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Investigation of Mutant DNA in Plasma of Patients with Colorectal Neoplasia

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TABLE OF CONTENTS:

Dedication

I dedicate this thesis with all my love and gratitude to my wife, Claire and my sons Charlie, Alex and William.

Declaration

I declare that this thesis was composed entirely by myself and the work presented is my own unless otherwise stated. I have included data generated in collaboration with colleagues within the research group, which allowed presentation of complete data on microsatellite analysis of plasma DNA.

Andrew JE Clark

Thesis Advisors

Professor MG Dunlop

Dr SM Farrington

Acknowledgements

The work presented in this thesis would not have been possible without the support and opportunities afforded to me by Professor Malcolm Dunlop. He has been invariably patient and generous with his time, knowledge and resources and I remain deeply in his debt. I am grateful to Professor Dunlop and the rest of his research group for providing a stimulating, challenging and robust scientific environment in which to venture into molecular biological research. My particular thanks go to Dr Susan Farrington again for her patience and knowledge as well as her instruction in molecular techniques that informed the progress of this work. Further invaluable technical support was kindly given to me by Stewart MacKay of the MRC Human Genetics Unit, particularly for his help with utilising automated robotic hardware and quantitative spectrofluorometry. My thanks also go to Mark Ong for continuing laboratory aspects of microsatellite analysis that allowed presentation of complete replicate data.

Fundamental to this project was the kind cooperation of clinical colleagues, notably the consultant surgeons of the Western General Hospital, Edinburgh for allowing their patients to be approached to participate in the study, and also the pathology department of the same hospital, particularly Dr A. Lessels, for giving of their time and expertise in providing access to pathology specimens.

My heartfelt thanks go to the patients who, almost without exception, kindly volunteered their time and clinical samples around what was a difficult and demanding time for them.

Abbreviations

AI	Allelic Imbalance
AJCC	American Joint Committeeon Cancer
APC	Adenomatous polyposis coli
bp	Base pair
CEA	Carcinoembryonic antigen
CNAPS	Circulating nucleic acids in plasma and serum
CRC	Colorectal cancer
CTC	Computerised tomography colonoscopy
DCC	Deleted in colon cancer
ds DNS	Double stranded DNA
°C	Degrees centigrade
EBV	Epstein barr virus
FAP	Familial adenomatous polyposis
FAM	5 carboxyfluorescein
FOBT	Faecal occult blood testing
HEX	6-carboxy-1,4-dichloro-2',4',5',7'tetrachlorofluorescein
HNPCC	Hereditary non polyposis colon cancer
ICT	Immunochemical tests
LOH	Loss of heterozygosity
mDNA	Mitochondrial DNA

MGB	Minor groove binding
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
MSS	Microsatellite stable
PBS	Phosphate buffered saline
RFLP	Restriction fragment length polymorphism
ROC	Receiver operator curve
SAP	Shrimp alkaline phosphatase
SNP	Single nucleotide polymorphism
TE	Tris EDTA
TGFβRII	Transforming growth factor β receptor two
VIC	4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein
WHO	World Health Organisation

ABSTRACT

Introduction:

Colorectal cancer is a major clinical and public health problem.Early stage colorectal cancer is amenable to surgical intervention with a high cure rate. Screening for the disease has now been rolled out throughout the UK, although current screening modality of faecal occult blood testing (FOBT) is suboptimal. Specific genetic alterations found in primary tumours, including mutations in proto-oncogenes, microsatellite instability and loss of heterozygosity (LOH), have been shown to be detectable in the host plasma. Colorectal cancer is an excellent paradigm in which to investigate the application of assays to detect tumour-related plasma DNA. There is substantial data regarding specific mutations and their frequency in colorectal cancer tissue and adenomas. The ability to detect early stage colorectal neoplasia using DNA plasma assays holds considerable promise as a non-invasive screening modality.

Materials and Methods:

Assay performance was assessed using colorectal cancer cell lines and archived material from genetic studies. Assays were then applied to a prospective cohort of 124 colorectal neoplasia cases and controls. Plasma DNA was quantified using a sensitive DNA binding fluorescent dye and detection platform. A well-characterised tumour-specific mutation was used as the target for the first assay. This mutation was in a poly A tract of the transforming growth factor beta receptor II (TGFβRII) gene and was assessed using a restriction fragment length polymorphism (RFLP) assay. Microsatellite analysis was performed using 3 polymorphic markers relevant to colorectal neoplasia in matched tumour, normal and plasma DNA samples. A novel assay was developed exploiting real-time fluorescent PCR amplification of single nucleotide polymorphisms in the adenomatous polyposis coli (APC) gene as a means to detect tumour-specific allelic imbalance. Assay performance was determined in spiking experiments, and performance assessed in clinical samples. A further iteration of the assay was developed to quantify tumour specific alleles in plasma by counting individual alleles PCR amplified from plasma DNA of cases and controls.

Results:

Quantification of total plasma DNA revealed a significant difference between cases and controls (area under the receiver operator curve: 0.7). Despite intensive efforts to overcome the problem, the TGFβRII RFLP assay was affected by technical difficulties when applied to

plasma DNA. The required high number of PCR cycles introduced artefact and limited its applicability. Microsatellite analysis demonstrated LOH in matched tumour and plasma samples with maximal sensitivity of 67% but specificity was only 32%. The quantitative PCR assay to detect APC LOH was able to detect 3-5ng of homozygous DNA introduced into 1 ml of heterozygous plasma, and accurately quantified LOH in tumour tissue. However, it was not able to discriminate cases from controls by assessment of plasma DNA. Counting of alleles in plasma DNA from a test case demonstrated matching LOH to that seen in the primary tumour. Allele counting of a further cohort suggested that this approach has a low false positive rate.

Discussion:

Analysis of plasma DNA is technically challenging due to a low abundance of partially fragmented mutant DNA sequences admixed with normal DNA in plasma with DNAases and PCR inhibitors. However total quantity of plasma DNA was higher in cancer cases compared to controls with good specificity for cancer at high DNA concentrations. Fluorescent microsatellite analysis of plasma DNA demonstrated encouraging overall sensitivity but poor specificity that waslikely at least partly a reflection of low DNA abundance and hence sampling error within aliquots of plasma DNA. A quantitative PCR approach was developed and validated with relevant levels of in vitro sensitivity, and improved discrimination of LOH in some clinical samples. Adaptation of this approach to count alleles individually is labour intensive and expensive but appears to address issues of sampling error due to abundance and hence improves specificity. These data, whilst highlighting some of the technical challenges in the field, demonstrate associations of plasma DNA in colorectal neoplasia that warrant further investigation .

CHAPTER 1:

INTRODUCTION

1.1 Colorectal Cancer

1.1.1 Colorectal Cancer Epidemiology and Environmental Risk factors

Colorectal cancer (CRC) is a leading cause of cancer death in Western populations. The World Health Organisation (WHO) estimates 945 000 new cases occur annually worldwide, with 492 000 deaths (1). In the UK there are around 30,000 new cases and 20,000 deaths annually from the disease (2)(Cancer Research Campaign, Cancer Stats: Incidences—UK March 2005).In Scotland in 2003 there were 3,365 incident cases of colorectal cancer and 1,582 deaths from the disease (3). 5 year survival rates in the period 1997-2001 were 40.8% for males and 40.7% for females (3). As a result, colorectal cancer is the third commonest cause of cancer behind lung and breast cancers, and second only to lung cancer as a cause of cancer death for both sexes combined. More than 50% of Western populations will develop an adenomatous polyp by the age of 70, and around 1 in 10 of these will develop cancer (4). Therefore around 5% of Western populations will develop colorectal cancer in their lifetime (5).

Survival rates from colorectal cancer have improved marginally over a number of decades, and improvements are attributable to a number of factors including improved peri-operative care and detection modalities as well as the development of adjuvant treatments. Despite these modest improvements, CRC remains a common and lethal disease with considerable clinical and public health impact.

However, the incidence and impact of colorectal cancer is dramatically lower in under developed countries (6), and the high incidence in developed countries has been suggested to be partly due to factors in Western diets.

There is conflicting evidence from epidemiological studies as to whether high fibre diets have a protective effect against the development of colorectal neoplasia. High fibre diets might be protective by bulking the stool and reducing intestinal transit time hence diluting dietary carcinogens and reducing the time that the colorectal mucosa is exposed to them. Case-control studies have suggested a protective effect of high fibre diet (7) which has not been bourne out in other studies (8;9). High vegetable consumption has also been associated with reduced colorectal neoplasia risk (10), particularly brassica vegetables (11). Conversely it has been suggested that high fat diets are implicated in increased risk of colorectal cancer although again reports have generated conflicting results. Whilst some data have suggested an association (12) this may be due to confounding lifestyle factors associated with a high fat diet. Interventional studies utilising low fat diets have failed to demonstrate alterations in the rate of colorectal neoplasia (13). Increased levels of alcohol consumption have also been linked with increased colorectal cancer risk, particularly in conjunction with associated dietary deficiencies (14).

Various other associations between colorectal neoplasia risk and dietary factors have been postulated including vitamins, salts and trace elements (15) and it seems likely that dietary risk is a function of complex interactions of multiple interrelated ingested substances, along with attendant lifestyle factors.(16;17). A summary report of diet and lifestyle factors was published by the world cancer research fund in 2007 highlighting the relative contributions of the above factors, as well as other parameters including abdominal fatness and height (http://www.dietandcancerreport.org/cup/current_progress/colorectal_cancer.php)

There is evidence that secondary bile acids may be carcinogenic, and as such the altered enterohepatic circulation following cholecystectomy may result in prolonged exposure to carcinogenic stimuli. Metaanalyses of the rate of colorectal cancer following colocystectomy have indeed shown a mildly elevated risk of colorectal cancer (18), although a cause and effect relationship has not been demonstrated.

1.1.2 Clinical Aspects of Colorectal Cancer

a) Presentation, Investigation and Staging

The clinical presentation of colorectal cancer is diverse and results in symptoms requiring attention either electively or as an emergency. Emergency presentation and operative intervention may be indicated on the basis of tumour obstruction, perforation or bleeding, whereby surgical management is associated with poorer outcomes both in short and long terms (19) and particularly with respect to the ageing population with this disease (20). The cardinal symptoms and signs of CRC that present electively include rectal bleeding, change in bowel habit, abdominal pain, anaemia and the finding of an abdominal mass, with the symptomatic picture being dependant on the anatomic site of the tumour. The relative frequencies of colorectal cancer sites are demonstrated figure 1.1.Figure 1.1

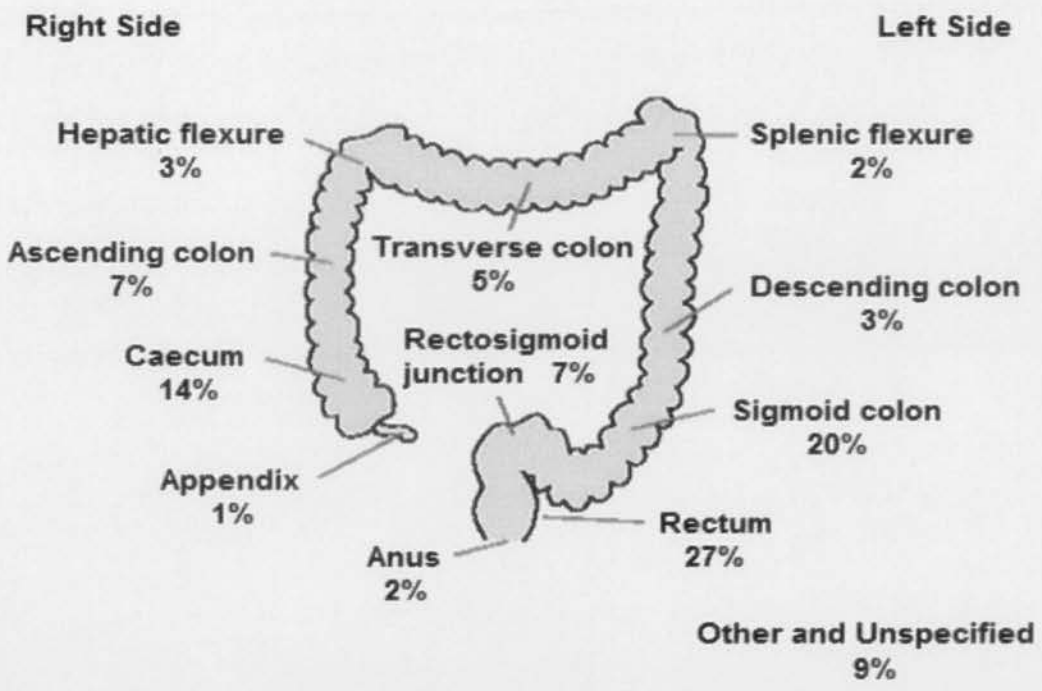


Figure 1.1 legend: Distribution frequency of colorectal cancer at different anatomic sites. (21)

The gold standard diagnostic test for colorectal cancer is direct visualisation and biopsy at colonoscopy, which is also able to exclude the presence of synchronous tumours. Investigation of lower gastrointestinal symptoms is also performed using double contrast barium enema, as well as an increasing role of computerised tomography (CT) colonography, discussed further in section 1.3.3.

Following a diagnosis clinical pre-operative disease staging is performed with imaging modalities, most commonly by CT scanning of chest and abdomen, although there remains a place for plain chest radiography and liver ultrasound to look for distant metastasis. There is an increasing role for magnetic resonance imaging (MRI) particularly in the assessment of local extent of rectal cancer to inform the use of neoadjuvant therapy (http://www.sign.ac.uk/pdf/sign67.pdf).

