c.949T>C	p.F317L	+	9:133748288-133748288
AKT1	ENST00000349310	36918	c.145G>A	p.E49K	-	14:105246455-105246455
ALK	NM_004304	28055	c.3522C>A	p.F1174L	-	2:29443695-29443695
ALK	NM_004304	28056	c.3824G>A	p.R1275Q	-	2:29432664-29432664
APC	NM_000038	13113	c.3927_3931delAAAGA	p.E1309fs*4	+	5:112175218-112175222
APC	NM_000038	13121	c.4099C>T	p.Q1367*	+	5:112175390-112175390
APC	NM_000038	13125	c.3340C>T	p.R1114*	+	5:112174631-112174631
APC	NM_000038	13127	c.4348C>T	p.R1450*	+	5:112175639-112175639
APC	NM_000038	13728	c.3907C>T	p.Q1303*	+	5:112175198-112175198
APC	NM_000038	13864	c.4393_4394delAG	p.S1465fs*3	+	5:112175684-112175685
APC	NM_000038	18561	c.4666_4667insA	p.T1556fs*3	+	5:112175957-112175958
APC	NM_000038	18701	c.3926_3930delAAAAG	p.E1309fs*4	+	5:112175217-112175221
APC	NM_000038	18702	c.3964G>T	p.E1322*	+	5:112175255-112175255
APC	NM_000038	18704	c.4312delA	p.T1438fs*35	+	5:112175603-112175603

APC	NM_000038	18719	c.3923_3924insA	p.E1309fs*6	+	5:112175214-112175215
APC	NM_000038	18758	c.4108A>T	p.K1370*	+	5:112175399-112175399
APC	NM_000038	18760	c.3916G>T	p.E1306*	+	5:112175207-112175207
APC	NM_000038	18764	c.3921_3925delAAAAG	p.E1309fs*4	+	5:112175212-112175216
APC	NM_000038	18775	c.3925G>T	p.E1309*	+	5:112175216-112175216
APC	NM_000038	18783	c.4316delC	p.P1439fs*34	+	5:112175607-112175607
APC	NM_000038	18834	c.4135G>T	p.E1379*	+	5:112175426-112175426
APC	NM_000038	18838	c.4391_4394delAGAG	p.E1464fs*8	+	5:112175682-112175685
APC	NM_000038	18852	c.2626C>T	p.R876*	+	5:112173917-112173917
APC	NM_000038	18861	c.4128T>A	p.Y1376*	+	5:112175419-112175419
APC	NM_000038	18862	c.4132C>T	p.Q1378*	+	5:112175423-112175423
APC	NM_000038	18873	c.4385_4386delAG	p.S1465fs*3	+	5:112175676-112175677
APC	NM_000038	18931	c.4392_4393delGA	p.S1465fs*3	+	5:112175683-112175684
APC	NM_000038	18960	c.3880C>T	p.Q1294*	+	5:112175171-112175171
APC	NM_000038	19021	c.4330C>T	p.Q1444*	+	5:112175621-112175621
APC	NM_000038	19072	c.3871C>T	p.Q1291*	+	5:112175162-112175162
APC	NM_000038	19098	c.4463delT	p.L1488fs*19	+	5:112175754-112175754
APC	NM_000038	19099	c.3949G>C	p.E1317Q	+	5:112175240-112175240
APC	NM_000038	19127	c.4394_4395delGT	p.S1465fs*3	+	5:112175685-112175686
APC	NM_000038	19145	c.3925_3929delGAAAA	p.E1309fs*4	+	5:112175216-112175220
APC	NM_000038	19236	c.4333delA	p.T1445fs*28	+	5:112175624-112175624
APC	NM_000038	19263	c.3922_3926delAAAGA	p.E1309fs*4	+	5:112175213-112175217
APC	NM_000038	19332	c.4391_4392delAG	p.S1465fs*3	+	5:112175682-112175683
APC	NM_000038	19349	c.4479_4480delGG	p.E1494fs*19	+	5:112175770-112175771
APC	NM_000038	19664	c.3919delA	p.I1307fs*1	+	5:112175210-112175210
APC	NM_000038	19688	c.4386_4387delGA	p.S1465fs*3	+	5:112175677-112175678
APC	NM_000038	19695	c.4660_4661insA	p.T1556fs*3	+	5:112175951-112175952
APC	NM_000038	23598	c.4483delA	p.S1495fs*12	+	5:112175774-112175774
APC	NM_000038	24946	c.4291delA	p.M1431fs*42	+	5:112175582-112175582
APC	NM_000038	25827	c.4048A>T	p.K1350*	+	5:112175339-112175339
APC	NM_000038	29331	c.4063delT	p.S1355fs*60	+	5:112175354-112175354
APC	NM_000038	41618	c.4463_4466delTATT	p.L1488fs*18	+	5:112175754-112175757
ATM	NM_000051	12791	c.7996A>G	p.T2666A	+	11:108204681-108204681
ATM	NM_000051	12792	c.5380C>T	p.L1794L	+	11:108173640-108173640

ATM	NM_000051	12793	c.2542G>C	p.E848Q	+	11:108137973-108137973
ATM	NM_000051	12951	c.7325A>C	p.Q2442P	+	11:108200958-108200958
ATM	NM_000051	20404	c.7328G>A	p.R2443Q	+	11:108200961-108200961
ATM	NM_000051	21624	c.9139C>T	p.R3047*	+	11:108236203-108236203
ATM	NM_000051	21626	c.9023G>A	p.R3008H	+	11:108236087-108236087
ATM	NM_000051	21636	c.8084G>C	p.G2695A	+	11:108205769-108205769
ATM	NM_000051	21642	c.9022C>T	p.R3008C	+	11:108236086-108236086
ATM	NM_000051	21825	c.1229T>C	p.V410A	+	11:108119823-108119823
ATM	NM_000051	21826	c.2572T>C	p.F858L	+	11:108138003-108138003
ATM	NM_000051	21918	c.5224G>C	p.A1742P	+	11:108172421-108172421
ATM	NM_000051	21919	c.5041A>G	p.I1681V	+	11:108170476-108170476
ATM	NM_000051	21920	c.5044G>T	p.D1682Y	+	11:108170479-108170479
ATM	NM_000051	21922	c.5821G>C	p.V1941L	+	11:108180945-108180945
ATM	NM_000051	21924	c.1058_1059delGT	p.C353fs*5	+	11:108117847-108117848
ATM	NM_000051	21930	c.8839A>T	p.T2947S	+	11:108225590-108225590
ATM	NM_000051	22481	c.8174A>T	p.D2725V	+	11:108206594-108206594
ATM	NM_000051	22485	c.8668C>G	p.L2890V	+	11:108218089-108218089
ATM	NM_000051	22499	c.1810C>T	p.P604S	+	11:108123551-108123551
ATM	NM_000051	22507	c.3925G>A	p.A1309T	+	11:108155132-108155132
BRAF	NM_004333	449	c.1391G>A	p.G464E	-	7:140481417-140481417
BRAF	NM_004333	450	c.1391G>T	p.G464V	-	7:140481417-140481417
BRAF	NM_004333	451	c.1397G>T	p.G466V	-	7:140481411-140481411
BRAF	NM_004333	452	c.1397G>C	p.G466A	-	7:140481411-140481411
BRAF	NM_004333	453	c.1397G>A	p.G466E	-	7:140481411-140481411
BRAF	NM_004333	455	c.1405G>C	p.G469R	-	7:140481403-140481403
BRAF	NM_004333	457	c.1405G>A	p.G469R	-	7:140481403-140481403
BRAF	NM_004333	458	c.1405_1406GG>TC	p.G469S	-	7:140481402-140481403
BRAF	NM_004333	459	c.1406G>T	p.G469V	-	7:140481402-140481402
BRAF	NM_004333	460	c.1406G>C	p.G469A	-	7:140481402-140481402
BRAF	NM_004333	461	c.1406G>A	p.G469E	-	7:140481402-140481402
BRAF	NM_004333	462	c.1742A>G	p.N581S	-	7:140453193-140453193
BRAF	NM_004333	463	c.1756G>A	p.E586K	-	7:140453179-140453179
BRAF	NM_004333	464	c.1760A>C	p.D587A	-	7:140453175-140453175
BRAF	NM_004333	465	c.1761C>G	p.D587E	-	7:140453174-140453174

BRAF	NM_004333	466	c.1781A>T	p.D594V	-	7:140453154-140453154
BRAF	NM_004333	467	c.1781A>G	p.D594G	-	7:140453154-140453154
BRAF	NM_004333	468	c.1785T>G	p.F595L	-	7:140453150-140453150
BRAF	NM_004333	469	c.1786G>C	p.G596R	-	7:140453149-140453149
BRAF	NM_004333	470	c.1789C>G	p.L597V	-	7:140453146-140453146
BRAF	NM_004333	471	c.1790T>G	p.L597R	-	7:140453145-140453145
BRAF	NM_004333	472	c.1796C>T	p.T599I	-	7:140453139-140453139
BRAF	NM_004333	473	c.1798_1799GT>AA	p.V600K	-	7:140453136-140453137
BRAF	NM_004333	474	c.1798_1799GT>AG	p.V600R	-	7:140453136-140453137
BRAF	NM_004333	475	c.1799_1800TG>AA	p.V600E	-	7:140453135-140453136
BRAF	NM_004333	476	c.1799T>A	p.V600E	-	7:140453136-140453136
BRAF	NM_004333	477	c.1799_1800TG>AT	p.V600D	-	7:140453135-140453136
BRAF	NM_004333	478	c.1801A>G	p.K601E	-	7:140453134-140453134
BRAF	NM_004333	1111	c.1390G>C	p.G464R	-	7:140481418-140481418
BRAF	NM_004333	1112	c.1396G>C	p.G466R	-	7:140481412-140481412
BRAF	NM_004333	1113	c.1405_1407GGA>AGC	p.G469S	-	7:140481401-140481403
BRAF	NM_004333	1119	c.1776A>G	p.I592M	-	7:140453159-140453159
BRAF	NM_004333	1120	c.1774A>G	p.I592V	-	7:140453161-140453161
BRAF	NM_004333	1121	c.1782T>A	p.D594E	-	7:140453153-140453153
BRAF	NM_004333	1123	c.1784T>C	p.F595S	-	7:140453151-140453151
BRAF	NM_004333	1125	c.1790T>A	p.L597Q	-	7:140453145-140453145
BRAF	NM_004333	1126	c.1789_1790CT>TC	p.L597S	-	7:140453145-140453146
BRAF	NM_004333	1127	c.1797_1799AGT>GAG	p.V600R	-	7:140453136-140453138
BRAF	NM_004333	1128	c.1797_1797A>TACTACG	p.T599_V600insTT	-	7:140453138-140453138
BRAF	NM_004333	1130	c.1798G>A	p.V600M	-	7:140453137-140453137
BRAF	NM_004333	1132	c.1803A>C	p.K601N	-	7:140453132-140453132
BRAF	NM_004333	1135	c.1813_1814AG>TT	p.S605F	-	7:140453121-140453122
BRAF	NM_004333	1136	c.1814G>A	p.S605N	-	7:140453121-140453121
BRAF	NM_004333	6137	c.1799T>G	p.V600G	-	7:140453136-140453136
BRAF	NM_004333	6262	c.1330C>T	p.R444W	-	7:140481478-140481478
BRAF	NM_004333	6265	c.1803A>T	p.K601N	-	7:140453132-140453132
BRAF	NM_004333	18443	c.1799T>C	p.V600A	-	7:140453136-140453136
BRAF	NM_004333	21609	c.1761C>A	p.D587E	-	7:140453174-140453174
BRAF	NM_004333	21612	c.1783T>C	p.F595L	-	7:140453152-140453152