Surgery remains the mainstay of colorectal cancer treatment and provides the only opportunity for cure. Excision of the affected portion of bowel along with its arterial supply

and attendant lymphatic drainage with appropriate oncological margins is coupled to technical considerations regarding restoration of intestinal continuity where appropriate. The ability to resect all macroscopic evidence of tumour and detailed histological examination of the resection provide fundamental information relating to prognosis.

Classical staging of colorectal cancer remains the most important predictor of survival following resection, and informs the role of adjuvant therapy discussed below. The initial staging described by Dukes (22) remains valid with more recent modifications or alternatively TNM (tumour, node, metastasis) classification given below in figure 1.2.

Figure 1.2:

Table 7.1: Staging of colorectal cancers

UICC/TNM		Modified Dukes'
Stage 0	Carcinoma in situ	A
Stage I	No nodal involvement, no distant metastasis Tumour invades submucosa (T1, N0, M0) Tumour invades muscularis propria (T2, N0, M0)	
Stage II	No nodal involvement, no distant metastasis Tumour invades into subserosa (T3, N0, M0) Tumour invades into other organs (T4, N0, M0)	B
Stage III	Nodal involvement, no distant metastasis 1 to 3 regional lymph nodes involved (any T, N1, M0) 4 or more regional lymph nodes involved (Any T, N2, M0)	C
Stage IV	Distant metastasis (any T, any N, M1)	D

Figure 1.2 legend: AJCC 5th edition TNM staging of colorectal cancer.

http://www.cancerstaging.org/products/ajccguide

A fundamental problem in impacting on the poor outcomes of colorectal cancer is that the disease commonly becomes symptomatic after the disease is no longer localised, reducing the possibility of surgical cure. For example in the non-screened arm of the Nottingham faecal occult blood test (FOBT) screening trial 11, 37, 23 % of cancer cases had Dukes A, B and C stage cancers respectively, with a further 24% have distant metastasis at presentation (23).

English data, from the national cancer intelligence network, are given in the table below along with the 5 year survival following diagnosis of each stage of disease.

Number of cases (1996-2006) and five-year relative survival of colorectal cancer patients (diagnosed 1996-2002) by stage at diagnosis, England. http://www.ncin.org.uk/publications/data_briefings/colorectal_cancer_survival_by_stage.aspx

Stage at diagnosis	Number of cases	Percentage of cases (%)	Percentage of cases excl.Unknown (%)	5-year relative survival (%)	Confidence interval (95%)
Dukes A	26,727	8.7	13.2	93.2	92.5 - 93.9
Dukes B	74,784	24.2	36.9	77.0	76.4 - 77.5
Dukes C	72,806	23.6	35.9	47.7	47.1 - 48.3
Dukes D	28,377	9.2	14.0	6.6	6.1 - 7.0
Unknown	106,040	34.3		35.4	35.0 - 35.8
Total	308,734	100.0	100.0	50.7	50.4 - 51.0

b) Adjuvant treatment

Over recent decades an increasing role has developed for non surgical treatment modalities. These treatments are aimed at reducing the chance of tumour recurrence after potentially curative surgery, or at symptomatic control where surgery cannot effect potential cure and is not considered the most appropriate method of palliation. The widespread introduction of adjuvant chemotherapy has occurred following many randomised controlled trials analysing subsets of pathological stage disease with various treatment regimes. Several studies have demonstrated a survival benefit for 5-fluoruracil based regimes in combination with other

agents (e.g. levamisole(24), folinic acid (25;26)) in patients with lymph node metastases. The role of adjuvant chemotherapy in patients without lymph node metastasis is less clear. This has resulted in national guidelines recommending that adjuvant chemotherapy should be available for patients with lymph node metastasis but not for those without (http://www.sign.ac.uk/guidelines/fulltext/67/section9.html#ref165). It is necessary to select patients that are most likely to benefit from adjuvant chemotherapy, which may result in an overall survival benefit of around 5-10%, whilst not exposing those who will not benefit from it to treatment toxicity. Currently the best selection is via classical disease staging, although biological parameters and biomarkers hold promise for more accurate selection of patients that need and will respond to chemotherapy. There is also compelling evidence to support the use of neo-adjuvant radiotherapy in selected cases of rectal cancer, which in conjunction with the surgical technique of total mesorectal excision results in reduced local recurrence rates (27). Recently an increasing role of surgical resection of liver metastases has evolved with 5 year survival rates of over 30% in carefully selected populations (28). (29)(30)

c) Approaches to Improving Colorectal Cancer Outcomes

There are several strategies with potential to impact on colorectal cancer mortality in the future. Primary disease prevention provides an ideal approach by reducing disease incidence to some degree. To this end there is substantial research activity into substances, such as aspirin, that reduce rates of colorectal neoplasia (31-33). Similarly identification of further dietary risk factors and altering ingestion rates in the general population may reduce the incidence of colorectal cancer, although clearly this is a very large public health undertaking. Secondary prevetion of colorectal cancer is already well developed with compelling evidence for colonscopic surveillance of patients who have previously developed colorectal cancer as well as groups of patients at high risk of the disease (34). There is further potential to develop

this approach by identification of more at risk groups with the discovery of additional risk alleles and modifier genes(35).

An alternative approach is to detect disease at an earlier stage when more amenable to surgical cure. To this end there is a wealth of evidence regarding preclinical detection of colorectal neoplasia by screening and the development of clinical biomarkers that are discussed further in sections 1.4 and 1.5, and are the basis of this thesis.

Additionally there are continuing efforts to improve the efficacy of treatments following a diagnosis of colorectal cancer including the evaluation of further chemotherapy agents and identification of patients likely to benefit from adjuvant treatments based on molecular and genetic profiling.

1.2 CRC genetics

Colorectal cancer is a disease caused by genetic mutation. Alteration of genetic sequences that code for essential cellular components and control mechanisms result in dysregulation of these genes and associated pathways. As such cellular processes are altered, resulting in the affected cell behaving abnormally and ultimately in exhibiting a cancerous phenotype. The stimulus for genetic mutation causing a change in cell phenotype is exposure to time and environmental risk factors. However the cells of different individuals are at greatly varying risk of the mutational stimulus resulting in genotypic and phenotypic alteration. As such the aetiology of colorectal cancer is multifactorial, with major contributions from genetics and environment. There is a large body of evidence highlighting the importance of genetics in CRC from descriptive data on cancer occurrence in relatives of affected individuals, and from cohort studies (36). However the relative contribution of genetic susceptibility to the entirety of colorectal cancer remains difficult to fully elucidate. A Scandinavian twin study demonstrated that 35% of all colorectal cancer could be attributed to genetic predisposition (37). Approximately 20% of all colorectal cancer cases occur in individuals with more than one first or second degree relative also affected (38). Within this proportion there exist subgroups of patients with an identifiable genetic mutation conferring greatly elevated CRC risk, constituting hereditary colorectal cancer syndromes. During the expansion of molecular genetic research over recent decades, these subgroups have been subject to extensive investigation. As such many genotype-phenotype characterisations have been identified, allowing tailoring of clinical management aimed at improving outcomes. Investigation of hereditary colorectal cancer syndromes has partially elucidated the pathogenesis of the totality of colorectal cancer, by identifying important genes in colorectal cancer and examining their relative frequencies and phenotypic correlations. Analysis of genetic

sequences from tumour tissue of colorectal and other cancers has identified a number of genes demonstrated to be important in carcinogenesis including oncogenes, tumour suppressor genes and mismatch repair genes discussed further below. Additionally genome wide association studies have identified susceptibility alleles, which engender a less marked degree of risk, and are the basis for cancer genetic variance.

Cancer genetic variance

1.2.1 Adenoma-carcinoma sequence:

There is considerable indirect evidence that colorectal cancer develops from adenomas, with a morphological progression from normal epithelium through small early adenomas, large dysplastic adenomas and frank carcinoma. This evidence includes the observation that the prevalence of adenomas precedes carcinomas by around 5 years (39) and adenomas are found at different colonic sites at similar relative frequencies to cancers (40). The benign adenomas of familial adenomatous polyposis (FAP) have almost identical morphology to sporadic colorectal adenomas and adenomas in FAP have an almost 100% rate of malignant transformation by the fourth decade of life. Also incidental benign polyps are frequently found within colonic specimens resected for cancer (41), and cancers are often surrounded by benign adenomatous tissue, from which they are thought to have arisen. A proportion of polyps have been shown to enlarge at serial follow up, and cancer shown to develop at the site of index polyp in a smaller proportion (42). Furthermore large adenomas are more commonly dysplastic (39), and have a higher malignant potential (43). Significantly removal of benign polyps has been shown to decrease CRC incidence in high risk populations (34;44), and in average risk individuals undergoing screening (45), whilst patients with demonstrated polyps have a higher colorectal cancer mortality rate (46).

1.2.2 Multistep theory of colorectal carcinogenesis

In the late 1980s Vogelstein (47) proposed a genetic mechanism for the development of colorectal neoplasia involving sequential mutation at a series of genes, and correlating with the morphological progression of colorectal adenomas. This group examined the frequencies of mutations at different stages of colorectal neoplasia, for four common genetic aberrations, namely Kirsten ras (K-ras) mutation and allelic deletions of chromosomes 5,17 and 18. They demonstrated that whilst K-ras mutations and chromosome 18 allelic losses were common in cancer and late stage adenomas, these alterations were rare in early adenomas. They concluded that these results suggested a model of colorectal carcinogenesis where the steps required involved the mutational activation of oncogene(s) coupled with the loss of function of tumour suppressor genes(47). Each mutation is postulated to confer an advantage to that cell, resulting in a disproportionate excess of the offspring of that cell and its subsequent generations; clonal expansion. Acquisition of further mutations at different loci within these cells result in development of further abnormal phenotype, such as uninhibited cell growth, loss of adhesion and ultimately the ability to metastasise, i.e. a malignant phenotype (48-50). This multi-step process involving mutational events on both oncogenes and tumour suppressor genes has been termed chromosomal or genetic instability. Genetic instability is a defining molecular signature of most human cancers (51), and is characterised molecularly by allelic imbalance, representing losses or gains of defined chromosomal regions (52), and is now generally accepted worldwide for the development of most cancers, with evidence that genetic instability occurs at an early stage of colorectal tumourigenesis (53). The fundamental genetic change of LOH occurs where one allele is contained in a chromosomal segment that is deleted due to mutation, resulting in only the other allele being functional and detectable.

With respect to the deleted region containing a tumour suppressor gene, function of the gene is lost when a further mutational event affects the remaining allele.

1.2.3 Tumour Heterogeneity

A controversial feature of colorectal neoplasia is the existence of intratumour heterogeneity. The multistep theory of carcinogenesis postulates that the step wise accumulation of mutation results in a progressive survival advantage for the affected cell or clone, and results in tumour progression and proliferation of that clone. As such early mutational events would be expected to be observed in all the subsequent clones, and hence a degree of tumour homogeneity(54). However there is evidence that intratumour heterogeneity exists in colorectal cancer. Studies analysing multiple portions of microdissected tumours or metastases have identified differing genetic aberrations from different portions of the same tumour. Whilst the extent of this phenomenon remains to be clearly elucidated, its potential to affect results of analysis of DNA extracted frombodily fluids which may be derived from any or all tumour clonesare clear and are discussed further in following sections.

1.2.4 Mutator phenotype pathway

Approximately 15% of colorectal cancers demonstrate an alternative molecular phenomenon of microsatellite instability (MSI) or replication error positivity (RER+), whereby the integrity of short repetitive tracts of DNA (microsatellites) is not maintained (55-57). This is due to dysfunction of the mismatch repair system, which has a role in the recognition, excision and repair of DNA replication errors. Hereditary MSI-H colorectal tumours are due to an inherited germ-line mutation in mismatch repair genes, whereas sporadic MSI-H

tumours result from acquired mutations in the same genes, with promoter methylation an important mechanism. However there is convincing evidence that the classical model of progression of adenoma to carcinoma is applicable to hereditary non polyposis colon cancer (HNPCC) CRC. Adenomas predate carcinoma in young HNPCC kindreds, and have also been shown to demonstrate similar molecular characteristics to HNPCC carcinomas, including MSI. Importantly surveillance with colonoscopic polypectomy reduces cancer incidence and improves survival (58), this being the rationale underpinning the identification of germline mutation carriers. Conversely sporadic MSI-H CRC has been suggested to occur from precursors other than the classical adenoma. These include hyperplastic and serrated polyps as well as aberrant crypt foci, on the basis of several factors including the absence of mutations in sporadic MSI-H CRC which are abundant in adenomas, and the occurrence of mutations associated with sporadic MSI-H in the above precursors (59;60). The ability to identify and treat premalignant precursors has been robustly shown to affect colorectal cancer survival.

When microsatellite stable (MSS), MSI-L and HNPCC MSI-H have been stringently excluded from analysis, the resultant sporadic MSI-H tumours have been shown to have reduced frequencies of genetic aberrations in genes including APC, K-ras and TP53, as well as less frequent LOH of chromosomes 5q, 17p and 18q (61-63). However the differential frequency of these mutations has not been demonstrated in other studies (64).

1.2.5 Promoter Methylation

Gene promoter regions may undergo aberrant methylation on CpG islands causing dysregulation of gene transcription. This process can result in epigenetic silencing of genes, and has been implicated in the pathogenesis of cancer (65). Many tumour suppressor genes

show evidence of methylation silencing, providing a pathway for tumour suppressor gene inactivation (66).

There is evidence that promoter methylation is an important mechanism in colorectal cancer, most specifically in sporadic MSI cancer (67), although aberrant methylation has been associated with left sided tumours and stage 4 disease (68), with additional evidence of relevance to prognosis since promoter methylation of genes involved in apoptosis ,such as hMLH1, can occur in tumour models of resistance to chemotherapeutic drugs (69) There is also increasing amounts of evidence that promoter methylation is involved more diffusely in carcinogenesis. For example P 16 methylation is a common feature in neoplasia (70), including as an early event in lung carcinogenesis (71), and 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers (72).

Abnormal methylation can be detected using methylation specific PCR, which is sensitive to in vitro dilution to between 1 in 500 and 1in 1000 (73;74). Treatment of DNA with bisulfite results in the conversion of unmethylated cytosine residues to uracil, whilst methylated residues remain unchanged. Therefore following bisulphite treatment the methylated and unmethylated sequences are different, allowing the design of specific primers that amplify only one or the other (75).

This technique allows assessment of the methylation status across a broad range of important genes, dysfunction of which have been implicated in carcinogenesis, and hence provides an attractive target for the development of molecular biomarkers.

1.2.6 Alternate Molecular Pathways

Since the completion of the work presented in this thesis there has been considerable further progress in understanding the molecular pathways of colorectal cancer. Whilst this largely

relates to the increasing understanding of the importance of CpG island methylator phenotype (CIMP), it has also clarified the interrelations of this phenomenon with the pathways indicated above. As such Jass proposed a new molecular classification of colorectal cancer(76), which characterises 5 molecular classes , and is presented in the table below with suggested relative frequencies of each class for the totality of colorectal cancer

Group	CIMP	MSI	Kras	APC	BRAF	Frequency
1	++	++	-	-	+	12%
2	+	+ or -	-	-	+	8%
3	-	+ or -	+	-	-	20%
4	-	-	-	+	-	57%
5	-	++	-	-	-	3%

From the above table it can be seen that upto 20% of colorectal cancers may demonstrate mutation in the B type Raf kinase gene, however this was not appreciatated until after the completion of this work hence there was no attempt to develop an assay to this marker

1.2.7 Specific Genes in Colorectal Cancer

In relation to colorectal cancer there is a large body of evidence supporting the multistep model of carcinogenesis and illustrated by mutations observed in genes important in colorectal cancer described below.

It is apparent that mutational rates at different loci vary, both in relation to patient demographics (age, sex, site) and also according to stage of disease, a consideration of direct relevance to biomarker discovery and performance

a) APC

Studies of affected families identified the APC gene, responsible for the FAP phenotype. The gene was localised to the region 5q21(77-81), and encodes a 312 kDa protein (82). The APC protein is multifunctional but primarily interacts with the intracellular protein β-catenin which transcriptionally activates downstream genes such as c-myc and cyclin D1. These pathways are implicated multiple processes including cellular adhesion and chromosome stability.

APC protein phosphorylates β-catenin (83) resulting in its ubiquitination and degradation, hence inhibiting cellular proliferation and promoting apoptosis (84). Dysfunction of this system therefore generates neoplastic cellular characteristics.

Inactivating mutations are classical of those of a tumour suppressor gene, with mutations of one allele and allelic loss of the other allele commonly found in colorectal tumours of FAP and sporadic neoplasms. Mutations frequently result in protein truncation, and whilst there is a relative excess of mutations in exon 15(85), they may occur at any point of the gene, making mutation detection laborious.

Aberrations of the APC gene are among the earliest genetic events in colorectal neoplasia (86), and has been postulated as a gatekeeper mutational event, resulting in adenoma formation (48). APC mutations have been observed in the earliest histological lesions associated with colorectal neoplasia (87), and are common in early adenomas (~50%)(48). APC mutations per se are clearly however not a universal event with some studies determining mutation rates of between 60–80% in colorectal cancers (85;88), and much

lower rates of 5q LOH in other reports (89). However the pathway is dysregulated in nearly all colorectal cancer with epigenetic silencing implicated as an alternative mechanism.

The biological importance of APC is great both in sporadic and familial colorectal cancer and is highlighted by the familial cancer syndrome FAP. FAP is an autosomal dominant syndrome, with very high penetrance, caused by germline mutation of the APC gene. Affected individuals develop multiple colorectal polyps at a young age with inevitable development of cancer without intervention, as well as a number of clinically important extracolonic manifestations. One inactivated APC allele is inherited from the affected parent, and phenotypic changes occur when the function of the second allele from the other parent is lost by somatic mutation. The inherited mutant allele has relevance to the phenotypic expression of disease, and provides one of the clearest examples of genotype-phenotype correlation. For example mutations between codons 1250-1464 produce profuse polyposis (90), whereas associated desmoid tumours occur with mutations between codons 1403-1578.

b) P53

The p53 gene is located on chromosome 17p, and is one of the most characterised tumour suppressor genes, having been called the guardian of the genome (91). p53 protein acts as a transcription factor, influencing multiple genes, and effects G1 cell cycle arrest (92), allowing either DNA repair or induction of apoptosis (93). Transcription and translation of wild type p53 is induced by cellular stress through the protein MDM2 (94). As such p53 helps to safeguard against the propagation of mutations to the next generation of a cell's offspring (95). Mutation of p53 can therefore result in genetic instability, increased cell survival and inappropriate cell growth, and has long been associated with the development of a variety of cancers. Germline p53 mutation is responsible for the neoplastic Li-Fraumeni syndrome, and

examples of sporadic p53 mutation are described for most human cancers, although the precise mutations may vary according to primary tumour site (96)

Detectable p53 mutations are common in colorectal neoplasms. It has been proposed that p53 mutation occurs at a relatively late stage of the adenoma-carcinoma sequence on the basis that mutation is relatively rare in early adenomas, but common in carcinomas. Mutations have been demonstrated in around a quarter of adenomas, in half of adenomas with high grade dysplasia, and upto three-quarters of colorectal cancers (47;97). In addition to p53 mutations occuring in 70% of advanced colorectal cancer the presence of the mutation has been reported as an adverse prognostic factor (98).

The mutations of the p53 gene observed in colorectal cancer are classical of those associated with a tumour suppressor. Therefore it is common to detect allelic loss of 17 p and mutation in the remaining allele of the p53 gene. Allelic deletions of the short arm of chromosome 17 are observed in around 75% of colorectal cancers, and the region of allelic loss has been mapped to that containing the p53 gene (99). In the same study the remaining alleles were shown to contain mutations in a highly conserved region of the p53 gene. Multiple p53 mutations have been observed and whilst there is not a specific mutational 'hotspot', alterations are commonly located in exons 5-8 of the gene (82)
Allelic deletions are also commonly observed on the long arm of chromosome 17 (17q) in colorectal cancer having been described in around 40-50% of colorectal cancers (100), and are commonly been assessed using surrogate analysis with the microsatellite marker D17S250.

c) Deleted in Colon Cancer (DCC)

Early landmark studies of colorectal cancer genetics identified that a region on the long arm of chromosome 18q underwent allelic loss in over 70% of cases (97;101). This region was subsequently shown to contain a gene, termed deleted in colon cancer (DCC), which encoded for a protein with similarities to known cell adhesion molecules. Expression was shown to be decreased in colorectal cancer, with mutations including point mutations and insertions, which coupled with the high prevalence of allelic loss intimated the presence of a tumour suppressor gene (49).

DCC appears to be important late in the adenoma carcinoma sequence, mutation occurring infrequently in small adenomas (13%), but commonly (73%) in cases with carcinoma (47). DCC loss has also been associated with poorer prognosis (102)

d) Ras

The Kirsten Ras gene is located on the chromosome 12p and encodes a 21 kDa protein, the function of which is involved in intracellular signalling and hence control of cellular processes such as proliferation (103). K-ras is primarily involved in GTP signalling by binding and allowing hydrolysis of GTP resulting in GDP that in turn inhibits ras protein activity. GTP initiated intracellular cascade is an integral step in the transmission of many mitogenic signals and effects transcription factors such as c-myc, whose dysregulation results in abnormal cellular proliferation (104). K-ras mutation results in overactivity of the protein, allowing continued or inappropriate signal transduction and a hyperproliferative cellular state. K-ras mutations are also associated with up regulation of other factors thought to be important in colorectal carcinogenesis such as cyclin D1 (105).

The frequency of K-ras mutations in colorectal neoplasia specimens has been shown to vary according to the stage of neoplasia, such that 47% of colon cancers, 58% of large adenomas but only 9% of small adenomas exhibit mutations (47). As such K-ras mutation is thought to be important in the transition from an early to a late adenoma, with the initial development of neoplasia being dependent on other genetic events. Clinically this is an important step as small adenomas are common and only a proportion are thought to undergo malignant progression, whereas late adenomas have high malignant potential and detection is considered a positive endpoint in screening tests.

The relative frequency of K-ras mutations in colorectal neoplasms and the restricted number of common mutational sites has made this gene an attractive marker to study. As such the marker is potentially informative in substantial proportion of cases using a limited number of molecular assays. K-ras is hence one of the most characterised potential biomarkers in the putative non-invasive molecular detection methods described in sections 1.4 and 1.5.

e) TGFβRII

An important example of MSI being implicated in tumour development is TGF-β receptor II. Transforming Growth Factor-β (TGF-β) is a cytokine growth factor that exhibits a negative effect on epithelial cell growth. TGF-β binds to receptors TGF-βRI and TGF-βRII at the cell surface, although the different receptor types may initiate the same intracellular messages or act as a complex such that mutation of either subunit can result in dysregualtion of signal

transduction (106). TGF-β signalling is transmitted from the cell surface to the nucleus via the SMAD family of signal transducer proteins. Activated TGF-β receptors phosphorylate pathway restricted SMADs, which then oligomerise with SMAD4 and translocate to the nucleus and effect transcription and hence the cellular response to TGF-β(107). Dysfunction of this system has been shown to result in epithelial carcinoma progression since:

1) TGF-β suppresses the growth of some epithelial cell lines
2) Certain cell lines can become unresponsive to TGF-β
3) Inhibition of TGF-β enhances the tumourigenicity of weakly tumourigenic cell lines. (108)

However it has been suggested that TGF-β has multiple roles in tumourigenesis, acting as a tumour suppressor at an early stage, but promoting invasion and metastasis in later stage neoplasms (109).

Of particular interest in colorectal cancer is the existence of a poly A tract within the coding region of the TGF-β RII gene. This poly A tract is a 10 base pair adenosine repeat (A_{10}) at codons 125-128 of its 565 codon open reading frame. In the presence of defective mismatch repair this repetitive tract is subject to short insertions or deletions, resulting in a frameshift mutation and hence truncation of the encoded protein, rendering it non-functioning. Analysis of mutations in this A_{10} repeat in tumour samples from microsatellite unstable colorectal cancers has shown a high mutational frequency. In one study of 111 MSI tumour cells, 100 (90%) demonstrated mutation of the A_{10} tract of the TGF-β RII gene (110). As such analysis of TGF-β RII is an attractive marker for analysis of MSI tumours.

1.3 Clinical Screening Modalities

Wilson and Jungner proposed criteria for disease screening in 1968 (111). These remain the relative requirements to be fulfilled for screening in asymptomatic populations for a given disease, and are outlined below along with their implications in colorectal cancer.

1) The disease must be important in clinical or public health terms. Clearly colorectal cancer meets this criterion on the basis of the evidence presented in the previous section.
2) There should be an available diagnostic test. The gold standard for diagnosis of colorectal cancer is visualisation at colonoscopy with histological confirmation by biopsy. Colonoscopy is widely available and routinely practised in first world healthcare systems, and has high levels of sensitivity and specificity. With regard to screening tests of the asymptomatic population, colonoscopy has yet to be evaluated by way of randomised controlled clinical trials. There are a number of alternative tests in this context, with differing sensitivity, specificity and other performance indicators, the evidence for which is presented in the following section.
3) The test should be acceptable to screened individuals. The acceptability of proposed screening modalities is again considered in the following section.
4) There should be an accepted treatment for disease once detected. Resectional surgery remains the mainstay of colorectal cancer treatment. Long-term survival is related to disease stage at presentation, and adjuvant treatment may be offered accordingly.
5) Diagnostic and treatment facilities should be available. Westernised societies have systems for disease management by necessity of the high disease incidence that becomes clinically apparent without screening. Screening for colorectal cancer has implications for the demand on these systems and their organisation, discussed under

each of the proposed screening modalities below. Developing countries with less advanced health care systems have lower incidence of colorectal cancer, shifting the cost to benefit ratio, and hence impacting on the rationale and practicalities of disease screening in these populations.

6) There should be a recognisable latent phase of the disease. There is a large body of evidence supporting the concept of the adenoma-carcinoma sequence, discussed in section 1.2. As such benign adenomas or polyps may be considered as pre-malignant neoplasms, and hence constitute a latent phase of disease. Endoscopic treatment of polyps is less invasive than resectional cancer surgery, has superior survival, and additionally may reduce subsequent cancer incidence (34)
7) The natural history of the disease should be known. Much remains to be elucidated in the detail of the adenoma carcinoma sequence, particularly in terms of which polyps progress to cancer and the time frame in which this occurs. However the principle of the adenoma-carcinoma sequence is widely accepted, and the clinical course following the development of frank malignancy is uniformly poor without surgical intervention.
8) There should be a consensus on treatment. There is uniformity in the principle of resectional surgery for non-metastatic colorectal cancer, and also in the application of endoscopic polypectomy for adenomas. The survival advantage of adjuvant treatment for lymph node positive colorectal cancer has been clearly demonstrated.
9) The cost of case finding and treatment must be economically balanced. The health economics of colorectal cancer screening is complex, and is considered in evaluating current screening modalities below.

10) Case finding should be a continuous process. This is the case within a defined age range in the UK FOBT screening programme, but not with proposed once off flexible sigmoidoscopy.

Other factors that are important when considering screening modalities are the safety of both the screening test and the action taken on the finding of a positive result, and also the accuracy of the test which affects the risk benefit analysis of screening.