BRAF	NM_004333	24642	c.1411G>T	p.V471F	-	7:140481397-140481397
BRAF	NM_004333	27912	c.1405_1407GGA>AGT	p.G469S	-	7:140481401-140481403
BRAF	NM_004333	30594	c.1801_1803delAAA	p.K601del	-	7:140453132-140453134
BRAF	NM_004333	33808	c.1798G>T	p.V600L	-	7:140453137-140453137
BRAF	NM_004333	53198	c.1785T>A	p.F595L	-	7:140453150-140453150
CDH1	NM_004360.2	19748	c.1108G>C	p.D370H	+	16:68846137-68846137
CDH1	NM_004360.2	19761	c.1196_1199delCTGA	p.T399fs*17	+	16:68847274-68847277
CDH1	NM_004360.2	28934	c.240_241insGGTG	p.V82fs*13	+	16:68835649-68835650
CDKN2A	NM_000077	12473	c.172C>T	p.R58*	-	9:21971186-21971186
CDKN2A	NM_000077	12731	c.171_172CC>TT	p.R58*	-	9:21971186-21971187
CDKN2A	NM_000077	13281	c.205G>T	p.E69*	-	9:21971153-21971153
CDKN2A	NM_000077	13486	c.181G>T	p.E61*	-	9:21971177-21971177
CSF1R	NM_005211	946	c.902T>A	p.L301*	-	5:149453044-149453044
CSF1R	NM_005211	947	c.2906A>G	p.Y969C	-	5:149433645-149433645
CSF1R	NM_005211	948	c.2906A>T	p.Y969F	-	5:149433645-149433645
CSF1R	NM_005211	949	c.2907T>G	p.Y969*	-	5:149433644-149433644
CSF1R	NM_005211	952	c.2905T>C	p.Y969H	-	5:149433646-149433646
CSF1R	NM_005211	954	c.902T>C	p.L301S	-	5:149453044-149453044
CSF1R	NM_005211	955	c.2907T>A	p.Y969*	-	5:149433644-149433644
CTNNB1	NM_001904	5661	c.94G>T	p.D32Y	+	3:41266097-41266097
CTNNB1	NM_001904	5662	c.110C>T	p.S37F	+	3:41266113-41266113
CTNNB1	NM_001904	5663	c.133T>C	p.S45P	+	3:41266136-41266136
CTNNB1	NM_001904	5664	c.121A>G	p.T41A	+	3:41266124-41266124
CTNNB1	NM_001904	5666	c.110C>A	p.S37Y	+	3:41266113-41266113
CTNNB1	NM_001904	5667	c.134C>T	p.S45F	+	3:41266137-41266137
CTNNB1	NM_001904	5668	c.94G>C	p.D32H	+	3:41266097-41266097
CTNNB1	NM_001904	5669	c.98C>T	p.S33F	+	3:41266101-41266101
CTNNB1	NM_001904	5670	c.101G>T	p.G34V	+	3:41266104-41266104
CTNNB1	NM_001904	5671	c.101G>A	p.G34E	+	3:41266104-41266104
CTNNB1	NM_001904	5672	c.94G>A	p.D32N	+	3:41266097-41266097
CTNNB1	NM_001904	5673	c.98C>A	p.S33Y	+	3:41266101-41266101
CTNNB1	NM_001904	5675	c.109T>G	p.S37A	+	3:41266112-41266112
CTNNB1	NM_001904	5676	c.122C>T	p.T41I	+	3:41266125-41266125
CTNNB1	NM_001904	5677	c.98C>G	p.S33C	+	3:41266101-41266101

CTNNB1	NM_001904	5679	c.110C>G	p.S37C	+	3:41266113-41266113
CTNNB1	NM_001904	5681	c.95A>G	p.D32G	+	3:41266098-41266098
CTNNB1	NM_001904	5684	c.100G>C	p.G34R	+	3:41266103-41266103
CTNNB1	NM_001904	5685	c.133T>G	p.S45A	+	3:41266136-41266136
CTNNB1	NM_001904	5686	c.100G>A	p.G34R	+	3:41266103-41266103
CTNNB1	NM_001904	5687	c.109T>C	p.S37P	+	3:41266112-41266112
CTNNB1	NM_001904	5688	c.121A>C	p.T41P	+	3:41266124-41266124
CTNNB1	NM_001904	5689	c.134C>G	p.S45C	+	3:41266137-41266137
CTNNB1	NM_001904	5690	c.95A>C	p.D32A	+	3:41266098-41266098
CTNNB1	NM_001904	5691	c.95A>T	p.D32V	+	3:41266098-41266098
CTNNB1	NM_001904	5692	c.134C>A	p.S45Y	+	3:41266137-41266137
CTNNB1	NM_001904	5701	c.122C>G	p.T41S	+	3:41266125-41266125
CTNNB1	NM_001904	5706	c.65T>C	p.V22A	+	3:41266068-41266068
CTNNB1	NM_001904	5716	c.121A>T	p.T41S	+	3:41266124-41266124
CTNNB1	NM_001904	5738	c.61G>A	p.A21T	+	3:41266064-41266064
CTNNB1	NM_001904	5747	c.37G>A	p.A13T	+	3:41266040-41266040
CTNNB1	NM_001904	6050	c.64_114del51	p.V22_G38del	+	3:41266067-41266117
CTNNB1	NM_001904	6064	c.74_97del24	p.W25_D32del	+	3:41266077-41266100
EGFR	NM_005228	6210	c.2240_2251del12	p.L747_T751>S	+	7:55242470-55242481
EGFR	NM_005228	6213	c.2582T>A	p.L861Q	+	7:55259524-55259524
EGFR	NM_005228	6218	c.2239_2247del9	p.L747_E749del	+	7:55242469-55242477
EGFR	NM_005228	6219	c.2248G>C	p.A750P	+	7:55242478-55242478
EGFR	NM_005228	6220	c.2238_2255del18	p.E746_S752>D	+	7:55242468-55242485
EGFR	NM_005228	6223	c.2235_2249del15	p.E746_A750del	+	7:55242465-55242479
EGFR	NM_005228	6224	c.2573T>G	p.L858R	+	7:55259515-55259515
EGFR	NM_005228	6225	c.2236_2250del15	p.E746_A750del	+	7:55242466-55242480
EGFR	NM_005228	6239	c.2156G>C	p.G719A	+	7:55241708-55241708
EGFR	NM_005228	6240	c.2369C>T	p.T790M	+	7:55249071-55249071
EGFR	NM_005228	6241	c.2303G>T	p.S768I	+	7:55249005-55249005
EGFR	NM_005228	6252	c.2155G>A	p.G719S	+	7:55241707-55241707
EGFR	NM_005228	6253	c.2155G>T	p.G719C	+	7:55241707-55241707
EGFR	NM_005228	6254	c.2239_2253del15	p.L747_T751del	+	7:55242469-55242483
EGFR	NM_005228	6255	c.2239_2256del18	p.L747_S752del	+	7:55242469-55242486
EGFR	NM_005228	6256	c.2254_2277del24	p.S752_I759del	+	7:55242484-55242507