Screening for CRC has been proposed by the following methods:

Faecal occult blood testing (FOBT)

Colonoscopy

Flexible sigmoidoscopy

Barium enema

Computerised tomographic colonoscopy

These approaches are based on various premises, and each present problems in addressing the balance of safety, sensitivity, specificity, acceptability and cost effectiveness. The rationale and available data for proposed forms of colorectal cancer screening are given in the following section. In addition there are some developmental approaches, based on knowledge of the molecular genetics of colorectal cancer, which are discussed subsequently.

For any screening programme to be effective, it must be embraced by the populations it is offered to. Lack of awareness of disease and or a screening test will result in its poor uptake, and hence limitation of its potential benefits and cost-effectiveness. A survey of knowledge

and attitudes has been performed in Europe (112). This highlighted a lack of knowledge of the risk factors for CRC, and of the existence of screening tests. Three quarters of over 20,000 people surveyed expressed interest in FOB screening if it were provided free, although this was a selected population, sufficiently motivated to undergo a health questionnaire interview. Perceived problems to screening were lack of awareness of risk (31%), youth (22%) and fear of a painful test (19%). Cultural factors are likely to play a role, in particular in relation to bowel symptoms, and this heterogeneity was demonstrated with 91% having a reluctance to discussion in Finland compared to 39% in Iceland. As with many diseases, those most at risk of poor outcomes may constitute those with the least knowledge of disease and motivation to be screened. These issue remain a major consideration in contemplating the implementation of CRC screening.

1.3.1 Faecal Occult Blood Testing (FOBT)

It has been known for a long time that colorectal neoplasms tend to bleed, although the rate of loss may be sufficiently low that blood is not detectable macroscopically in the faeces, and for many years attempts have been made to detect lesser degrees of, or occult, blood loss. A fundamental difficulty in detecting occult blood loss is that colorectal neoplasms may bleed only intermittently, or not at all, hence limiting the sensitivity of this approach (113). However the approach was popularised in the 1960's with the use of guaiac tests, which detect pseudoperoxidase activity present in haem. In an early study of 8 patients with colorectal cancer, only one had a negative FOB test (114), although it was recognised that multiple samples were required, and a positive test might occur due to any cause of haem being present in the stool. This included the presence of animal related haem, which stimulated meat-free diet restriction in a subsequent study. This study demonstrated that the majority of cancers detected by FOB testing were confined to the bowel wall and hence potentially curable by surgery (115).

The observation that patients with surgically curable colorectal cancer frequently had positive FOB tests, coupled with its safety and low cost, stimulated interest in this as a screening modality. An additional postulated benefit was that FOBT might detect a proportion of benign polyps, a positive FOBT resulting in a colonoscopy and polypectomy. According to the adenoma-carcinoma sequence, it might therefore be expected that in the long term there would be less remaining polyps in the screened groups in which cancer might develop, and hence FOBT might be expected to reduce the subsequent incidence of colorectal cancer. There is now a wealth of published data, of varying grades, on the impact of FOBT on colorectal cancer mortality and incidence. There are many series and case-control studies

supporting benefits of FOBT in selected populations (116;117) ((118). However with regard to its usefulness as a screening test the highest quality data is provided by population based randomised, controlled trials of which there are a number that have not only published their complete initial results, but also with mature, long-term follow up data.

a) Types of FOB Test

Different studies have employed variations of FOBT that warrant consideration prior to examining the evidence generated from those trials. The most reliable and high quality, long term data have been generated using guaiac tests, generally not hydrated, with follow-up of a positive test by colonic imaging. As such the results of smaller series with modifications of the type of test employed must be interpreted with caution. A joint committee representing the WHO and the World Organisation for Digestive Endoscopy (119) concluded that no single type of FOBT was applicable across all populations, and that the balance between sensitivity and specificity should take into account the availability of colonoscopy services. Guaiac tests are prone to false negative results for a variety of reasons, including the presence of peroxidase containing food in the faeces, which can be reduced by dietary restriction (120). Rehydrated FOBT results in a higher positivity rate, hence improving sensitivity but decreasing specificity as demonstrated by the high positivity and hence colonoscopy rate in the Minnesota study described below (121), and similar results have been demonstrated elsewhere (122). Whilst increased sensitivity is desirable, in the context of population screening the sacrificed specificity results in increased demand for colonoscopy that impact both the safety of and ability to deliver a screening program. Practical difficulties in interpreting the test can be lessened by alteration of the developing agent, such as with HOSENSA (120), although the impact on sensitivity and specificity may be similar to that of rehydrated Hemoccult (123).

Immunochemical tests (ICTs) have the theoretical advantage of being unaffected by other sources of faecal peroxidase, but are substantially more expensive. There is a lack of data comparing ICTs to guaiac tests with respect to CRC mortality and ICTs have not to date formed the basis for implemented screening pilots or programs. There are data that ICTs may have marginally better sensitivity than guaiac tests, that is not at the expense of specificity,(122-124) but the levels are comparable and the additional expense and need for laboratory development appear to have limited the use of these tests. However studies employing guaiac and ICT tests have suggested that a combination of the two types of test might improve accuracy (125). Additionally automated reading ICT tests are now available, with comparable performance characteristics, which may improve usability (126). Blood related markers other than haemoglobin have been examined in stool in including calprotectin (127)and lysozyme (128). However these do not appear to result in improved cancer detection rates when compared to FOB testing (129).

b) Large Randomised Controlled Trials

The results from the three largest RCTs of FOBT are summarised in the table below:

	Size	Positivity Rate %	% Localised Cases vs controls	Interval	Relative Reductio
USA	48,000	2.4 (unrehydrated) 9.8 (rehydrated)	59 vs 53	Annual Biennial	33% 21%
UK	150,000	2.1 (unrehydrated)	52 vs 44	Biennial	15%
Denmark	62,000	1.0 (unrehydrated)	56 vs 48	Biennial	18%

i) Minnesota

The first large randomised controlled trial of FOBT was the Minnesota study, published in 1993 (121). This was a study of over 46,000 volunteers, aged 50-80, who were randomly assigned to receive either standard care (control group), annual FOB testing or

biennial FOB testing, with 83% of tests rehydrated. 46,551 subjects aged 50-80 were offered either 6 or 11 FOBTs over the course of the study period.

ii) Nottingham

A larger population based was the Nottingham trial with nearly 153,000 recruited participants, aged 50-74, generated through general practitioner registers (23). There were approximately 75,000 controls, who were not contacted or given information about the study having been randomly allocated to be controls, although eligible members of the same household were allocated to the same arm of the trial. 75,000 individuals in the biennial screening group were sent haemoccult, and asked to test 2 samples from each of either 3 or 6 consecutive stools. FOB tests were not rehydrated and dietary restriction was employed prior to retesting only for participants with a borderline initial result. The median follow-up was 7.8 years.

iii) Denmark

A further population based from Denmark enrolled 140,000 45-75 year olds, allocating 30,967 to the screening arm of the trial to receive biennial FOB screening (130).

c) Outcomes

i) Mortality

The primary outcome measure of screening programmes is that of death rates both in terms of disease specific and overall mortality. In the Minnesota study colorectal cancer mortality was significantly improved, with the cumulative 13 year CRC mortality per 1000 improved from 8.83 (7.26-10.40)) in the control arm, compared to 5.88 (4.61-7.15) in the annual screening group compared, with a non significant difference in the biennial screening group (8.33 (6.82-9.84)) (121). In the Nottingham study, the disease specific survival was significantly better for individuals in the screening group compared to the control group ($p<0.0001$). However all cause mortality was not different between groups, presumably reflecting that death caused by colorectal cancer in the population is an infrequent event (2% of all deaths) and hence for a study to have sufficient power to demonstrate an effect on overall mortality,

the sample size would have to be very large (131). The size of the survival advantage in the screening group depended on whether the cause of death was taken as that on the death certificate, or that verified by the study group. Using verified deaths there was a 15% reduction in disease specific mortality- odds ratio 0.85 (95% CI: 0.74-0.98) p=0.026. Using certified cause of death the confidence interval just straddled 1.0. The greatest CRC mortality reduction was seen in those in the screening group who accepted their first invitation for FOBT, with a 39% reduction (odds ratio 0.61 (95% CI: 0.50-0.74), whereas those who refused their first FOB test had a non significant increase in their CRC mortality. The difference in colorectal cancer mortality started to become apparent at 3-4 years after recruitment into the study.

In the Nottingham study the same number of cancers were detected by FOB screening (236) as were diagnosed subsequently after a negative FOB test (false negatives), demonstrating the limitations of the test. However the stage distribution was significantly improved in the screened group. There was both a higher proportion of earliest stage disease 20% versus 11% ($p<0.001$), and a lower proportion of late stage disease (Dukes C and D) 46% versus 52% ($p<0.01$). The incidence of advanced CRC was also lowest in those who accepted their first screening invitation, with a ratio of 0.76 (95% CI: 0.63-0.93).

Ahlquist (132) has argued that the deleterious effects of screening are responsible for the absence of an overall mortality reduction in the screened groups, with the benefit being restricted to disease specific survival. He suggested that false negative results are falsely reassuring resulting in delayed presentation and poorer outcomes for those with cancers missed by the poor sensitivity of FOBT. However analysis of interval cancers in the screening group, i.e. those who declined testing or those with a falsely negative test, demonstrates that these cancers have a similar stage distribution to controls, and that survival following interval cancer is actually better than cancers in the control group ($p<0.01$)(131).

However there is clearly a difference between individuals who were motivated to attend for screening and had a falsely reassuring test, than those who did not take up the invitation to be screened, yet these patients were included together in the analysis of the interval cancers. Additionally Ahlquist postulated that screening detects a subset of 'innocuous' cancers that were not destined to injure that patient in their remaining lifetime, and hence produced an 'overdiagnosis' bias of indolent cancers in the screened population. The authors of the Nottingham study refuted this with data on screening participants who subsequently died between 30 days and 2 years after surgery. This was a small group the majority of whom (26/36) died of recurrent colorectal cancer. Analysing those initially found to have either advanced adenomas or Dukes A cancers, there were 6 individuals, all of whom died of causes unrelated to colorectal cancer. They postulated that there were only 6 patients in their very large trial who would likely have died of other causes before presentation of their colorectal neoplasia, and hence that 'overdiagnosis' was not a significant problem. Subsquently the Nottingham group reported on all deaths within the trial groups, showing that significantly more people died from verified colorectal cancer in the control group compared to the FOB arm (133).

ii) Test Performance

The Nottingham study did not generate figures for sensitivity or specificity of FOB testing, however the positive predictive value was 12% for colorectal cancer and 46% for all neoplasia.

The overall sensitivity in the Minnesota study was around 85%, with improved performance with multiple tests, rehydrating specimens and dietary restriction. However whilst this sensitivity is impressive, the positive predictive value of FOB testing for CRC was only 2.2% in this study, which is likely partially a reflection of the rehydration of the FOBT slides (121), compromising specificity for improved sensitivity.

Other studies that have specifically assessed the performance characteristics of FOBT have generated sensitivity figures of around 50% (134), with generally high specificity at over 90% depending on the type of test used (125).

iii) Colonoscopy Rates and Risk

Whilst FOBT in itself is an extremely safe test, the implication of a positive result is to undergo bowel visualisation, predominantly by colonoscopy, which is not without risk. Indeed any benefits of screening by FOBT are derived from interventional investigation and treatment.

In the Minnesota study 38% of those screened annually, and 28% of those screened biennially underwent colonoscopy at least once, a much higher colonoscopy rate than in other randomised controlled trials where the colonoscopy rates were under 5%. Critics of the Minnesota study suggest that observed benefits in the screening group occurred as a result of nearly a third of that arm undergoing colonoscopy rather than as a result of the effectiveness of FOBT (121).

In the Nottingham study 4% of patients (1778 out of 44450) who underwent at least 1 FOBT underwent colonoscopy on at least 1 occasion. Whilst this a greatly reduced proportion to that seen in the Minnesota study, when extrapolated to the general population as a whole this constitutes a dramatic increase in demand for colonoscopy.

In the Nottingham trial the complication rate of colonoscopy was 0.5%, the majority of which required surgery, but none of which resulted in death. 5 patients died within 30 days of surgery for screen detected colorectal cancer, and a further 2 died without any surgery (131). However these figures included only colonoscopies that directly resulted from a positive FOBT. Clearly positive findings at initial endoscopy might clinically warrant further procedures, with a similar level of risk, but the figures from these follow-on procedures were not included in this analysis.

However one of the strengths of FOBT is its high specificity, hence resulting in relatively few unnecessary colonoscopies compared to other modalities, albeit at the expense of sensitivity.

iv) Uptake

The acceptability of a screening modality is fundamental to its potential to have impact on the disease in any population. This may be especially the case for colorectal cancer screening using modalities reliant on faeces, which may have a level of stigma associated with it that varies across societies.

Compliance was high in the first randomised controlled trial to report, with 75% of the annual screening group and 78% in the biennial group accepting screening. There was a level of drop-out from this with 83 and 84% of test positive subjects undergoing diagnostic follow-up either by colonoscopy or flexible sigmoidoscopy and barium enema (121). However the Minnesota trial studied a volunteer population, who by their nature constitute an interested and motivated group hence limiting the applicability of these figures to population screening. Indeed in the population based Nottingham trial only 38.2% of the screening group completed all the FOBTs they were offered, with 21.4% completing at least one but not all and 40.4% of invited subjects did not complete any FOBT.

As such, uptake by the general population was less than 60%, for any test at all, and just over a third for complete compliance with testing. Therefore around half of the potential population benefit of screening could not be realised. The reasons for the low uptake are likely multifactorial, as reflected in the wide variation in compliance rates in participants from different general practices (29%-74%). Partly it is likely that poor disease awareness, and lack of understanding of the screening test and process may cause a degree of apathy in

the asymptomatic population. These considerations are likely to be less of a factor now than during the time frame of the study period due to increased publicity of colorectal cancer screening, and health promotion with regard to colorectal cancer in general. This may partly be reflected in the increased compliance between the pilot study (36.9%) and the study proper (57.0%)

However it seems likely that there is a reluctance to perform faeces based testing in the community. Clearly having to collect individual stools, and then smear portions of that stool onto slides is not an attractive proposition, particularly for people who feel well and do not perceive themselves to be at great risk of a given disease. Use of tests which are easy to use and are self reported by the individual have not been shown to increase compliance rates (135). Additionally a requirement to undergo dietary restriction has been shown, in some studies to impact on acceptability, rising where not required from 53% to 66% (136), although a meta-analysis suggested no difference in completion or positivity rates with mild dietary restriction for non-rehydrated guaiac tests.

Initial compliance rates in the Danish study were higher than in the UK at 67%. The Danish study did not re-offer screening to those who refused the first invitation. Unsurprisingly therefore their screening group became a selected and motivated one, with 90% accepting repeat screening.

d) Metaanalysis

There have been meta-analyses of data from controlled trials of FOB screening with haemoccult (137;138) including using intention to treat analysis, and analysis according to whether screening was actually performed.

Identified were the 3 randomised trials described above, plus a further scandanavian study (139), and 2 non-randomised trails (140;141). In all trials there was part of the protocol that

included dietary restriction. The majority of trails had non-rehydrated tests giving a lower positivity rate and higher positive predictive value for cancer (5.6-17.7%), compared to rehydrated tests (positive predictive value 2.2-4.2%). The sensitivity of FOBT ranged from 46% unhydrated (130) to 92% rehydrated(121), and was defined as screen detected cancers over all cancers in screening group within 2 years. Cancer related mortality reductions in each of the trials were comparable and formal testing confirmed a lack of heterogeneity. Therefore mortality results were combined to give a reduction in disease related mortality of 16% (relative risk 0.84 95% CI: 0.77-0.93) rising to 23% for those who actually underwent screening (relative risk 0.77, 95% CI: 0.57-0.89). The number needed to screen in order to prevent 1 death from colorectal cancer over 10 years was 1173 (741-2807), and would relate to between 20 to 800 colonoscopies per life prolonged. At the time of the meta-analysis only medium term data was available (8-13 years) and at that time there was no apparent effect of screening on CRC incidence. Since then longer term results have been published and are described below.

e) Long Term Follow Up

The long-term data for the Minnesota study was published in 2000 (45), totalling 18 years of follow-up. Whilst at 12 years follow-up there was no significant reduction in colorectal cancer incidence, by 18 years there was a statistically significant reduction in cancer incidence in those subjects undergoing either annual (cumulative incidence ratio 0.80 (95% CI: 0.70-0.90) or biennial (0.83 (95% CI: 0.73-0.94)) FOBT. Completeness of 18 year follow-up data was excellent, being over 90% in each of the three groups, and cause of death was accurately ascertained for essentially all participants who died. It is important to highlight that although the study completed in1992, those patients who had positive findings upto that point, continued to have surveillance as deemed clinically appropriate. Therefore

the 'protective' effect of screening was not derived only from the period of study, but also from continued follow-up and endoscopic intervention. These benefits occurred despite a period during the study of around 4 years, where screening did not occur, and which presumably would likely attenuate the effects of screening on colorectal cancer incidence. The longer term mortality results from the Nottingham study (142) demonstrated that the reduced CRC mortality in the screened group (on an intention to treat basis) was maintained at a median follow-up of 11 years, being a 13% reduction in the screening group as a whole, rising to 27% for those who actually accepted screening.

f) Psychological Aspects of Screening

Screening tests may have psychological impact on individuals offered testing, and particularly those with positive tests(143). With relation to FOB screening, individuals' psychiatric morbidity has been evaluated by questionnaire, and found not to differ before the offer of screening compared to 3 months afterwards (144). The anxiety of a positive test result may be unavoidable for true-positives, but is a particular disadvantage for poorly specific tests by creating unwarranted anxiety in false-positives. However this anxiety was shown to be short lived in a small sample of individuals, falling the day after the index investigation that demonstrated them to be false-positive (Parker MA).

g) Implementation and Cost Effectiveness

A Dutch group concluded that screening with FOBT met criteria for disease screening and should be implemented on a national basis (145). In addition to the large population based trials of FOB screening described above, there is now data derived from screening pilot schemes in areas of the UK (2), such thatthere is nationwide implementation of population

based FOB screening for CRC in the UK. Many international health agencies have issued guidelines for colorectal cancer screening including the WHO (119).

Clearly widespread implementation of screening programmes requires the screening test to be cost effective. Evaluation of data from the Nottingham trial suggested that FOB screening has comparable cost-effectiveness to breast cancer screening, which is widely established in developed countries. Combination of the impact and costs of FOB screening in a mathematical model to generate quality adjusted life-year gained, estimated cost-effectiveness to be similar in the short term and superior in the long term compared to breast cancer screening (146). Later analysis of the complete data from the same trial, calculated the cost of screening to be £5290 per cancer detected and £1584 per life year gained (147). This compares favourably with costs of breast cancer screening elsewhere in Europe, which had costs of £10365 per cancer detected and £8561 per life year saved (148). Clearly the accuracy and particularly the specificity of the means of testing impact cost-effectiveness, and the costings given above where generated from non-rehydrated tests with high specificity.

Data from an earlier American study (149) again suggested that annual FOB screening was affordable, with a cost of $20 per person screened per year, including follow-up testing of all positive findings. Addition of 5 yearly sigmoidoscopy to screening increased the cost 2.5 times taking into account only direct costs. Estimated cost of each life day gained was $57, equivalent to $20805 per life year (~ £12238 at 1.7 dollars to the pound). Clearly the health economics differ between the UK, where services are free at point of access, and the USA. The cost of screening tests in the USA does not however appear to be the main determining factor in uptake of CRC screening (150).

Despite the cost effectiveness of CRC screening compared to other screening programmes, implementation of national screening programmes certainly alters the need for CRC services. Some of the effects of FOBT screening studies have been beneficial in this regard, including decreasing the amount of emergency admissions with the disease (151). However demand on diagnostic and follow-up services is substantially increased by individuals returning a positive FOBT. A study of UK pilot study areas demonstrated a 20-30% increase in colonoscopy activity, with associated increases in pathology and outpatient demand (152). There is a further 28% increase due to follow up colonoscopy after positive findings at index investigation. Whilst there is a suggestion that this demand improves the quality of colonoscopy services, and may reduce cancer incidence in the long term, this dramatic increase in workload necessitates planning and provision of resource in areas implementing population screening, which may be predicted by modelling(153).

Modelling of implementation in Canada (154) using an actuarial model in the age group 50-74, postulating screening from 2000-2010 using unrehydrated tests on either an annual of biennial basis. Over 10 years they estimated that 4,444 and 2,827 CRC cancer deaths would be prevented for annual and biennial screening respectively, needing 3,400 and 2,700 FOB tests respectively to prevent each death.

FOBT screening has now been rolled out in the UK. Prior to this,large scale pilot schemes had been undertaken in a population of around 1 million, with close to half that offered screening (2). 56.8% of participants offered screening accepted, and the test positive rate was 1.9% (and hence over 5000 colonoscopies). Encouragingly 48% of the 552 screen detected cancers were Dukes A (with 92 of these being polyp cancers) and only 1% had distant metastasis at diagnosis.

Therefore population based FOBT screening is a reality, and despite the limitations of the test, and the attendant health delivery and cost implications, there are clear clinical and public health benefits as demonstrated from several randomised controlled trials.

1.3.2 Endoscopy

One approach to colorectal cancer screening is by direct visualisation of the colorectal mucosa at endoscopy. Colonoscopy remains the gold standard for detection of colorectal neoplasms in clinical practice, with utility for foreshortened examinations by way of rigid and flexible sigmoidoscopy. Extrapolation of these techniques to screening asymptomatic populations is not however straightforward on account of the degree of risk associated with these tests as well as the costs and ability to deliver these investigations, which require skilled operators to perform, on a large scale.

a) Sigmoidoscopy

The extent of sigmoidoscopy is limited to essentially the left colon for flexible sigmoidoscopy and the rectum for rigid sigmoidoscopy. These approaches can therefore only expect to detect a proportion of all colorectal cancers, although left sided cancers are preponderant, and particularly so within certain epidemiological groups.

i) Detection rates

The MRC/ICRF prospective randomised controlled trial of screening asymptomatic populations using once only flexible sigmoidoscopy trial has recently reported(155).Initial results of the yields of testing were published previously, both for the whole study (156) as well as a subset (157). Adenomas were detected in 10% and 12.1% of screened individuals in the subset and whole study respectively. Cancer detection rate was 0.7% and 0.3% respectively with a referral rate for colonoscopy for the subset of 6% (similar to that of FOBT). 55% and 62% respectively of screen detected cancers were Duke's stage A. Projections of the impact of flexible sigmoidoscopy screening in the UK suggested that it would prevent 5500 cases and 3500 deaths annually, thus saving 40,000 years of life (158).

Proposed examination interval has been 5 years, with little postulated benefit from more frequent examinations, from data generated from a cohort study that generated annual CRC incidence for the first 5 years after a negative sigmoidoscopy (159). However flexible sigmoidoscopy is unable to detect neoplasms that are not left sided (160), and also distal colonic findings are not a good predictor of more proximal neoplasms, resulting in a sensitivity of 44% in one study (161).

ii) Protective effect

Previously case controlled studiesof sigmoidoscopy suggested a reduction in the risk of developing fatal cancer of around 60%, limited to cancers within the reach of the endoscope (162)..Another study(164) investigated whether flexible lower GI endoscopy protected against the development

of cancer, showing a risk reduction with an odds ratio of developing cancer of 0.51 (95% CI; 0.44-0.58) for colon cancer and 0.55 (95% CI; 0.47-0.64) for rectal cancer.

There s now however level 1 evidence of the protective effect of once only screening flexible sigmoidoscopy from the MRC trial (155), with a 23% overall reduction in colorectal cancer incidence at median 11 years follow up, and with numbers needed to be screened to prevent cancer diagnosis or death of 191 and 489 respectively.This benefit was greater still when analysed for those who underwent screening, and for distal tumours.

iii) Other considerations

Compliance for flexible sigmoidoscopy based screening programmes is likely to be poorer than that of non-invasive screening tests on account of the potential for discomfort and the

need for bowel preparation. A study of over 6000 asymptomatic patients compared screening by FOBT with screening by a combination of FOBT and flexible sigmoidoscopy (165). Whilst they concluded that the yield for neoplasia was 4 times greater with the combination of tests, compliance was 48% for the FOB portion of the combined arm, but only 20% agreed to flexible sigmoidoscopy. A study comparing FOBT and flexible sigmoidoscopy in 55-56 year olds, had an attendance rate of 59% for the FOBT arm compared to 49% in the flexible sigmoidoscopy arm (166). The initial compliance results of the ICRF/MRC randomised trial demonstrated that 23246 offered screening flexible sigmoidoscopy , around 60% expressed interest, but only 75% of these attended, giving an uptake of only 45% (157), although for the whole study attendance was stated as 71% (156). Recently an assessment of the barriers to screening flexible sigmoidoscopy identified a lack of awareness of bowel cancer, and fear of test invasiveness and of cancer diagnosis as relevant factors across ethnic groups(167).However of 4422 individuals attending flexible sigmoidoscopy screening in the context of a multicentred randomised controlled trial, 99% reported that they were glad they had the test, and 91% that they had only mild or no pain (168).

Flexible sigmoidoscopy is variable in aspects of the examination including the amount of colon visualised, the degree of discomfort caused to patients and the interpretation of results. Whilst guidelines have been generated to minimise heterogeneity of examination quality (169), considerable variation exists. Within a large randomised controlled trial there was wide variation in the adenoma detection rates between centres, thought to relate to endoscopist performance and related to screening experience (170). In relation to patient factors, a study in asymptomatic volunteers demonstrated that the procedure was classed as difficult in 33% of cases (171), and that length of insertion related to being female and having previous

surgery, as well as poor bowel preparation. Indeed women who have undergone hysterectomy may have technically more difficult procedure (172), resulting in significantly more pain and shorter distance of insertion, also resulting in a significant trend toward lower relative detection rates.

Factors resulting in sub-optimal flexible sigmoidoscopy will impact on the efficacy of flexible sigmoidoscopy as a screening test, and these considerations should be included in projections of performance and cost-effectiveness.

Flexible sigmoidoscopy is relatively expensive in comparison to FOBT. The direct costs of performing once-off flexible sigmoidoscopy have been published and were estimated at £56 rising to £91 when considering the cost of subsequent management, with additional cost also incurred to the patient (173). A mathematical model of the costs of flexible sigmoidoscopy screening suggested the cost would be around £80 per person invited, or £117 for those undergoing screening given an optimisitic compliance rate of 70%, and estimated the cost per life year saved to be £2889 (174). This compares to estimated cost, within a different time-frame in a different health care system, of $20 per person screened per year, including follow-up testing of all positive findings (149). Within the UK there is evidence that flexible sigmoidoscopy with or without FOB testing is a potentially cost effective strategy for the early detection of colorectal cancer (175).

b) Colonoscopy

There are no randomised controlled trials of population screening using colonoscopy, however it has been adopted as the modality for screening in some countries including the

USA and Germany (176)according to guidelines from several leading gastroenterology societies (177).