EGFR	NM_005228	6268	c.2257C>T	p.P753S	+	7:55242487-55242487
EGFR	NM_005228	12366	c.2572C>A	p.L858M	+	7:55259514-55259514
EGFR	NM_005228	12367	c.2237_2254del18	p.E746_S752>A	+	7:55242467-55242484
EGFR	NM_005228	12369	c.2240_2254del15	p.L747_T751del	+	7:55242470-55242484
EGFR	NM_005228	12370	c.2240_2257del18	p.L747_P753>S	+	7:55242470-55242487
EGFR	NM_005228	12376	c.2307_2308ins9	p.V769_D770insASV	+	7:55249009-55249010
EGFR	NM_005228	12377	c.2319_2320insCAC	p.H773_V774insH	+	7:55249021-55249022
EGFR	NM_005228	12378	c.2310_2311insGGT	p.D770_N771insG	+	7:55249012-55249013
EGFR	NM_005228	12381	c.2319_2320ins9	p.H773_V774insNPH	+	7:55249021-55249022
EGFR	NM_005228	12382	c.2239_2248TTAAGAGAAG>C	p.L747_A750>P	+	7:55242469-55242478
EGFR	NM_005228	12383	c.2239_2251>C	p.L747_T751>P	+	7:55242469-55242481
EGFR	NM_005228	12384	c.2237_2255>T	p.E746_S752>V	+	7:55242467-55242485
EGFR	NM_005228	12387	c.2239_2258>CA	p.L747_P753>Q	+	7:55242469-55242488
EGFR	NM_005228	12419	c.2238_2252>GCA	p.L747_T751>Q	+	7:55242468-55242482
EGFR	NM_005228	12422	c.2238_2248>GC	p.L747_A750>P	+	7:55242468-55242478
EGFR	NM_005228	12429	c.2573_2574TG>GT	p.L858R	+	7:55259515-55259516
EGFR	NM_005228	12678	c.2237_2251del15	p.E746_T751>A	+	7:55242467-55242481
EGFR	NM_005228	12728	c.2236_2253del18	p.E746_T751del	+	7:55242466-55242483
EGFR	NM_005228	12986	c.2429G>A	p.G810D	+	7:55249131-55249131
EGFR	NM_005228	12988	c.2125G>A	p.E709K	+	7:55241677-55241677
EGFR	NM_005228	13003	c.2310_2311insAAC	p.D770_N771insN	+	7:55249012-55249013
EGFR	NM_005228	13004	c.2310_2311insGGC	p.D770_N771insG	+	7:55249012-55249013
EGFR	NM_005228	13180	c.2188C>T	p.L730F	+	7:55242418-55242418
EGFR	NM_005228	13181	c.2198C>T	p.P733L	+	7:55242428-55242428
EGFR	NM_005228	13182	c.2203G>A	p.G735S	+	7:55242433-55242433
EGFR	NM_005228	13183	c.2225T>C	p.V742A	+	7:55242455-55242455
EGFR	NM_005228	13184	c.2236G>A	p.E746K	+	7:55242466-55242466
EGFR	NM_005228	13186	c.2255C>A	p.S752Y	+	7:55242485-55242485
EGFR	NM_005228	13188	c.2281G>A	p.D761N	+	7:55242511-55242511
EGFR	NM_005228	13192	c.2428G>A	p.G810S	+	7:55249130-55249130
EGFR	NM_005228	13427	c.2126A>C	p.E709A	+	7:55241678-55241678
EGFR	NM_005228	13428	c.2311_2312ins9	p.D770_N771insSVD	+	7:55249013-55249014
EGFR	NM_005228	13432	c.2193G>A	p.W731*	+	7:55242423-55242423
EGFR	NM_005228	13433	c.2318A>G	p.H773R	+	7:55249020-55249020

EGFR	NM_005228	13549	c.2235_2251>AG	p.E746_T751>A	+	7:55242465-55242481
EGFR	NM_005228	13551	c.2235_2252>AAT	p.E746_T751>I	+	7:55242465-55242482
EGFR	NM_005228	13553	c.2572_2573CT>AG	p.L858R	+	7:55259514-55259515
EGFR	NM_005228	13554	c.2312_2315ACCC>GCGTGGACAACCG	p.N771_P772>SVDNR	+	7:55249014-55249017
EGFR	NM_005228	13556	c.2253_2276del24	p.S752_I759del	+	7:55242483-55242506
EGFR	NM_005228	14243	c.2234A>G	p.K745R	+	7:55242464-55242464
EGFR	NM_005228	18419	c.2200G>A	p.E734K	+	7:55242430-55242430
EGFR	NM_005228	18425	c.2156G>A	p.G719D	+	7:55241708-55241708
EGFR	NM_005228	18441	c.2154_2155GG>TT	p.G719C	+	7:55241706-55241707
EGFR	NM_005228	18442	c.2241_2244AAGA>CCCG	p.L747_R748>FP	+	7:55242471-55242474
EGFR	NM_005228	21683	c.323G>A	p.R108K	+	7:55211080-55211080
EGFR	NM_005228	21687	c.866C>T	p.A289V	+	7:55221822-55221822
EGFR	NM_005228	21690	c.1793G>T	p.G598V	+	7:55233043-55233043
EGFR	NM_005228	21984	c.2281G>T	p.D761Y	+	7:55242511-55242511
EGFR	NM_005228	23571	c.2238_2252del15	p.L747_T751del	+	7:55242468-55242482
EGFR	NM_005228	24869	c.2235_2252del18	p.E746_T751del	+	7:55242465-55242482
ERBB2	NM_004448	681	c.2335_2336ins9	p.S779_P780insVGS	+	17:37881006-37881007
ERBB2	NM_004448	682	c.2322_2323ins12	p.M774_A775insAYVM	+	17:37880993-37880994
ERBB2	NM_004448	683	c.2263_2264TT>CC	p.L755P	+	17:37880219-37880220
ERBB2	NM_004448	685	c.2326G>A	p.G776S	+	17:37880997-37880997
ERBB2	NM_004448	12552	c.2326_2327insTTT	p.G776>VC	+	17:37880997-37880998
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ERBB2	NM_004448	12556	c.2340_2341ins9	p.P780_Y781insGSP	+	17:37881011-37881012
ERBB2	NM_004448	12558	c.2325_2326ins12	p.A775_G776insYVMA	+	17:37880996-37880997
ERBB2	NM_004448	13170	c.2305G>C	p.D769H	+	17:37880261-37880261
ERBB2	NM_004448	14060	c.2264T>C	p.L755S	+	17:37880220-37880220
ERBB2	NM_004448	14062	c.2329G>T	p.V777L	+	17:37881000-37881000
ERBB2	NM_004448	14064	c.2329G>A	p.V777M	+	17:37881000-37881000
ERBB2	NM_004448	14065	c.2524G>A	p.V842I	+	17:37881332-37881332
ERBB2	NM_004448	18609	c.2327G>T	p.G776V	+	17:37880998-37880998
ERBB2	NM_004448	20959	c.2324_2325ins12	p.A775_G776insYVMA	+	17:37880995-37880996
ERBB2	NM_004448	21985	c.2632C>T	p.H878Y	+	17:37881440-37881440
ERBB4	NM_005235	12833	c.908C>A	p.S303Y	-	2:212578349-212578349
ERBB4	NM_005235	20392	c.419C>T	p.T140I	-	2:212812157-212812157

ERBB4	NM_005235	48361	c.2804A>T	p.K935I	-	2:212288942-212288942
ERBB4	NM_005235	48362	c.2791G>T	p.D931Y	-	2:212288955-212288955
ERBB4	NM_005235	48363	c.1853A>C	p.H618P	-	2:212530066-212530066
ERBB4	NM_005235	48364	c.1784A>T	p.D595V	-	2:212530135-212530135
ERBB4	NM_005235	48365	c.1042G>T	p.V348L	-	2:212576857-212576857
ERBB4	NM_005235	48366	c.916C>A	p.R306S	-	2:212578341-212578341
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ERBB4	NM_005235	48368	c.731C>G	p.T244R	-	2:212589811-212589811
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FBXW7	NM_033632.1	22932	c.1393C>T	p.R465C	-	4:153249385-153249385
FBXW7	NM_033632.1	22965	c.1394G>A	p.R465H	-	4:153249384-153249384
FBXW7	NM_033632.1	22971	c.832C>T	p.R278*	-	4:153258983-153258983
FBXW7	NM_033632.1	22973	c.1177C>T	p.R393*	-	4:153250883-153250883
FBXW7	NM_033632.1	22974	c.1436G>A	p.R479Q	-	4:153247366-153247366
FBXW7	NM_033632.1	22975	c.1513C>T	p.R505C	-	4:153247289-153247289
FBXW7	NM_033632.1	22979	c.1745C>T	p.S582L	-	4:153245446-153245446
FBXW7	NM_033632.1	23000	c.1514G>T	p.R505L	-	4:153247288-153247288
FGFR1	NM_000604	601	c.374C>T	p.S125L	-	8:38285938-38285938
FGFR1	NM_000604	12834	c.754C>A	p.P252T	-	8:38282209-38282209
FGFR2	NM_000141.2	36901	c.929A>G	p.K310R	-	10:123279503-123279503
FGFR2	NM_000141.2	36902	c.1647T>G	p.N549K	-	10:123258034-123258034
FGFR2	NM_000141.2	36903	c.755C>G	p.S252W	-	10:123279677-123279677
FGFR2	NM_000141.2	36904	c.1124A>G	p.Y375C	-	10:123274794-123274794
FGFR2	NM_000141.2	36905	c.1115C>G	p.S372C	-	10:123274803-123274803
FGFR2	NM_000141.2	36906	c.1144T>C	p.C382R	-	10:123274774-123274774
FGFR2	NM_000141.2	36912	c.1647T>A	p.N549K	-	10:123258034-123258034
FGFR3	NM_000142	714	c.742C>T	p.R248C	+	4:1803564-1803564
FGFR3	NM_000142	715	c.746C>G	p.S249C	+	4:1803568-1803568
FGFR3	NM_000142	719	c.1948A>G	p.K650E	+	4:1807889-1807889
FGFR3	NM_000142	720	c.1949A>T	p.K650M	+	4:1807890-1807890
FGFR3	NM_000142	721	c.1172C>A	p.A391E	+	4:1806153-1806153
FGFR3	NM_000142	726	c.1948A>C	p.K650Q	+	4:1807889-1807889
FGFR3	NM_000142	729	c.2381_2381T>GA	p.L794fs*23	+	4:1808949-1808949
FGFR3	NM_000142	731	c.1949A>C	p.K650T	+	4:1807890-1807890

FGFR3	NM_000142	24802	c.2089G>T	p.G697C	+	4:1808331-1808331
FGFR3	NM_000142	24842	c.1138G>A	p.G380R	+	4:1806119-1806119
FGFR3	NM_000142	29438	c.1921G>A	p.D641N	+	4:1807862-1807862
FLT3	Z26652	783	c.2503G>T	p.D835Y	-	13:28592642-28592642
FLT3	Z26652	784	c.2504A>T	p.D835V	-	13:28592641-28592641
FLT3	Z26652	785	c.2503G>C	p.D835H	-	13:28592642-28592642
FLT3	Z26652	786	c.2039C>T	p.A680V	-	13:28602329-28602329
FLT3	Z26652	787	c.2505T>A	p.D835E	-	13:28592640-28592640
FLT3	Z26652	788	c.2505T>G	p.D835E	-	13:28592640-28592640
FLT3	Z26652	796	c.2503_2505delGAT	p.D835del	-	13:28592640-28592642
FLT3	Z26652	797	c.2506_2508delATC	p.I836del	-	13:28592637-28592639
FLT3	Z26652	850	c.2520_2521insGGATCC	p.S840_N841insGS	-	13:28592624-28592625
FLT3	Z26652	19522	c.1775T>C	p.V592A	-	13:28608281-28608281
FLT3	Z26652	19686	c.2508C>G	p.I836M	-	13:28592637-28592637
FLT3	Z26652	19692	c.2525A>G	p.Y842C	-	13:28592620-28592620
FLT3	Z26652	19737	c.1803_1804ins81	p.L601_K602ins27	-	13:28608252-28608253
FLT3	Z26652	19790	c.1807_1808ins18	p.K602_W603insYEYDLK	-	13:28608248-28608249
FLT3	Z26652	19836	c.2508_2510delCAT	p.I836del	-	13:28592635-28592637
FLT3	Z26652	24531	c.2509_2510AT>CC	p.M837P	-	13:28592635-28592636
FLT3	Z26652	25248	c.2492G>A	p.G831E	-	13:28592653-28592653
FLT3	Z26652	27906	c.1796A>T	p.Y599F	-	13:28608260-28608260
FLT3	Z26652	27979	c.1788_1789ins36	p.E596_Y597ins12	-	13:28608267-28608268
FLT3	Z26652	28042	c.1352C>T	p.S451F	-	13:28610138-28610138
FLT3	Z26652	28044	c.1715A>G	p.Y572C	-	13:28608341-28608341
FLT3	Z26652	28047	c.2501G>A	p.R834Q	-	13:28592644-28592644
FLT3	Z26652	28771	c.1811_1812ins30	p.W603_E604insDREYEYDLKW	-	13:28608244-28608245
FLT3	Z26652	28921	c.1798_1799ins45	p.Y599_D600ins15	-	13:28608257-28608258
GNAS	NM_000516.3	27887	c.601C>T	p.R201C	+	20:57484420-57484420
GNAS	NM_000516.3	27895	c.602G>A	p.R201H	+	20:57484421-57484421
HNF1A	NM_000545.3	21471	c.617G>T	p.W206L	+	12:121431413-121431413
HNF1A	NM_000545.3	21477	c.817A>G	p.K273E	+	12:121432070-121432070
HNF1A	NM_000545.3	21478	c.618G>T	p.W206C	+	12:121431414-121431414
HRAS	NM_005343	480	c.34G>A	p.G12S	-	11:534289-534289
HRAS	NM_005343	481	c.34G>T	p.G12C	-	11:534289-534289

HRAS	NM_005343	482	c.34G>C	p.G12R	-	11:534289-534289
HRAS	NM_005343	483	c.35G>T	p.G12V	-	11:534288-534288
HRAS	NM_005343	484	c.35G>A	p.G12D	-	11:534288-534288
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HRAS	NM_005343	488	c.37G>T	p.G13C	-	11:534286-534286
HRAS	NM_005343	489	c.38G>T	p.G13V	-	11:534285-534285
HRAS	NM_005343	490	c.38G>A	p.G13D	-	11:534285-534285
HRAS	NM_005343	496	c.181C>A	p.Q61K	-	11:533875-533875
HRAS	NM_005343	498	c.182A>T	p.Q61L	-	11:533874-533874
HRAS	NM_005343	499	c.182A>G	p.Q61R	-	11:533874-533874
HRAS	NM_005343	500	c.182A>C	p.Q61P	-	11:533874-533874
HRAS	NM_005343	501	c.182_183AG>GT	p.Q61R	-	11:533873-533874
HRAS	NM_005343	502	c.183G>T	p.Q61H	-	11:533873-533873
HRAS	NM_005343	503	c.183G>C	p.Q61H	-	11:533873-533873
HRAS	NM_005343	33695	c.182_183AG>GA	p.Q61R	-	11:533873-533874
HRAS	NM_005343	52978	c.182_183AG>TA	p.Q61L	-	11:533873-533874
HRAS	NM_005343	52979	c.181_182CA>AG	p.Q61R	-	11:533874-533875
IDH1	NM_005896.2	28746	c.395G>A	p.R132H	-	2:209113112-209113112
IDH1	NM_005896.2	28747	c.394C>T	p.R132C	-	2:209113113-209113113
IDH1	NM_005896.2	28748	c.394C>A	p.R132S	-	2:209113113-209113113
IDH1	NM_005896.2	28749	c.394C>G	p.R132G	-	2:209113113-209113113
JAK2	ENST00000381652	12600	c.1849G>T	p.V617F	+	9:5073770-5073770
JAK2	ENST00000381652	25834	c.1848_1849TG>CT	p.V617F	+	9:5073769-5073770
JAK3	NM_000215	34213	c.2164G>A	p.V722I	-	19:17945696-17945696
JAK3	NM_000215	34214	c.1715C>T	p.A572V	-	19:17948009-17948009
KDR	NM_002253	21091	c.743C>G	p.A248G	-	4:55980348-55980348
KDR	NM_002253	32294	c.2617G>A	p.G873R	-	4:55962507-55962507
KDR	NM_002253	32339	c.824G>T	p.R275L	-	4:55979623-55979623
KDR	NM_002253	48460	c.3629C>T	p.P1210L	-	4:55953807-55953807
KDR	NM_002253	48461	c.3434G>A	p.G1145E	-	4:55955111-55955111
KDR	NM_002253	48462	c.3418C>A	p.L1140M	-	4:55955127-55955127
KDR	NM_002253	48463	c.2951G>C	p.S984T	-	4:55960989-55960989

KDR	NM_002253	48464	c.2917G>T	p.A973S	-	4:55961023-55961023
KDR	NM_002253	48465	c.1426G>T	p.V476L	-	4:55972964-55972964
KDR	NM_002253	48875	c.3922G>T	p.G1308*	-	4:55946257-55946257
KDR	NM_002253	48977	c.4063_4065delCCT	p.P1355del	-	4:55946114-55946116
KIT	NM_000222	1146	c.154G>A	p.D52N	+	4:55561764-55561764
KIT	NM_000222	1169	c.1651_1665del15	p.P551_V555del	+	4:55593585-55593599
KIT	NM_000222	1210	c.1667_1672delAGTGGA	p.W557_K558del	+	4:55593601-55593606
KIT	NM_000222	1216	c.1669T>A	p.W557R	+	4:55593603-55593603
KIT	NM_000222	1217	c.1669_1674delTGGAAG	p.W557_K558del	+	4:55593603-55593608
KIT	NM_000222	1219	c.1669T>C	p.W557R	+	4:55593603-55593603
KIT	NM_000222	1221	c.1669T>G	p.W557G	+	4:55593603-55593603
KIT	NM_000222	1234	c.1672_1680del9	p.K558_V560del	+	4:55593606-55593614
KIT	NM_000222	1239	c.1672_1686del15	p.K558_E562del	+	4:55593606-55593620
KIT	NM_000222	1247	c.1675_1677delGTT	p.V559del	+	4:55593609-55593611
KIT	NM_000222	1251	c.1675G>A	p.V559I	+	4:55593609-55593609
KIT	NM_000222	1252	c.1676T>A	p.V559D	+	4:55593610-55593610
KIT	NM_000222	1253	c.1676T>G	p.V559G	+	4:55593610-55593610
KIT	NM_000222	1255	c.1676T>C	p.V559A	+	4:55593610-55593610
KIT	NM_000222	1257	c.1679T>A	p.V560D	+	4:55593613-55593613
KIT	NM_000222	1260	c.1679T>G	p.V560G	+	4:55593613-55593613
KIT	NM_000222	1290	c.1727T>C	p.L576P	+	4:55593661-55593661
KIT	NM_000222	1294	c.1735_1737delGAT	p.D579del	+	4:55593669-55593671
KIT	NM_000222	1299	c.1755C>T	p.P585P	+	4:55593689-55593689
KIT	NM_000222	1304	c.1924A>G	p.K642E	+	4:55594221-55594221
KIT	NM_000222	1306	c.2143_2145delAGC	p.S715del	+	4:55597495-55597497
KIT	NM_000222	1310	c.2446G>T	p.D816Y	+	4:55599320-55599320
KIT	NM_000222	1311	c.2446G>C	p.D816H	+	4:55599320-55599320
KIT	NM_000222	1314	c.2447A>T	p.D816V	+	4:55599321-55599321
KIT	NM_000222	1321	c.2466T>A	p.N822K	+	4:55599340-55599340
KIT	NM_000222	1322	c.2466T>G	p.N822K	+	4:55599340-55599340
KIT	NM_000222	1323	c.2474T>C	p.V825A	+	4:55599348-55599348
KIT	NM_000222	1324	c.2515G>A	p.E839K	+	4:55602694-55602694
KIT	NM_000222	1326	c.1509_1510insGCCTAT	p.Y503_F504insAY	+	4:55592185-55592186
KIT	NM_000222	1332	c.1669_1683del15	p.W557_E561del	+	4:55593603-55593617

KIT	NM_000222	12706	c.1961T>C	p.V654A	+	4:55594258-55594258
KIT	NM_000222	12708	c.2009C>T	p.T670I	+	4:55595519-55595519
KIT	NM_000222	18681	c.2467T>G	p.Y823D	+	4:55599341-55599341
KIT	NM_000222	18896	c.1673_1687del15	p.K558_E562del	+	4:55593607-55593621
KIT	NM_000222	27909	c.1656_1673del18	p.Y553_K558>	+	4:55593590-55593607
KIT	NM_000222	28026	c.1621A>C	p.M541L	+	4:55593464-55593464
KRAS	NM_004985	513	c.34_36GGT>TGC	p.G12C	-	12:25398283-25398285
KRAS	NM_004985	515	c.35_36GT>TC	p.G12V	-	12:25398283-25398284
KRAS	NM_004985	516	c.34G>T	p.G12C	-	12:25398285-25398285
KRAS	NM_004985	517	c.34G>A	p.G12S	-	12:25398285-25398285
KRAS	NM_004985	518	c.34G>C	p.G12R	-	12:25398285-25398285
KRAS	NM_004985	520	c.35G>T	p.G12V	-	12:25398284-25398284
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KRAS	NM_004985	526	c.37_39GGC>CGT	p.G13R	-	12:25398280-25398282
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KRAS	NM_004985	528	c.37G>A	p.G13S	-	12:25398282-25398282
KRAS	NM_004985	529	c.37G>C	p.G13R	-	12:25398282-25398282
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KRAS	NM_004985	531	c.38_39GC>AT	p.G13D	-	12:25398280-25398281
KRAS	NM_004985	532	c.38G>A	p.G13D	-	12:25398281-25398281
KRAS	NM_004985	533	c.38G>C	p.G13A	-	12:25398281-25398281
KRAS	NM_004985	534	c.38G>T	p.G13V	-	12:25398281-25398281
KRAS	NM_004985	543	c.64C>A	p.Q22K	-	12:25398255-25398255
KRAS	NM_004985	546	c.175G>A	p.A59T	-	12:25380283-25380283
KRAS	NM_004985	549	c.181C>A	p.Q61K	-	12:25380277-25380277
KRAS	NM_004985	550	c.181C>G	p.Q61E	-	12:25380277-25380277
KRAS	NM_004985	551	c.182A>C	p.Q61P	-	12:25380276-25380276
KRAS	NM_004985	552	c.182A>G	p.Q61R	-	12:25380276-25380276
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KRAS	NM_004985	554	c.183A>C	p.Q61H	-	12:25380275-25380275
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KRAS	NM_004985	12703	c.57G>C	p.L19F	-	12:25398262-25398262
KRAS	NM_004985	12721	c.38_39GC>TT	p.G13V	-	12:25398280-25398281