There are theoretical advantages of screening using colonoscopy, including its high cancer detection rate along with its evident sensitivity and specificity. That said, there is a definite failure rate, with one study of back to back colonoscopies for 183 patients showing an overall miss rate for adenomas of 24%, albeit predominantly for adenomas less than 5mm in size (178). That said, whilst there is a potential for FOBT to detect left sided tumours more sensitively than right sided ones(179), complete colonoscopy visualises the entire colorectal mucosal surface. Colonoscopy with polypectomy has the advantage of being therapeutic, with removal of adenomatous polyps implicated in a reduction in CRC incidence. Evidence to support this includes a cohort study of patients who had undergone polypectomy, compared to patients who did not undergo polypectmy and a population based reference group, resulting in an up to 90% reduction in incidence (34) as well as reduction in incidence in high risk groups such as HNPCC (180). Clearly these groups are at higher CRC risk than the asymptomatic population, but in the absence of data from a randomised asymptomatic population trial aimed at detecting an incidence reduction rather than initial cancer detection, the inference remains that polypectomy reduces colorectal cancer incidence.

There have been a number of case-controlled studies of screening colonoscopy.

A retrospective study of 40-49 year olds determined the prevalence of colorectal lesions found at screening colonoscopy, for 906 asymptomatic individuals, in which 3.5% had advanced neoplasms and none had cancer. They extrapolated that between 250 and 1000 examinations would be required to detect one cancer in this age group, and concluded that the low yield supported screening not being offered to individuals under 50. (181).

A further study of women aged 50-79, consecutively referred for CRC screening, and also 40-49 year olds where a first degree relative had CRC, assessed colonoscopic screening of 'average risk' women for colorectal neoplasia (182). This study excluded patients with a positive FOB within 6 months, symptoms that might be attributable to CRC, or colonic visualisation within 10 years. The study used distal colorectal findings as a surrogate for flexible sigmoidoscopy. Of 1593 eligible patients, 1483 (93.1%) participated, although all those approached had been referred for screening, therefore these acceptance rates are not suitable for extrapolation to the general population. The colonoscopy completion rate was 98.7% with no complications of colonoscopy. 4.9% of patients had either large / advanced polyps or CRC. The yield increased with age and with a family history of CRC.
Distal colorectal findings would have had a yield of only 1.7% (missing 3.2%), and this 'flexible sigmoidoscopy' yield did not vary with age or family history.
If positive distal findings had gone on to trigger colonoscopy, then 94% of cases of advanced proximal neoplasia would have been missed (47/50), therefore they concluded that distal findings at flexible sigmoidoscopy are not a good predictor of those needing a colonoscopy. The study authors concluded that colonoscopy was the preferred method of screening women.

The parallel study to the one in females described above was in men aged 50-75 (183). Men have a higher prevalence of colorectal neoplasia (160), and hence benefits might be expected to be greater.Taking the results from Lieberman's and Schoenfeld's studies and comparing men and women without a family history and with a negative FOBT, the diagnostic yield in men was significantly higher (66.3%) than in women (35.2%) ($p<0.001$). The study of males was of a fairly highly selected group of men (3196 enrolled from 17,732 patients screened for enrolment) had a yield of 10.5% for advanced adenomas and 1% for cancer. Of those which

advanced proximal colorectal neoplasia, 48.4% had some sort of adenoma in the distal colon, falling to 37.9% if the distal colon was quantified as the sigmoid and rectum only.

However whilst colonoscopy remains the gold standard for diagnosis of colorectal cancer, including as a follow up test for other positive screening modalities, there are a number of considerations when applying it to screening asymptomatic populations.

Clearly colonoscopy is invasive, requires bowel preperation, and can be uncomfortable for patients, all of which are likely to impact on acceptability and hence compliance. There are studies suggesting colonoscopy is a safe form of screening with major morbidity related to colonoscopy in 0.3% (9 of 3196) procedures and 0.1% in only diagnostic procedures (184), none of which were perforations. However perforation rate at colonoscopy is generally accepted to be in the region of 0.1% along with a small but undeniable risk of mortality (185). Therefore higher morbidity and mortality would be expected to occur as a result of colonoscopic population screening compared to non-invasive methods.

There are also delivery and cost implications for colonoscopic screening, although whilst colonoscopy is expensive when compared with sigmoidoscopy in absolute terms, there is evidence for better for cost per neoplasm detected (186).

Given the safety, cost and logistical implications of population based colonoscopic screeningthe UK screening programme, and others, advocate the use of therapeutic colonoscopy for other screening test positive individuals.

1.3.3 CT Colonography

Over recent years adaptations of conventional CT have been developed that allow more detailed visualisation of the colon, including detecting intraluminal pathology. This technique has variously been termed virtual colonoscopy (VC) and CT colonography (CTC), and has been evaluated in its ability to detect colorectal neoplasms. The accuracy of the test for detecting pathology is fundamental to its potential utility as a screening modality, but there are a number of other theoretical advantages and disadvantages of this method of colonic imaging. These are discussed in more detail below, during discussion of the data from CTC trials. Briefly the potential advantages include that CTC is considered less invasive, does not require sedation and has a lower risk of complication when compared to conventional colonoscopy. The disadvantages compared to colonoscopy include the inability to derive histological material or be therapeutic at the same examination, radiation dosage, and a relative poor availability in current colorectal practices. However as colonoscopy has not been evaluated as a screening modality by way of a randomised controlled trial, comparisons with conventional colonoscopy should perhaps be limited.

A brief consideration of the technical requirements of CT colonography is helpful when considering the applicability of this test to the asymptomatic general population.Whilst there are many modifications of examination protocols, the general principles include the performance of a high resolution CT scan following some form of bowel preparation. The images are reconstructed using software and interpreted by a radiologist with training and /or experience in the technique.

The characteristics of the hardware appear to have great relevance to the performance of CT colonography as discussed when considering the accuracy data below. As such multi slice scanners appear to have better performance, as do more advanced software analysis

packages that allow 3 dimensional, virtual fly-through reconstruction. The difference in scanners and analysis software is the main rationale in the literature to try to explain the disparate accuracy results in clinical trials of CT colonography.

Each of the larger trials of CT colonography have utilised standard bowel preparation as part of their protocol. Bowel cleansing improves data quality, as for example residual stool can be mistaken for neoplastic masses. The main disadvantage of requiring bowel preparation is reduced acceptability to patients, and may require inpatient preparation in elderly or infirm patients. The advantage however of having a fully prepared bowel is that confirmatory or therapeutic colonoscopy can be undertaken either the same day or without the need for further bowel preparation, which is not the case after barium enema or indeed if rectal contrast has been used as an adjunct to CT colonography. Various techniques have been investigated in order to try to decrease the amount of colonic preparation needed before CT colonography. Orally ingested agents, such as barium, have been used to bind retained faeces, and have been termed tagging agents. Whilst in some studies tagging agents have been administered in addition to bowel preparation, in others bowel preparation has been substituted with tagging agents (187). Digital subtraction software processing allows subtraction of labelled stool from imaging. The proposition of obviating the need for bowel preparation would make CT colonography more attractive both as a diagnostic test and a potential screening modality, however it seems likely that convenience would be at the expense of image quality to some degree, and it appears that image quality is fundamental to test accuracy and hence applicability as described in analysis of clinical trials below.

Also standard practice to date is colonic distension using rectal insufflation of gas, such as carbon dioxide. Colonic distension improves image quality, but is unpleasant for the patient. Discomfort is not considered sufficient to require routine sedation, which is heralded as an advantage over conventional colonoscopy. Certainly this is the case in terms of

recovery time and patient convenience in being able to be alone the night of the examination, and in view of negating the cost of recovery facilities and staff. However unmodulated discomfort may be a factor in suboptimal patient acceptability. A number of drugs have been administered in an attempt to improve colonic distension and reduce colonic spasm, but are yet to be of proven benefit. Use of intravenous contrast can help differentiate benign and malignant strictures but has the attendant risks of the administration of contrast media. Initial reports in small numbers of symptomatic patients suggested sensitivity for large polyps and cancers of around 90% (188;189), although the sensitivity for smaller polyps was around 50% or lower. Additionally these studies suggested a false positive rate of 19% (188), and a large proportion had technical difficulties resulting in a suboptimal examination, with 28% unable to hold their breath sufficiently and a further 8% were deemed to have insufficient bowel preparation.

Since these initial reports larger scale studies have been performed and generated conflicting results, with the results summarised in the table below

Study	***Protocol***	***Sensitivity***	***Specificity***
Cotton	2D	>10mm = 55%	90%
Johnson	3D, prep	>10mm = 63%	90%
Pickhardt	3D, prep and tagging	>10mm = 96% >8mm = 92%	>8mm = >95%

The results from these studies demonstrate that performance characteristics for CT colonography have been varaible. Sensitivity and specificity appear to depend fundamentally on polyp size. If it is assumed that a cut off of >10mm is clinically useful then CTC is likely to be a good screening test. Certainly conventional colonoscopy has a four and a half times greater yield at detecting and recovering subcentimeter polyps (193). There is some debate as to whether this arbitrarydistinction is valid. Some studies have grouped lesions sized 5-9mm together, but Pickhardt suggests that the threshold for referral to colonoscopy (and polypectomy) may lie within this range e.g. at 8mm. The threshold level for referring for colonoscopy would obviously have major implications for the resultant colonoscopy work-load (e.g. 6mm: 29.7%, 8mm: 13.5%, 10mm: 7.5%, would get colonoscopy (192)).Clearly technical considerations effect test performance. There is a learning curve with data interpretation, and also technical difficulties including insufficiently distended bowel (194), as well as aspects of continually evolving data interpretation and image analysis software that may partially explain the suboptimal results of early studies.

An additional feature of CT colonography is the detection of extracolonic pathology or 'incidentalomas'. One study demonstrated thatt 11% of 264 CT colonographies had 'important' extracolonic findings, 7% had further investigations, and just over 2% had surgery on the basis of the findings (195). Further work-up cost an average of $28 per examination. Therefore these findings are relatively common, causing further costs to be considered. However 3 extracolonic malignancies were overlooked at CT colonography, presumably since this is not the ideal modality for detecting extracolonic pathology. The frequency is high in this study and lower in asymptomatic populations, where important extracolonic findings were detected in 4.5% of patients (56), including cancer in 5 patients (0.4%) and 2 abdominal aortic aneurysms that were subsequently repaired (192). This is

presumably as extracolonic disease may have accounted for symptoms felt to warrant colonoscopy and hence inclusion in trials of symptomatic patients. In asymptomatic female populations there is an appreciable (4.1%) rate of detecting adnexal masses, although this is not a good predictor of ovarian cancer, and normal adnexal findings at CT colonography was not protective against the development of ovarian cancer in the short term(196). Additionally it has been suggested that 81% of colorectal cancers can be accurately staged by the initial CT colonography (197).

CTC is non-invasive and hence considered to be low risk, excluding the fact that positive findings require follow up conventional colonoscopy with attendant risks. However CT exposes patients to a significant radiation dose estimated at a median dose of 8.8mSv (198), that may predispose to secondary cancers (199). Studies have propsed a reduction in CTC radiation dose, for example 50 CT colonographies at 100 mAs, and then added noise to the data to correspond to scans performed at 50 and 30 mAs. Whilst they suggested that image quality decreased appreciably with reducing dose, the sensitivity for polyp detection remained unchanged with lower radiation doses. Whilst it is a very attractive proposition to reduce the radiation dose, particularly when considering exposing the asymptomatic, and generally disease free population to these levels, caution must be used when interpreting this data. Firstly and most importantly the quality of the CT colongraphy images appear, unsurprisingly, to be fundamental to the quality of the test in the context of screening. Technological and image quality arguments are held to be the difference between CT colonography being a poor screening test (190) and a good screening test (192). Sacrificing radiation dose for test accuracy would be counter-intuitive. Secondly the above-mentioned study was small, and did not actually perform scans at lower dosage levels, but only simulated them. A slightly larger study of 158 patients with indication for colonoscopy

underwent low-dose multi-detector row helical CT colonography, with radiation doses of 1.8 mSv for men and 2.4mSv for women. These lower dose scans had a sensitivity profile similar to the larger studies already discussed, which was again polyp size dependant. Performance was a sensitivity of 96.0%, specificity of 96.6% and a positive predictive value of 94.1%.(200).

Although perforation has been described (201), it is generally not considered as a risk of CT colonography.

Despite these caveats, if test accuracy was shown to not be compromised by reduced radiation dose, in a large series within the asymptomatic population, then the argument for CT colonography as a screening modality would be enhanced further. That said, despite the technique being available for some time now, there remains debate about the applicability of the technique to thegeneral population (202), although there is a trial underway from the Netherlands directly comparing CT versus conventional colonoscopy as a means of population screening(203).(201)

There has been a metaanalysis of CT colonography (204), which included 33 studies of 6393 patients. They included several protocols and importantly a number of different technological variables in a variety of symptomatic and asymptomatic study populations. As discussed above it is not surprising therefore that the sensitivity results for different size categories of polyps were heterogeneous. Reassuringly however specificity was homogenously high at over 90 % for all 3 categories of polyp size and increasing with increasing size category. The authors recognise the heterogeneity of their included studies, however they conclude that CT colonography cannot be advocated for screening until a number of the heterogeneity issues are resolved. It would seem more progressive however to suggest that independent corroboration of the results from studies using the most advanced techniques and hardware, might allow advocation of CT colonography as a screening tool.

A further consideration for CT colonography as a screening tool is patient acceptability. 54.3% of patients recalled greater discomfort with virtual colonoscopy, compared to 38.1% for optical colonoscopy ($p<0.001$), although only 8.2% rated the virtual colonscopy as severe. In terms of overall acceptance, under 10% rated their experience of virtual colonoscopy as less than good.A total of 68.3% rated virtual colonoscopy as more acceptable than actual colonoscopy. It is hard to attribute this increase in acceptability compared to the proportion that found the test more uncomfortable. Certainly bowel preparation was the same for the tests. There may be an effect of avoidance of sedation and the recovery time from this, however this is a selected study group, who are highly motivated towards disease screening, and it is conceivable that the potential extracolonic information might sway opinion to some degree. That said there is evidence that CT colongraphy is the preferred method of colonic visulaisation for screening by patients with one study suggesting that 36% of study participants would have forgone screening if CT colonography had not been available(205).