KRAS	NM_004985	12729	c.180_181TC>CA	p.Q61K	-	12:25380277-25380278
KRAS	NM_004985	14209	c.35_36GT>AC	p.G12D	-	12:25398283-25398284
KRAS	NM_004985	19404	c.436G>A	p.A146T	-	12:25378562-25378562
KRAS	NM_004985	20818	c.57G>T	p.L19F	-	12:25398262-25398262
KRAS	NM_004985	87281	c.36_37TG>AT	p.G13C	-	12:25398282-25398283
KRAS	NM_004985	87298	c.180_181TC>AA	p.Q61K	-	12:25380277-25380278
MET	NM_000245	690	c.3742T>C	p.Y1248H	+	7:116423413-116423413
MET	NM_000245	691	c.3803T>C	p.M1268T	+	7:116423474-116423474
MET	NM_000245	696	c.3334C>T	p.H1112Y	+	7:116417463-116417463
MET	NM_000245	699	c.3743A>G	p.Y1248C	+	7:116423414-116423414
MET	NM_000245	700	c.3757T>G	p.Y1253D	+	7:116423428-116423428
MET	NM_000245	703	c.3335A>G	p.H1112R	+	7:116417464-116417464
MET	NM_000245	706	c.504G>T	p.E168D	+	7:116339642-116339642
MET	NM_000245	707	c.3029C>T	p.T1010I	+	7:116411990-116411990
MET	NM_000245	710	c.1124A>G	p.N375S	+	7:116340262-116340262
MLH1	NM_000249.2	26085	c.1151T>A	p.V384D	+	3:37067240-37067240
MPL	NM_005373.1	18918	c.1544G>T	p.W515L	+	1:43815009-43815009
MPL	NM_005373.1	19193	c.1543_1544TG>AA	p.W515K	+	1:43815008-43815009
NOTCH1	NM_017617.2	12771	c.4802T>C	p.L1601P	-	9:139399344-139399344
NOTCH1	NM_017617.2	12772	c.4724T>C	p.L1575P	-	9:139399422-139399422
NOTCH1	NM_017617.2	13042	c.4781T>C	p.L1594P	-	9:139399365-139399365
NOTCH1	NM_017617.2	13046	c.4757T>C	p.L1586P	-	9:139399389-139399389
NOTCH1	NM_017617.2	13047	c.4735_4737delGTG	p.V1579del	-	9:139399409-139399411
NOTCH1	NM_017617.2	13048	c.5036T>C	p.L1679P	-	9:139397768-139397768
NOTCH1	NM_017617.2	13053	c.4796G>C	p.R1599P	-	9:139399350-139399350
NOTCH1	NM_017617.2	24673	c.4724T>C	p.L1575P	-	9:139399422-139399422
NPM1	NM_002520.4	17559	c.863_864insTCTG	p.W288fs*12	+	5:170837547-170837548
NPM1	NM_002520.4	17571	c.863_864insCATG	p.W288fs*12	+	5:170837547-170837548
NPM1	NM_002520.4	17573	c.863_864insCCTG	p.W288fs*12	+	5:170837547-170837548
NRAS	NM_002524	561	c.34G>C	p.G12R	-	1:115258748-115258748
NRAS	NM_002524	562	c.34G>T	p.G12C	-	1:115258748-115258748
NRAS	NM_002524	563	c.34G>A	p.G12S	-	1:115258748-115258748
NRAS	NM_002524	564	c.35G>A	p.G12D	-	1:115258747-115258747
NRAS	NM_002524	565	c.35G>C	p.G12A	-	1:115258747-115258747

NRAS	NM_002524	566	c.35G>T	p.G12V	-	1:115258747-115258747
NRAS	NM_002524	569	c.37G>C	p.G13R	-	1:115258745-115258745
NRAS	NM_002524	570	c.37G>T	p.G13C	-	1:115258745-115258745
NRAS	NM_002524	571	c.37G>A	p.G13S	-	1:115258745-115258745
NRAS	NM_002524	572	c.38_39GT>TC	p.G13V	-	1:115258743-115258744
NRAS	NM_002524	573	c.38G>A	p.G13D	-	1:115258744-115258744
NRAS	NM_002524	574	c.38G>T	p.G13V	-	1:115258744-115258744
NRAS	NM_002524	575	c.38G>C	p.G13A	-	1:115258744-115258744
NRAS	NM_002524	577	c.52G>A	p.A18T	-	1:115258730-115258730
NRAS	NM_002524	579	c.181_182CA>AG	p.Q61R	-	1:115256529-115256530
NRAS	NM_002524	580	c.181C>A	p.Q61K	-	1:115256530-115256530
NRAS	NM_002524	581	c.181C>G	p.Q61E	-	1:115256530-115256530
NRAS	NM_002524	582	c.182A>C	p.Q61P	-	1:115256529-115256529
NRAS	NM_002524	583	c.182A>T	p.Q61L	-	1:115256529-115256529
NRAS	NM_002524	584	c.182A>G	p.Q61R	-	1:115256529-115256529
NRAS	NM_002524	585	c.183A>T	p.Q61H	-	1:115256528-115256528
NRAS	NM_002524	586	c.183A>C	p.Q61H	-	1:115256528-115256528
NRAS	NM_002524	12725	c.181_182CA>TT	p.Q61L	-	1:115256529-115256530
NRAS	NM_002524	12730	c.180_181AC>TA	p.Q61K	-	1:115256530-115256531
NRAS	NM_002524	30646	c.182_183AA>TG	p.Q61L	-	1:115256528-115256529
NRAS	NM_002524	33693	c.182_183AA>GG	p.Q61R	-	1:115256528-115256529
NRAS	NM_002524	53223	c.181_183CAA>AAG	p.Q61K	-	1:115256528-115256530
PDGFRA	NM_006206	736	c.2525A>T	p.D842V	+	4:55152093-55152093
PDGFRA	NM_006206	737	c.2524_2535del12	p.D842_H845del	+	4:55152092-55152103
PDGFRA	NM_006206	739	c.1682T>A	p.V561D	+	4:55141036-55141036
PDGFRA	NM_006206	741	c.1678_1692del15	p.R560_S564del	+	4:55141032-55141046
PDGFRA	NM_006206	743	c.2021C>T	p.T674I	+	4:55144547-55144547
PDGFRA	NM_006206	12396	c.2524G>T	p.D842Y	+	4:55152092-55152092
PDGFRA	NM_006206	12397	c.2524_2526GAC>TAT	p.D842Y	+	4:55152092-55152094
PDGFRA	NM_006206	12398	c.2524_2525GA>AT	p.D842I	+	4:55152092-55152093
PDGFRA	NM_006206	12399	c.2536G>T	p.D846Y	+	4:55152104-55152104
PDGFRA	NM_006206	12400	c.2527_2538del12	p.I843_D846del	+	4:55152095-55152106
PDGFRA	NM_006206	12401	c.2524_2532del9	p.D842_M844del	+	4:55152092-55152100
PDGFRA	NM_006206	12402	c.2530_2541del12	p.M844_S847del	+	4:55152098-55152109

PDGFRA	NM_006206	12405	c.2521_2526delAGAGAC	p.R841_D842del	+	4:55152089-55152094
PDGFRA	NM_006206	12407	c.2528_2539del12	p.I843_S847>T	+	4:55152096-55152107
PDGFRA	NM_006206	12408	c.2526_2538>G	p.D842_D846>E	+	4:55152094-55152106
PDGFRA	NM_006206	12411	c.2524_2536>A	p.D842_D846>N	+	4:55152092-55152104
PDGFRA	NM_006206	12412	c.2525_2538>GA	p.D842_D846>G	+	4:55152093-55152106
PDGFRA	NM_006206	12414	c.2525_2535>TT	p.D842_H845>V	+	4:55152093-55152103
PDGFRA	NM_006206	12415	c.2526_2541>GGCC	p.D842_S847>EA	+	4:55152094-55152109
PDGFRA	NM_006206	21973	c.1659_1664delGAGGTA	p.R554_Y555del	+	4:55141013-55141018
PDGFRA	NM_006206	22413	c.2472C>T	p.V824V	+	4:55152040-55152040
PDGFRA	NM_006206	22416	c.1975A>T	p.N659Y	+	4:55144146-55144146
PDGFRA	NM_006206	28053	c.1694_1695insA	p.S566fs*6	+	4:55141048-55141049
PIK3CA	NM_006218.1	746	c.263G>A	p.R88Q	+	3:178916876-178916876
PIK3CA	NM_006218.1	754	c.1035T>A	p.N345K	+	3:178921553-178921553
PIK3CA	NM_006218.1	757	c.1258T>C	p.C420R	+	3:178927980-178927980
PIK3CA	NM_006218.1	759	c.1616C>G	p.P539R	+	3:178936074-178936074
PIK3CA	NM_006218.1	760	c.1624G>A	p.E542K	+	3:178936082-178936082
PIK3CA	NM_006218.1	762	c.1625A>T	p.E542V	+	3:178936083-178936083
PIK3CA	NM_006218.1	763	c.1633G>A	p.E545K	+	3:178936091-178936091
PIK3CA	NM_006218.1	764	c.1634A>G	p.E545G	+	3:178936092-178936092
PIK3CA	NM_006218.1	765	c.1635G>T	p.E545D	+	3:178936093-178936093
PIK3CA	NM_006218.1	766	c.1636C>A	p.Q546K	+	3:178936094-178936094
PIK3CA	NM_006218.1	767	c.1637A>C	p.Q546P	+	3:178936095-178936095
PIK3CA	NM_006218.1	771	c.3073A>G	p.T1025A	+	3:178952018-178952018
PIK3CA	NM_006218.1	773	c.3129G>T	p.M1043I	+	3:178952074-178952074
PIK3CA	NM_006218.1	774	c.3139C>T	p.H1047Y	+	3:178952084-178952084
PIK3CA	NM_006218.1	775	c.3140A>G	p.H1047R	+	3:178952085-178952085
PIK3CA	NM_006218.1	776	c.3140A>T	p.H1047L	+	3:178952085-178952085
PIK3CA	NM_006218.1	777	c.3145G>A	p.G1049S	+	3:178952090-178952090
PIK3CA	NM_006218.1	778	c.2102A>C	p.H701P	+	3:178938860-178938860
PIK3CA	NM_006218.1	6147	c.1636C>G	p.Q546E	+	3:178936094-178936094
PIK3CA	NM_006218.1	12458	c.1634A>C	p.E545A	+	3:178936092-178936092
PIK3CA	NM_006218.1	12459	c.1637A>G	p.Q546R	+	3:178936095-178936095
PIK3CA	NM_006218.1	12461	c.3062A>G	p.Y1021C	+	3:178952007-178952007
PIK3CA	NM_006218.1	12464	c.3204_3205insA	p.N1068fs*4	+	3:178952149-178952150

PIK3CA	NM_006218.1	12591	c.3127A>G	p.M1043V	+	3:178952072-178952072
PIK3CA	NM_006218.1	12597	c.3145G>C	p.G1049R	+	3:178952090-178952090
PIK3CA	NM_006218.1	17442	c.1624G>C	p.E542Q	+	3:178936082-178936082
PIK3CA	NM_006218.1	25041	c.1637A>T	p.Q546L	+	3:178936095-178936095
PIK3CA	NM_006218.1	27133	c.1633G>C	p.E545Q	+	3:178936091-178936091
PIK3CA	NM_006218.1	27374	c.1635G>C	p.E545D	+	3:178936093-178936093
PTEN	NM_000314.4	4894	c.952_955delCTTA	p.L318fs*2	+	10:89720801-89720804
PTEN	NM_000314.4	4898	c.950_953delTACT	p.V317fs*3	+	10:89720799-89720802
PTEN	NM_000314.4	4903	c.954_957delTACT	p.L318fs*2	+	10:89720803-89720806
PTEN	NM_000314.4	4929	c.17_18delAA	p.K6fs*4	+	10:89624243-89624244
PTEN	NM_000314.4	4937	c.16_17delAA	p.K6fs*4	+	10:89624242-89624243
PTEN	NM_000314.4	4958	c.955_958delACTT	p.T319fs*1	+	10:89720804-89720807
PTEN	NM_000314.4	4986	c.741_742insA	p.P248fs*5	+	10:89717716-89717717
PTEN	NM_000314.4	4990	c.968_969insA	p.N323fs*2	+	10:89720817-89720818
PTEN	NM_000314.4	4994	c.963_964insA	p.T321fs*3	+	10:89720812-89720813
PTEN	NM_000314.4	5025	c.742_743insC	p.P248fs*5	+	10:89717717-89717718
PTEN	NM_000314.4	5026	c.742_743insA	p.P248fs*5	+	10:89717717-89717718
PTEN	NM_000314.4	5036	c.202T>C	p.Y68H	+	10:89685307-89685307
PTEN	NM_000314.4	5039	c.518G>A	p.R173H	+	10:89711900-89711900
PTEN	NM_000314.4	5089	c.517C>T	p.R173C	+	10:89711899-89711899
PTEN	NM_000314.4	5149	c.511C>T	p.Q171*	+	10:89711893-89711893
PTEN	NM_000314.4	5150	c.640C>T	p.Q214*	+	10:89717615-89717615
PTEN	NM_000314.4	5151	c.1003C>T	p.R335*	+	10:89720852-89720852
PTEN	NM_000314.4	5775	c.1002_1003CC>TT	p.R335*	+	10:89720851-89720852
PTEN	NM_000314.4	5801	c.968delA	p.N323fs*21	+	10:89720817-89720817
PTEN	NM_000314.4	5816	c.867delA	p.V290fs*1	+	10:89720716-89720716
PTEN	NM_000314.4	5823	c.963delA	p.T321fs*23	+	10:89720812-89720812
PTEN	NM_000314.4	5888	c.723_724insTT	p.E242fs*15	+	10:89717698-89717699
PTEN	NM_000314.4	23626	c.962_963insA	p.N323fs*2	+	10:89720811-89720812
PTEN	NM_000314.4	30622	c.795delA	p.K267fs*9	+	10:89717770-89717770
PTEN	NM_000314.4	43098	c.969delT	p.N323fs*21	+	10:89720818-89720818
PTEN	NM_000314.4	53243	c.964_964delA	p.N323fs*21	+	10:89720813-89720813
PTEN	NM_000314.4	87314	c.797_797delA	p.K267fs*9	+	10:89717772-89717772
PTPN11	NM_002834.3	13000	c.226G>A	p.E76K	+	12:112888210-112888210

PTPN11	NM_002834.3	13011	c.181G>T	p.D61Y	+	12:112888165-112888165
PTPN11	NM_002834.3	13013	c.205G>A	p.E69K	+	12:112888189-112888189
PTPN11	NM_002834.3	13014	c.214G>A	p.A72T	+	12:112888198-112888198
PTPN11	NM_002834.3	13016	c.226G>C	p.E76Q	+	12:112888210-112888210
PTPN11	NM_002834.3	13017	c.227A>G	p.E76G	+	12:112888211-112888211
PTPN11	NM_002834.3	13019	c.218C>T	p.T73I	+	12:112888202-112888202
PTPN11	NM_002834.3	13020	c.1504T>C	p.S502P	+	12:112926884-112926884
PTPN11	NM_002834.3	13022	c.182A>T	p.D61V	+	12:112888166-112888166
PTPN11	NM_002834.3	13025	c.227A>T	p.E76V	+	12:112888211-112888211
PTPN11	NM_002834.3	13026	c.227A>C	p.E76A	+	12:112888211-112888211
PTPN11	NM_002834.3	13027	c.1508G>C	p.G503A	+	12:112926888-112926888
PTPN11	NM_002834.3	13028	c.179G>T	p.G60V	+	12:112888163-112888163
PTPN11	NM_002834.3	13035	c.215C>A	p.A72D	+	12:112888199-112888199
PTPN11	NM_002834.3	14271	c.1508G>T	p.G503V	+	12:112926888-112926888
RB1	NM_000321	868	c.2242G>T	p.E748*	+	13:49039164-49039164
RB1	NM_000321	869	c.1980_1983delCCGG	p.L660fs*2	+	13:49033843-49033846
RB1	NM_000321	879	c.1072C>T	p.R358*	+	13:48942685-48942685
RB1	NM_000321	883	c.2117G>T	p.C706F	+	13:49037877-49037877
RB1	NM_000321	887	c.1654C>T	p.R552*	+	13:48955538-48955538
RB1	NM_000321	888	c.1666C>T	p.R556*	+	13:48955550-48955550
RB1	NM_000321	890	c.409G>T	p.E137*	+	13:48919244-48919244
RB1	NM_000321	892	c.1735C>T	p.R579*	+	13:49027168-49027168
RB1	NM_000321	915	c.596T>A	p.L199*	+	13:48923148-48923148
RET	NM_020975	965	c.2753T>C	p.M918T	+	10:43617416-43617416
RET	NM_020975	966	c.1900T>C	p.C634R	+	10:43609948-43609948
RET	NM_020975	968	c.1894_1899delGAGCTG	p.E632_L633del	+	10:43609942-43609947
RET	NM_020975	974	c.1901G>A	p.C634Y	+	10:43609949-43609949
RET	NM_020975	975	c.1902C>G	p.C634W	+	10:43609950-43609950
RET	NM_020975	977	c.2647_2648GC>TT	p.A883F	+	10:43615568-43615569
RET	NM_020975	978	c.1892A>G	p.D631G	+	10:43609940-43609940
RET	NM_020975	981	c.2646_2648AGC>TTT	p.A883F	+	10:43615567-43615569
RET	NM_020975	982	c.1895_1897delAGC	p.E632_L633>V	+	10:43609943-43609945
RET	NM_020975	983	c.1893_1898delCGAGCT	p.D631_L633>E	+	10:43609941-43609946
RET	NM_020975	984	c.1834_1860del27	p.F612_C620del	+	10:43609078-43609104