1.3.4 Summary

There is clearly a rationale for colorectal cancer screening. Several modalities have been adopted based on differing quality of evidence, but as yet all approaches are suboptimal in some respects.

Overall uptake of current colorectal cancer screening methods are poor, as highlighted by an American study, the rates of colorectal cancer screening compliance were markedly lower than that for other cancers, with compliance of 77.2% - 91.7% for cervical smear tests versus 9.9% - 35.2% for FOB testing. This study also noted a wide range of uptake levels within the study population, with a 3.6-fold difference in the range of estimates for FOB testing (9.9% to 35.2%). This study was unable to demonstrate significant associations with educational status or race with FOB testing(206). There is a lack of data to elucidate why CRC screening uptake is sub-optimal, although is likely to be multifactorial. It seems likely that part of the reason for poor uptake is stigma surrounding tests involving analysis of the lower gastrointestinal tract or faeces directly.

There is clearly a need for improved screening tests for colorectal cancer. There remains considerable effort in both improving the effectiveness of current modalities, but also in molecular biology translational research. Particular interest has centred on the detection of tumour derived DNA in body fluids, discussed in the following section.These approachesmay have greater overall impact if they can also improve compliance rates , for example by the generation of a blood test.

Since this work was undertaken there has been considerable interest in the development of biomarkers, including the categorisation of differentbiomarkers according to their proposed clinical application. Briefly biomarkers might be intended to supplement currently available tests (add-on), be intended to identify individuals whop might benefit from further tests (triage) and those intended to replace currently available modalities (substitute). Alternatively biomarkers can be classified according to the potential clinical information they provide including prognostic, early detection/screening, prediction and pharmacodynamic. Increasingly it is appreciated that these different classes of biomarkers are best served by different performance characteristics.

1.4 Molecular Detection in Stool

Analysis of DNA extracted from stool has been investigated as a method for translating the knowledge of the molecular genetics of colorectal cancer into a clinically useful test, ultimately with a view to screening for disease. The ability to detect specific mutations in collected stool samples may reflect exfoliated neoplastic cells from a primary tumour, and hence constitute a marker of cancer and adenomas.

The theoretical advantages of this approach are great in that testing is non invasive, does not require bowel preparation, may reflect changes throughout the colon and has the potential to have superior sensitivity and specificity compared with FOB testing.

Certainly there are limitations of FOB as a test that limit its effectiveness to impact on disease outcomes. Despite this there is a significant and appreciable impact of FOB testing on disease related mortality in asymptomatic population screening groups, discussed above. It seems likely that a more accurate test, particularly in terms of sensitivity would increase these benefits even further. Detailed below is the evidence from clinical studies aimed at determining the sensitivity and specificity of stool based molecular tests. It is important to note that one of the main limitations of FOB testing is acceptability to those offered testing, with uptake of around 50-60%. Whilst advocates of stool based molecular tests argue that this is likely to be largely in part due to the unreliability of FOBT (207), it seems more likely that an appreciable factor is the fact that the test is stool based, with the attached social stigmata in certain societies. As such molecular stool testing can only possibly circumvent some of the difficulties with FOB testing, although a sensitive and specific clinical test would be a significant step forward.

1.4.1 Pathophysiology of Faecal Molecular Markers

Looking in stool for DNA that is derived from neoplastic cells has further theoretical advantage over FOB in that it constitutes a direct marker of the neoplasm rather than of blood hence likely improving specificity, and it is expected that cell exfoliation is a more constant process than occult bleeding (207). Gastrointestinal mucosa has a high turnover rate, occurring completely every few days, although not all old coloncytes are removed by exfoliation (208). Neoplastic coloncytes have been shown to have a higher rate of exfoliation, and neoplastic cells and neoplastic DNA are relatively enriched in effluent to account for 14-24% of human DNA recovered from stool(209;210). Additionally cell density in the mucous layer overlying a colorectal cancer has been shown to be appreciably higher than that over normal epithelium (211). This enrichment of target material has been suggested to be the result of several pathophysiological processes including impaired apoptosis (212), cell adhesion (213), phagocytic removal and increased cellular proliferation (214). Additionally shed neoplastic colonocytes may have an ongoing survival advantage by being relatively spared the programmed cell death that normal cells undergo when separated from the basement membrane (211).

Whilst this apparent enrichment of neoplasia related targets would appear to make stool an ideal target for molecular approaches, there are a number of inherent difficulties in analysing faecal DNA:

1.4.2 Difficulties of Faecal Molecular Markers

Colonocytes may be trapped in the mucous layer, predominantly on the stool surface (215), and hence not evenly distributed. Therefore adequate sized stool samples are required and should include a portion of stool surface. Also processing may require agents to release neoplastic material from the mucous layer to optimise target recovery (207).

Several assays have analysed intact colonocytes recovered from stool, with high sensitivity and specificity in selected cases of colorectal cancer (216;217). However there is evidence that colonocytes undergo intraluminal lysis, and hence whole colonocyte assays are less sensitive at detecting proximal cancers than distal ones. Additionally storage of faeces for more than 1 day prior to analysis also results in lysis and decreased test accuracy (216).

These same technical difficulties also apply to approaches aimed at exploiting abnormal gene expression in colorectal neoplasia by detecting altered levels of mRNA, since mRNA is not stable in faeces and therefore the collection and processing of intact coloncytes is required (216).

DNA is relatively stable in stool, in comparison to mRNA. However the proportion of DNA derived from the host is estimated to be 0.01%, with the remainder being derived from dietary sources (218). Added to this is the presence of inhibitors of PCR within stool, resulting in difficulties across studies at isolating amplifiable quantities of host target DNA. Leading authors in the field advocate the use of a sequence specific hybrid capture technique, and report sufficient quantities to allow consistent analysis of DNA from faeces (210;219).

1.4.3 Faecal DNA

Given the technical considerations discussed above there has been a relative focus on the detection of DNA in stool. Investigators have assessed both absolute levels of human DNA in

stool and specific mutations related to colorectal cancer as discussed in the section on the molecular genetics of the disease.

a) Total DNA and Long DNA

Quantitative assessment of total human DNA has been shown to be of some discriminatory value in determining patients with colorectal cancer from those without, in an attempt to exploit the relative enrichment of neoplastic DNA in stool. When applying the necessarily stringent specificity for a test proposed as a population screening test, sensitivity is around 50% (218;219). Combined quantitative and qualitative assessment has also been performed by analysing the quantity of different length DNA extracted from stool. Apoptosis results in DNA being degraded by endonucleases resulting in small fragments. If exfoliated neoplastic coloncytes undergo relatively little apoptosis as a reflection of their neoplastic characteristics, then it might be expected that the DNA recovered from stool of a patient with cancer might be less degraded, or occur in longer fragments, 'long DNA'. Using over 200 base pairs as the criterion of long DNA, the sensitivity of this assay was 64% for cancer and 55% for adenomas in a small selected cohort of patients (210). It may be that this methodology is also less sensitive for proximal tumours as it seems likely that some degradation occurs during faecal transit, and has technical implications for collection and storage of samples. Additonally faecal long DNA may be detectable in patients with inflammatory conditions (220) or with altered colonocyte proliferation post adenoma (221), and may be persistently elevated following resection of tumour, hence reducing specificity for cancer and clinical utility. This said in subset analysis of multitarget assays, long DNA was the single most informative marker for cancer (210), and was not affected by tumour site.

b) Specific Mutations

i) K-ras

Mutations of K-ras were amongst the first genetic aberrations sought in stool (222), on account of the proportion of colorectal tumours exhibiting these changes and the well characterised mutational hot spot at codons 12 and 13. Many institutions have examined stool for the presence of matched ras mutations to those seen in the primary tumour, using a variety of PCR related techniques (222-233). These matched changes can be reproducibly detected in between 50 and 100% of cases examined, but the sensitivity of K-ras assays in isolation is under 50% on account of the mutational frequency at these loci in sporadic colorectal cancer. As such the work on K-ras mutation detection in stool was important in demonstrating that tumour derived mutations were indeed detectable in stool, however in isolation would not be sufficiently sensitive to provide a clinically useful test. Furthermore, work has suggested that K-ras mutations are not specific for cancer, and can be detected in both normal and benign gastrointestinal conditions (234;235). A landmark study using multiple stool markers demonstrated a sensitivity and specificity of mutation detection for cancer of 91% and 93% when K-ras was included in the panel of markers, but improved specificity that was not at the expense of sensitivity when K-ras was omitted from the panel (210). As such K-ras mutation in stool cannot be considered either sufficiently sensitive or specific.

ii) APC

Whilst mutational events involving the APC gene are considered fundamental in tumourigenesis, the frequency of detectable mutations in sporadic colorectal cancer is similar to that of the K-ras gene. This is likely to partly result from the large number of inactivating

mutations described and the diffuse locations of the mutations with the gene, such that detecting a mutation involves analysis of large numbers of base pairs. Therefore detection of APC mutations in stool is a less attractive technical proposition than alternative approaches. However methodologies have been developed which do not require analysis of large portions of the gene, but which have the ability to detect a proportion of APC mutational events. Vogelstein's group applied one such novel methodology called digital protein truncation to stool DNA. This is based on the principle that many APC mutations result in generation of a stop codon and hence protein truncation upon translation, and that PCR of stool DNA that has a high level of integrity (i.e. long DNA) can then be subjected to in vitro translation and then analysis of transcribed products. Using this technique they were able to detect APC mutations in stool with a sensitivity of 57% (in both polyps and cancers) and specificity of 100% (28 controls) (209). Again, analysis of APC alone will result in suboptimal sensitivity, but many multi-panel assays include some form of assessment of APC mutations, discussed further below.

iii) p53

A study of 25 patients determined detectable mutant p53 in stool samples using a PCR single strand conformation polymorphism assay, claiming a sensitivity of 64%. However this was 64% of the 11 patients with a comparable mutation in the primary tumour, and hence the sensitivity for the whole study population was only 28% (236). Further analysis of p53 mutation in stool has occurred as part of a multi-assay panel.

iv) Microsatellite Analysis

Microsatellite analysis of stool DNA is most commonly employed as part of a multi-target panel in an attempt to detect microsatellite unstable tumours (210;225;231;233;237-239).

Microsatellite analysis alone has been suggested to be suboptimal. Utilisation of the polymorphic BAT-26 to detect microsatellite unstable cancers, predominating in the proximal colon was suggested as an adjunctive screening modality to flexible sigmoidoscopy to detect distal tumours. A strategic limitation of this approach is that whilst MSI is more common in proximal tumours than distal ones, the majority of proximal cancers are microsatellite stable and hence cannot be addressed by this approach. However a small study demonstrated detectable BAT-26 changes in stool in 17 of 18 (out of 46 cases in total) patients with BAT-26 alterations in their primary tumour (240).

There has been little investigation of LOH in stool either at microsatellite or other markers. This is despite apparently good results in a report from Japan, looking at several markers including APC, p53 and hMLH1. This study compared stool DNA to neoplastic tissue and normal blood DNA in sporadic and HNPCC cases, and determined a sensitivity of 96.7% and a specificity of 100% using only p53 and APC markers (239).

v) Methylation

The importance of epigenetic changes in tumourigenesis is increasingly recognised. The phenomenon of promoter hypermethylation provides a strategic opportunity to detect the totality of colorectal cancer with similar technical methods. Application of methylation sensitive PCR to stool DNA has been shown to be feasible (207), although reports to date, examining SFRP2 methylation, have suboptimal specificity as well as sensitivity (77%) (241).

c) Multitarget assays

(210;225;231;233;237-239)

In view of the limitations of each individual marker in stool, there has been a relative focus in applying a panel of markers to stool DNA, in an attempt to improve sensitivity and overall test accuracy. To this end there is a commercial kit, Pre-GenePlus (Lab Corp, Burlington, NC) constituting analysis of K-ras, APC, p53, microsatellite analysis and DNA integrity (long DNA). Results from different institutions vary, however test specificity is generally reported as over 95%. Unsurprisingly test sensitivity varies for cancer compared to adenoma detection, with cancer detection rates between 62-91%. Adenoma detection has greater variation and is quoted at a sensitivity between 26 and 82%,

These results give encouragement to this approach to colorectal cancer screening, and are theoretically superior to FOBT performance characteristics, although are yet to be tested in such a large scale setting (242). Clearly stool DNA testing is far more involved than FOBT, and hence more expensive, although modelling suggests that the multitarget panel might compare favourably with the cost of screening by colonoscopy (243). Stool DNA analysis does not address some of the practical deficiencies of stool testing as alluded to above. There is limited data on uptake and acceptability, although high compliance and preference rates were achieved against colonoscopy and FOBT in a highly selected and motivated population of patients who had already attended for screening (244).

1.5 Circulating DNA

Plasma DNA was first described in 1947(245;246)but the field of free circulating DNA was pioneered by two plant biologists, Anker and Stroun, initially through their work on a plant tumour, crown gall. This tumour is caused by bacterial DNA translocating into plant cells. Affected plants develop secondary distant tumours which, since plant cells are unable to circulate on account of their cell walls (247), stimulated the hypothesis of circulating, free, non cell associated DNA. This observation led to experiments on frog hearts (248) and human lymphocytes (249) suggesting release of free DNA in higher organisms.