RET	NM_020975	1049	c.1895_1918>TGCGGC	p.E632_A640>VRP	+	10:43609943-43609966
RET	NM_020975	21338	c.2304G>C	p.E768D	+	10:43613840-43613840
SMAD4	NM_005359.3	14057	c.733C>T	p.Q245*	+	18:48584560-48584560
SMAD4	NM_005359.3	14096	c.1333C>T	p.R445*	+	18:48603032-48603032
SMAD4	NM_005359.3	14105	c.1394_1395insT	p.A466fs*28	+	18:48603093-48603094
SMAD4	NM_005359.3	14110	c.989A>C	p.E330A	+	18:48591826-48591826
SMAD4	NM_005359.3	14111	c.1028C>G	p.S343*	+	18:48591865-48591865
SMAD4	NM_005359.3	14113	c.1490G>A	p.R497H	+	18:48604668-48604668
SMAD4	NM_005359.3	14115	c.1569C>G	p.C523W	+	18:48604747-48604747
SMAD4	NM_005359.3	14118	c.502G>T	p.G168*	+	18:48581198-48581198
SMAD4	NM_005359.3	14121	c.1015_1029del15	p.F339_S343del	+	18:48591852-48591866
SMAD4	NM_005359.3	14122	c.1082G>A	p.R361H	+	18:48591919-48591919
SMAD4	NM_005359.3	14124	c.1341_1365del25	p.Q448fs*20	+	18:48603040-48603064
SMAD4	NM_005359.3	14129	c.1543A>T	p.R515*	+	18:48604721-48604721
SMAD4	NM_005359.3	14134	c.1576G>T	p.E526*	+	18:48604754-48604754
SMAD4	NM_005359.3	14135	c.1051G>C	p.D351H	+	18:48591888-48591888
SMAD4	NM_005359.3	14163	c.931C>T	p.Q311*	+	18:48586262-48586262
SMAD4	NM_005359.3	14174	c.1072G>T	p.G358*	+	18:48591909-48591909
SMAD4	NM_005359.3	14177	c.1546_1553delCAGAGCAT	p.S517fs*7	+	18:48604724-48604731
SMAD4	NM_005359.3	14216	c.363_364insA	p.C123fs*2	+	18:48575169-48575170
SMAD4	NM_005359.3	14249	c.1156G>C	p.G386R	+	18:48593405-48593405
SMARCB1	NM_003073.2	991	c.141C>A	p.Y47*	+	22:24133990-24133990
SMARCB1	NM_003073.2	992	c.472C>T	p.R158*	+	22:24143240-24143240
SMARCB1	NM_003073.2	993	c.601C>T	p.R201*	+	22:24145582-24145582
SMARCB1	NM_003073.2	1002	c.118C>T	p.R40*	+	22:24133967-24133967
SMARCB1	NM_003073.2	1057	c.1148delC	p.P383fs*3	+	22:24176357-24176357
SMARCB1	NM_003073.2	29382	c.1143delG	p.A382fs*5	+	22:24176352-24176352
SMO	NM_005631.3	13145	c.595C>T	p.R199W	+	7:128845101-128845101
SMO	NM_005631.3	13146	c.1604G>T	p.W535L	+	7:128850341-128850341
SMO	NM_005631.3	13147	c.970G>A	p.A324T	+	7:128846040-128846040
SMO	NM_005631.3	13148	c.1210G>A	p.V404M	+	7:128846374-128846374
SMO	NM_005631.3	13150	c.1918A>G	p.T640A	+	7:128851593-128851593
SRC	NM_005417	1369	c.1591C>T	p.Q531*	+	20:36031762-36031762
STK11	NM_000455	12924	c.842delC	p.P281fs*6	+	19:1221319-1221319

STK11	NM_000455	12925	c.109C>T	p.Q37*	+	19:1207021-1207021
STK11	NM_000455	18652	c.996G>A	p.W332*	+	19:1223059-1223059
STK11	NM_000455	20857	c.787_790delTTGT	p.F264fs*22	+	19:1221264-1221267
STK11	NM_000455	20871	c.837delC	p.P281fs*6	+	19:1221314-1221314
STK11	NM_000455	20874	c.180C>G	p.Y60*	+	19:1207092-1207092
STK11	NM_000455	20957	c.581A>T	p.D194V	+	19:1220488-1220488
STK11	NM_000455	21212	c.169delG	p.E57fs*7	+	19:1207077-1207077
STK11	NM_000455	21355	c.842C>T	p.P281L	+	19:1221319-1221319
STK11	NM_000455	21359	c.595G>A	p.E199K	+	19:1220502-1220502
STK11	NM_000455	21360	c.1062C>G	p.F354L	+	19:1223125-1223125
STK11	NM_000455	25229	c.595G>T	p.E199*	+	19:1220502-1220502
STK11	NM_000455	25847	c.580G>A	p.D194N	+	19:1220487-1220487
STK11	NM_000455	27322	c.180delC	p.Y60fs*1	+	19:1207092-1207092
STK11	NM_000455	28298	c.841_842>T	p.P281fs*6	+	19:1221318-1221319
STK11	NM_000455	48786	c.587G>T	p.G196V	+	19:1220494-1220494
TP53	NM_000546	6530	c.723delC	p.C242fs*5	-	17:7577558-7577558
TP53	NM_000546	6545	c.741_742CC>TT	p.R248W	-	17:7577539-7577540
TP53	NM_000546	6549	c.743G>T	p.R248L	-	17:7577538-7577538
TP53	NM_000546	6932	c.733G>A	p.G245S	-	17:7577548-7577548
TP53	NM_000546	10645	c.527G>T	p.C176F	-	17:7578403-7578403
TP53	NM_000546	10647	c.404G>T	p.C135F	-	17:7578526-7578526
TP53	NM_000546	10648	c.524G>A	p.R175H	-	17:7578406-7578406
TP53	NM_000546	10654	c.637C>T	p.R213*	-	17:7578212-7578212
TP53	NM_000546	10656	c.742C>T	p.R248W	-	17:7577539-7577539
TP53	NM_000546	10659	c.817C>T	p.R273C	-	17:7577121-7577121
TP53	NM_000546	10660	c.818G>A	p.R273H	-	17:7577120-7577120
TP53	NM_000546	10662	c.743G>A	p.R248Q	-	17:7577538-7577538
TP53	NM_000546	10663	c.916C>T	p.R306*	-	17:7577022-7577022
TP53	NM_000546	10667	c.646G>A	p.V216M	-	17:7578203-7578203
TP53	NM_000546	10670	c.469G>T	p.V157F	-	17:7578461-7578461
TP53	NM_000546	10690	c.473G>A	p.R158H	-	17:7578457-7578457
TP53	NM_000546	10704	c.844C>T	p.R282W	-	17:7577094-7577094
TP53	NM_000546	10705	c.586C>T	p.R196*	-	17:7578263-7578263
TP53	NM_000546	10709	c.722C>G	p.S241C	-	17:7577559-7577559

TP53	NM_000546	10710	c.892G>T	p.E298*	-	17:7577046-7577046
TP53	NM_000546	10714	c.473G>T	p.R158L	-	17:7578457-7578457
TP53	NM_000546	10716	c.329G>T	p.R110L	-	17:7579358-7579358
TP53	NM_000546	10722	c.853G>A	p.E285K	-	17:7577085-7577085
TP53	NM_000546	10731	c.707A>G	p.Y236C	-	17:7577574-7577574
TP53	NM_000546	10735	c.638G>A	p.R213Q	-	17:7578211-7578211
TP53	NM_000546	10742	c.578A>G	p.H193R	-	17:7578271-7578271
TP53	NM_000546	10758	c.659A>G	p.Y220C	-	17:7578190-7578190
TP53	NM_000546	10769	c.820G>T	p.V274F	-	17:7577118-7577118
TP53	NM_000546	10779	c.818G>T	p.R273L	-	17:7577120-7577120
TP53	NM_000546	10790	c.455C>T	p.P152L	-	17:7578475-7578475
TP53	NM_000546	10808	c.488A>G	p.Y163C	-	17:7578442-7578442
TP53	NM_000546	10810	c.725G>T	p.C242F	-	17:7577556-7577556
TP53	NM_000546	10812	c.722C>T	p.S241F	-	17:7577559-7577559
TP53	NM_000546	10813	c.394A>G	p.K132E	-	17:7578536-7578536
TP53	NM_000546	10817	c.747G>T	p.R249S	-	17:7577534-7577534
TP53	NM_000546	10863	c.833C>T	p.P278L	-	17:7577105-7577105
TP53	NM_000546	10889	c.536A>G	p.H179R	-	17:7578394-7578394
TP53	NM_000546	10891	c.814G>A	p.V272M	-	17:7577124-7577124
TP53	NM_000546	10893	c.824G>A	p.C275Y	-	17:7577114-7577114
TP53	NM_000546	10939	c.832C>T	p.P278S	-	17:7577106-7577106
TP53	NM_000546	11063	c.711G>T	p.M237I	-	17:7577570-7577570
TP53	NM_000546	11073	c.1024C>T	p.R342*	-	17:7574003-7574003
TP53	NM_000546	11081	c.733G>T	p.G245C	-	17:7577548-7577548
TP53	NM_000546	11148	c.476C>T	p.A159V	-	17:7578454-7578454
TP53	NM_000546	11196	c.734G>T	p.G245V	-	17:7577547-7577547
TP53	NM_000546	11218	c.464C>A	p.T155N	-	17:7578466-7578466
TP53	NM_000546	11224	c.394A>C	p.K132Q	-	17:7578536-7578536
TP53	NM_000546	11232	c.842A>G	p.D281G	-	17:7577096-7577096
TP53	NM_000546	11249	c.537T>G	p.H179Q	-	17:7578393-7578393
TP53	NM_000546	11291	c.1006G>T	p.E336*	-	17:7574021-7574021
TP53	NM_000546	11305	c.809T>C	p.F270S	-	17:7577129-7577129
TP53	NM_000546	11606	c.31G>C	p.E11Q	-	17:7579882-7579882
TP53	NM_000546	43559	c.517G>T	p.V173L	-	17:7578413-7578413

TP53	NM_000546	43657	c.569C>T	p.P190L	-	17:7578280-7578280
TP53	NM_000546	43683	c.758C>T	p.T253I	-	17:7577523-7577523
TP53	NM_000546	43827	c.581T>C	p.L194P	-	17:7578268-7578268
TP53	NM_000546	43836	c.475G>C	p.A159P	-	17:7578455-7578455
TP53	NM_000546	43989	c.596G>A	p.G199E	-	17:7578253-7578253
TP53	NM_000546	43990	c.610G>A	p.E204K	-	17:7578239-7578239
TP53	NM_000546	44017	c.869G>A	p.R290H	-	17:7577069-7577069
TP53	NM_000546	44037	c.322G>A	p.G108S	-	17:7579365-7579365
TP53	NM_000546	44151	c.535C>A	p.H179N	-	17:7578395-7578395
TP53	NM_000546	44162	c.635_636delTT	p.F212fs*3	-	17:7578213-7578214
TP53	NM_000546	44241	c.592G>T	p.E198*	-	17:7578257-7578257
TP53	NM_000546	44539	c.584T>G	p.I195S	-	17:7578265-7578265
TP53	NM_000546	44613	c.455C>A	p.P152Q	-	17:7578475-7578475
TP53	NM_000546	44673	c.284C>T	p.S95F	-	17:7579403-7579403
TP53	NM_000546	44769	c.755T>C	p.L252P	-	17:7577526-7577526
TP53	NM_000546	44782	c.520A>T	p.R174W	-	17:7578410-7578410
TP53	NM_000546	44877	c.584T>A	p.I195N	-	17:7578265-7578265
TP53	NM_000546	44908	c.743_744GG>AA	p.R248Q	-	17:7577537-7577538
TP53	NM_000546	45286	c.475G>T	p.A159S	-	17:7578455-7578455
TP53	NM_000546	45307	c.309C>A	p.Y103*	-	17:7579378-7579378
VHL	NM_000551.2	14305	c.266T>A	p.L89H	+	3:10183797-10183797
VHL	NM_000551.2	14311	c.499C>T	p.R167W	+	3:10191506-10191506
VHL	NM_000551.2	14346	c.266T>C	p.L89P	+	3:10183797-10183797
VHL	NM_000551.2	14368	c.473T>A	p.L158Q	+	3:10191480-10191480
VHL	NM_000551.2	14407	c.388G>C	p.V130L	+	3:10188245-10188245
VHL	NM_000551.2	14410	c.440delT	p.F148fs*11	+	3:10188297-10188297
VHL	NM_000551.2	17612	c.481C>T	p.R161*	+	3:10191488-10191488
VHL	NM_000551.2	17658	c.286C>T	p.Q96*	+	3:10183817-10183817
VHL	NM_000551.2	17735	c.444delT	p.F148fs*11	+	3:10188301-10188301

APPENDIX 2: Tumour and Plasma DNA sequencing results using Ion-Torrent PGM™ Platform

Tumour Sequencing Results in Case 1											
Chromosome	**Position**	**Gene Name**	**Reference**	**Variant**	**Variant Frequency**	**Total Coverage**	**Reference Coverage**	**Variant Coverage**	**cDNA change**	**Amino Acid change**	**Cosmic ID**
3	178938877	PIK3CA	G	A	17	934	777	157	not reported, share high similarity with Chromosome 22		
4	1807894	FGFR3	G	A	100	2004	4	2000	benign		
4	55141055	PDGFRA	A	G	99	2603	15	2587	benign		
7	55249063	EGFR	G	A	100	3371	4	3367	benign		
10	43613843	RET	G	T	100	1439	0	1436	benign		
12	25398284	KRAS	C	A	22	3256	2543	703	c.35G>T	p.G12V	520
17	7578406	TP53	C	T	32	1121	759	362	c.524G>A	p.R175H	10648

Time Point 1 Plasma Sequencing Results in Case 1											
Chromosome	**Position**	**Gene Name**	**Reference**	**Variant**	**Variant Frequency**	**Total Coverage**	**Reference Coverage**	**Variant Coverage**	**cDNA change**	**Amino Acid change**	**Cosmic ID**
3	178938877	PIK3CA	G	A	18	530	435	95	not reported, share high similarity with Chromosome 22		
4	1807894	FGFR3	G	A	100	1361	5	1356	benign		
4	55141055	PDGFRA	A	G	100	1225	1	1224	benign		
7	55249063	EGFR	G	A	100	1338	5	1333	benign		
10	43613843	RET	G	T	100	662	0	661	benign		
10	123274819	FGFR2	T	C	4	2477	2367	110	c.1099A>G	p.K367E	de novo

Tumour Sequencing Results in Case 2											
Chromosome	**Position**	**Gene Name**	**Reference**	**Variant**	**Variant Frequency**	**Total Coverage**	**Reference Coverage**	**Variant Coverage**	**cDNA change**	**Amino Acid change**	**Cosmic ID**
3	178938877	PIK3CA	G	A	37	583	370	213	not reported, share high similarity with Chromosome 22		
4	1807894	FGFR3	G	A	100	1158	3	1155	benign		
4	55141055	PDGFRA	A	G	100	1370	3	1366	benign		
5	112175770	APC	G	A	99	4245	62	4182	common polymorphism		
7	55249063	EGFR	G	A	50	2164	1081	1082	benign		
7	55259450	EGFR	C	T	54	1879	862	1017	synonymous/benign variant		
10	43613843	RET	G	T	100	1365	1	1361	benign		
12	25398284	KRAS	C	T	33	1433	956	477	c.35G>A	p.G12D	521
17	7577105	TP53	G	A	1	1444	1428	16	c.833C>T	p.P278L	10863
17	7578442	TP53	T	C	26	711	525	186	c.488A>G	P.Y163C	10808
17	37881000	ERBB2	G	A	1	2489	2460	29	tolerated		
18	48591904	SMAD4	C	G	33	1668	1108	555	c.1067C>G	p.P356R	de novo

Time Point 1 Plasma Sequencing Results in Case 2											
Chromosome	**Position**	**Gene Name**	**Reference**	**Variant**	**Variant Frequency**	**Total Coverage**	**Reference Coverage**	**Variant Coverage**	**cDNA change**	**Amino Acid change**	**Cosmic ID**
3	178938877	PIK3CA	G	A	24	509	385	124	not reported, share high similarity with Chromosome 22		
4	1807894	FGFR3	G	A	100	1871	2	1868	benign		
4	55141055	PDGFRA	A	G	100	1244	1	1243	benign		
4	153249384	FBXW7	C	T	69	2540	799	1740	c.1394G>A	p.R465H	22965
5	112175770	APC	G	A	99	4667	52	4612	common polymorphism		
7	55249063	EGFR	G	A	21	2310	1822	488	benign		
7	55259450	EGFR	C	T	82	2687	485	2202	synonymous/benign variant		
10	43613843	RET	G	T	100	1033	1	1029	benign		
17	7578406	TP53	C	T	55	776	350	425	c.524G>A	p.R175H	10648

Time Point 2 Plasma Sequencing Results in Case 2											
Chromosome	**Position**	**Gene Name**	**Reference**	**Variant**	**Variant Frequency**	**Total Coverage**	**Reference Coverage**	**Variant Coverage**	**cDNA change**	**Amino Acid change**	**Cosmic ID**
4	153249384	FBXW7	C	T	52	908	432	476	c.1394G>A	p.R465H	22965
11	108236201	ATM	G	C	6	1198	1133	65	c.9137G>C	p.S3046T	de novo
17	7578406	TP53	C	T	52	155	76	79	c.524G>A	p.R175H	10648
17	7579398	TP53	C	A	4.8	147	140	7	c.289G>T	p.V97F	de novo

Tumour Sequencing Results in Case 3											
Chromosome	**Position**	**Gene Name**	**Reference**	**Variant**	**Variant Frequency**	**Total Coverage**	**Reference Coverage**	**Variant Coverage**	**cDNA change**	**Amino Acid change**	**Cosmic ID**
4	1807894	FGFR3	G	A	100	265	0	265	synonymous/benign variant		
4	55141055	PDGFRA	A	G	100	2150	8	2142	benign		
4	55953853	KDR	G	A	4.8	3871	3684	186	tolerated		
5	112175216	APC	G	A	1	2434	2405	29	tolerated		
5	112175612	APC	C	G	7	1088	1014	72	tolerated		
5	112175615	APC	C	A	10	1084	970	113	tolerated		
5	112175634	APC	C	G	8	532	487	44	tolerated		
5	112175770	APC	G	A	46	3605	1932	1672	common polymorphism		
7	116411990	MET	C	T	47	3769	1999	1770	c.3029C>T	p.T1010I	707
7	140453136	BRAF	A	T	11	2419	2162	256	c.1799T>A	p.V600E	476
10	43613843	RET	G	T	100	1777	2	1773	benign		
12	25398284	KRAS	C	T	2	3068	3008	58	c.35G>A	p.G12D	521
17	7579358	TP53	C	T	1	358	354	4	c.329G>A	p.R110H	46115

Time Point 1 Plasma Sequencing Results in Case 3											
Chromosome	**Position**	**Gene Name**	**Reference**	**Variant**	**Variant Frequency**	**Total Coverage**	**Reference Coverage**	**Variant Coverage**	**cDNA change**	**Amino Acid change**	**Cosmic ID**
4	1807894	FGFR3	G	A	100	1251	1	1250	benign		
4	55141055	PDGFRA	A	G	100	866	1	865	benign		
5	112175770	APC	G	A	10	2657	2391	266	common polymorphism		
7	116411990	MET	C	T	40	2019	1200	817	c.3029C>T	p.T1010I	707
10	43613843	RET	G	T	100	1586	2	1583	benign		
13	28608281	FLT3	A	G	1	398	394	4	c.1775T>C	p.V592A	19522
17	7577539	TP53	G	A	68	2383	756	1617	c.742C>T	p.R248W	10656

Tumour Sequencing Results in Case 4											
Chromosome	**Position**	**Gene Name**	**Reference**	**Variant**	**Variant Frequency**	**Total Coverage**	**Reference Coverage**	**Variant Coverage**	**cDNA change**	**Amino Acid change**	**Cosmic ID**
4	1807894	FGFR3	G	A	100	1325	1	1323	benign		
4	55141055	PDGFRA	A	G	100	1149	3	1146	benign		
5	112175770	APC	G	A	1	3676	3636	39	common polymorphism		
7	116339672	MET	C	T	60	2333	920	1402	synonymous/benign variant		
10	43613843	RET	G	T	48	487	255	232	benign		
10	123274819	FGFR2	T	C	4.7	1924	1832	91	c.1099A>G	p.K367E	de novo
12	25398285	KRAS	C	A	56	943	412	529	c.34G>T	p.G12C	516
17	7577105	TP53	G	A	1	2037	2010	27	c.833C>T	p.P278L	10863
17	7578395	TP53	G	A	1	510	504	6	c.535C>T	p.H179Y	10768
17	7578526	TP53	C	T	63	822	295	520	c.404G>A	p.C135Y	10801

Time Point 1 Plasma Sequencing Results in Case 4											
Chromosome	**Position**	**Gene Name**	**Reference**	**Variant**	**Variant Frequency**	**Total Coverage**	**Reference Coverage**	**Variant Coverage**	**cDNA change**	**Amino Acid change**	**Cosmic ID**
4	1807894	FGFR3	G	A	100	1408	0	1408	synonymous/benign variant		
4	55141055	PDGFRA	A	G	100	1221	0	1221	benign		
7	116339672	MET	C	T	56	3222	1408	1799	synonymous/benign variant		
10	43613843	RET	G	T	49	1099	555	543	benign		
12	25398285	KRAS	C	A	32	876	593	282	c.34G>T	p.G12C	516
17	7578526	TP53	C	T	16	1975	1647	323	c.404G>A	p.C135Y	10801