Detectable levels of free circulating DNA were subsequently demonstrated in both healthy humans and those with diseases. High levels were seen in the presence of benign diseases, but the highest levels were found in cancer patients (250). Furthermore the concentration of DNA was highest in patients with metastatic cancer and serum DNA concentration was shown to be affected by anti-cancer therapy (251). Nearly a decade later the first evidence was generated that the detectable serum DNA of cancer patients was neoplastic in origin, using strand stability assays (252). These findings led to studies aimed at demonstrating the same genetic aberrations in plasma and serum that were present in primary tumours. Initial studies focused on mutation of the ras oncogene in a number of gastrointestinal tract cancers with some success, discussed further below.

The ability to detect small quantities of tumour derived DNA in the blood stream provides a target for translating knowledge of the molecular genetics of cancer into clinically useful tests. Should low copy number mutant DNA be detectable in plasma or serum, it might infer the presence of a primary tumour harbouring the same mutation. As such the presence of mutant DNA in the circulation might be a surrogate for the presence of neoplasia elsewhere. Blood is in contact with all other bodily systems and is easily accessible for sampling. Therefore analysis of free circulating DNA potentially provides an essentially non-invasive

way of detecting a broad range of both primary and recurrent tumours and in principle might have a role in tumour detection and therefore screening. Additionally alterations in circulating DNA might predict tumour behaviourand hence be of usefor monitoring of recurrence, response to therapy and informing prognosis.

Accordingly over recent years there has been intensive investigation of multiple genetic parameters in a broad range of tumour types. The approaches to utility of plasma and serum DNA are presented below, with demonstration of the wide variety of genetic changes that have been identified.

1.5.1 Quantification / detectabilty

The discovery that different diseases are associated with altered levels of plasma DNA, has led to the hypothesis that the absolute quantity of detectable DNA might be a marker of those diseases, specifically cancer. The early studies of plasma DNA concentration employed various techniques for quantification. Landmark studies from the USA (250;251;253), used a radioimmune assay, with the antibody derived from the serum of a patient with SLE. They determined the sensitivity of the assay as 25 ng of DNA per millilitre of plasma, with the tested serum not requiring extraction or purification. In their initial study the concentration range 0-50ng / ml was designated as normal, with a specificity of 93% from 55 controls (251). Sensitivity was 50%, with half of 173 patients with various cancers having free DNA levels within the normal range. Whilst there was no observed correlation between site or size of primary tumour and free DNA concentration, although there was a significantly higher mean concentration in patients with metastatic disease. Additionally the free DNA concentration was altered by therapy, to degrees varying with the type of primary, and was associated with clinical response to therapy. The authors suggested that the high proportion of

cancer patients with normal free DNA concentrations indicated low diagnostic value, but that utility might be found in monitoring response to treatment. Addition of an assay for the DNA binding protein C3DP did not improve accuracy, and levels of DNA and C3DP did not correlate with each other (253). This paper did however document the presence of an inhibitor of DNAse activity in the sera of cancer patients, but this inhibition was not due to the presence of C3DP. A follow up study of 386 patients with a variety of benign and malignant gastrointestinal diseases demonstrated a significantly higher serum DNA level in cancer patients (412 ng/ml (+/- 63ng)) compared to benign diseases (118 ng/ml (+/- 14ng)). The sensitivity of a high serum DNA indicating malignant disease varied according to site of primary tumour, but was highest for pancreatic cancer, where 90% of patients had levels elevated above 100ng /ml (leon 1981 Eur J Cancer).

Another early study of 37 patients with advanced malignancy showed detectable levels of plasma DNA in 10 of 37 patients, compared to none in 50 normal controls. Plasma DNA required extraction and processing, during which an estimated 65% was lost. Values in cancer patients ranged from 0.15-12 micrograms / ml plasma, and was of size 0.5 kb to 21 kb (254).

The inaccuracy of serum DNA concentration to reliably detect patients harbouring tumours from those with benign disease or no disease, gave insight into the lack of knowledge of the biological processes involved in the release of free DNA into the circulation. Additionally these studies began to highlight the technical difficulties of applying molecular techniques to low copy number DNA in the presence of the milieu of substances present within plasma and serum.

More recently attempts to utilise free DNA concentrations have continued using advanced molecular techniques. For example plasma [DNA] assessed by quantitative real time PCR for hTERT has been performed for 100 non small cell lung cancers and age/sex/smoking

matched controls. Mean values were 8 times higher in cancer cases (24.3 vs 3.1 ng / ml), with the area under the receiver operating characteristic curve being 0.94, suggesting that plasma [DNA] may have utility in itself (255). However, as part of the multicentre European Prospective Investigation into Cancer and Nutrition (EPIC) trial, plasma DNA levels were analysed from 776 controls, 359 cancers (including lung cancer) and 49 individuals who subsequently died of COPD. A high plasma [DNA] was associated with an increased odds ratio of death from COPD (2.53, 95% CI: 1.06-6.02), but with no significant increase risk of cancers (256). This would suggest that an absolute increase in plasma [DNA] is not specific to cancer. Additionally they demonstrated high plasma [DNA] from certain centres involved in the study, likely to reflect sample handling and processing, and highlighting the variability in assessment of absolute plasma [DNA].

Further evidence for the role of sample processing in determining absolute plasma [DNA] was provided by a study of thoracic malignancies, both oesophageal and lung cancers (257). Plasma samples from cases of oesophageal cancers, that had been previously collected and stored, had higher plasma [DNA] than controls. However this was not the case for comparable cancer cases whose samples had been collected prospectively and treated uniformly. They concluded that quantitative analysis of plasma [DNA] is of limited diagnostic value, and that utility required qualitative analysis of mutant plasma DNA. A more recent study in non-small cell lung cancer demonstrated that a circulating DNA cut off of above 104.5 ng/ml resulted in a 52% sensitivity and 95% specificity for the detection of cancer(258)

A further study of prostate disease demonstrated that whilst patients with prostate cancer had higher mean levels than controls, those with benign prostate disease had significantly higher plasma [DNA] than those proven to have cancer. They concluded that absolute plasma [DNA] was not of value in the management of prostate cancer (259), although these findings

were not supported in another study (260). Another prostatic study demonstrated elevated plasma DNA levels in patients with benign prostatic disease, but those with localised cancer had levels within the normal range, suggesting absolute levels have no utility in prostatic disease (261). A small study in breast cancer demonstrated no significant difference in plasma [DNA] between patients with and without distant metastasis (262). In terms of prognosis a study of ovarian cancers, using quantification by RT-PCR, demonstrated an increased risk of death with increased levels of circulating DNA (263).

A large study quantified plasma [DNA] using picogreen technology, which binds to double stranded DNA and releases fluorescence proportional to the quantity of DNA present within the sample. The plasma [DNA] from 122 patients with neoplasms was compared to that from 164 patients with non-neoplastic disease and 44 controls. There was a significant difference in the mean plasma [DNA] between groups. The area under the receiver operator curve was 0.90 for healthy controls and 0.74 for those with benign diseases. With specificity set at 100%, the maximum sensitivity achieved was 57%, with the sensitivity being lower when comparing cancer cases to benign diseases as opposed to healthy controls (52). Picogreen was also used to determine plasma DNA concentrations in lung cancer patients compared to patients with benign lung diseases and controls (264). There was a significantly different mean [plasma DNA] between each group and sensitivity of 96% for cancer patients compared to controls and 73% for cancer cases compared to benign diseases, utilising a receiver-operator curve.

It appears from the above evidence that there is a clear trend for the presence of higher circulating DNA levels in patients with malignancy when compared to those without. However the relationship does not appear to be straightforward since not all cancer patients have raised levels and there is discrepancy in results according to tumour site and

investigating groups. Additionally it appears that a number of benign diseases may also result in altered circulating DNA levels(265) hence impacting on test specificity and overall accuracy. It is apparent that sample collection standardisation. Additionally studies employing more recent quantification techniques have generated more discriminatory results. This approach seems inherently worth further investigation since analysis is straightforward, inexpensive and utilises readily accessible bodily fluids and hence of great potential applicability to large numbers of individuals.

1.5.2 Tumour Specific Mutations

With the discovery of tumour derived DNA in the circulation there has been a large body of work investigating the presence of tumour derived DNA in plasma examining a wide range of types of genetic aberration found in primary tumours, and detailed below.

a) EBV

An adaptation of pure quantification of circulating DNA has been extensively investigated in relation to nasopharyngeal cancer. This malignancy is associated with Epstein Barr virus (EBV) infection, the virion for which is incorporated into host human DNA, and replicated during tumour clonal expansion. As such EBV DNA quantification equates to detecting genetic aberrations associated with the tumour, rather than measuring plasma [DNA] per se.

Several studies have demonstrated the ability to detect EBV DNA in plasma. One study from Thailand, using nested PCR, demonstrated detectable EBV DNA in plasma or serum in 98 of

167 nasopharyngeal cancer cases prior to treatment, compared to 10 of 77 normal controls (266). A group from Hong Kong have employed quantitative real time PCR to not only show detectability, but also demonstrate temporal and quantitative relationships. In a small cross-sectional study they demonstrated that 10 patients with tumour recurrence had a significantly higher plasma EBV [DNA] than 15 patients remaining in clinical remission, whose plasma EBV [DNA] remained negligible. In a parallel study 17 patients were followed up, all of whom had detectable plasma EBV [DNA] prior to radiotherapy. 16 patients showed an initial reduction in plasma EBV [DNA]. In those who developed recurrence, the plasma EBV [DNA] rose gradually to levels deemed high prior to the development of clinically manifest recurrence. Those cases who remained in remission maintained persistently low plasma EBV [DNA] (267). Furthermore plasma EBV [DNA] was shown to be an independent prognostic indicator for early clinical events (RR 3.8 (95% CI:1.6-9.2) for each ten fold increase in plasma EBV [DNA]), and also in a further cohort to be a significant variable associated with death from the disease (268). These results demonstrated the possibility of using plasma DNA as a marker of prognosis, response to therapy and recurrence. Similar results have been replicated in a study of 99 patients with stage III and IV disease, but without distant metastasis. Plasma EBV DNA was detectable in 94 of 99 patients and in none of 40 controls or 20 patients cured of disease. Higher plasma EBV [DNA] was associated with increasing disease stage, and poorer survival. Additionally genotyping of paired tumour and plasma DNA samples suggested that the free circulating EBV DNA originated from the primary tumour (269). In a temporal analysis of prospective cohorts, 65% and 96% of cases with locoregional and distant recurrence respectively had elevated plasma EBV [DNA], on average 100 days prior to the development of clinically detectable abnormalities (270)
The Hong Kong group have recently addressed the issue of using plasma EBV [DNA] to determine the risk of treatment failure, with a view to utilising plasma EBV [DNA] as a

factor in determining clinical treatment. They assessed the pre-treatment plasma EBV [DNA] in 90 patients with early stage nasopharyngeal carcinoma, who went on to receive single modality therapy on account of their early stage disease on standard clinical assessment. Over a mean follow-up of nearly 4 years, 7 patients developed distant metastasis, and 12 developed locoregional recurrence. The pre-treatment plasma EBV [DNA] was significantly higher in those who went on to develop distant disease, and the probability of distant failure was significantly higher in patients with a relatively low plasma EBV [DNA] of 4000 copies / ml plasma. They concluded that patients with early clinical stage disease but with raised plasma EBV [DNA] pre-treatment might warrant therapy that is classically reserved for more advanced cases (271). Most recently there is evidence that plasma EBV DNA clearance rates may be a prognostic marker for nasopharyngeal cancer, with cases with a short plasma DNA half life having a significantly higher complete response rate and overall survival (272)

This approach which employs essentially both quantitative and qualitative (tumour specific) assessment is as close to clinical application as the field of circulating DNA has come. However this disease is in an essentially priviledged position of having a ubiquitous mutation associated with viral infection, and hence is a unique but not reproducible scenario for other malignancies. It is however likely to represent the fore-runner for clinical application of tests in this field and likely to give helpful insights in terms of applicability, logistics and tumour derived plasma DNA biology.

b) Ras gene

The ras oncogene family is commonly mutated in a range of human cancers. In Kirsten ras the point mutations responsible for gene dysregulation are most commonly found at

mutational hot-spots in codons 12 and 13. The k-ras gene hence became an early target to exploit the presence of tumour derived DNA in plasma. Many groups have intensively investigated ras mutations in plasma in a broad range of tumours, particularly gastrointestinal malignancies.

In pancreatic cancer, by way of example, 90% of cancers exhibit K-ras mutations (273), and these appear to occur at an early stage of cancer development (274). Whilst there is evidence that these mutations can be detected in pancreatic fluid (275), this is difficult to sample clinically, and the ability to detect these changes in blood provides a less invasive and more attractive proposition.

A study of 21 patients with irresectable pancreatic cancer demonstrated detectable K-ras mutations in the plasma of 81% of them, with no changes being detected in a small number of controls or with benign pancreatic disease. Additionally in 4 patients there were detectable mutations in plasma DNA prior to a diagnosis being able to be established by conventional means (276). A similar study found K-ras mutations in 71% of primary tumours and in the matched plasma of 60% of these. The lower sensitivity may reflect that the patients in this study had treatable disease, and indeed the persistence of detectable plasma ras mutations was associated with poor prognosis (277) A subsequent larger study of 44 cases of pancreatic cancer and 60 controls with either benign or no disease, demonstrated K-ras mutations in only 27% of matched plasma DNA samples (278). Plasma positivity was significantly associated with tumour stage and the presence of metastasis, and was an independent prognostic factor. 5% of patients with chronic pancreatitis also had detectable mutant DNA in plasma, suggesting poor specificity.

A more recent study employed a sensitive mutation specific mismatch ligation assay to analyse 28 pancreatic cancer cases. Whilst 93% of primary tumours exhibited K-ras mutations, these changes were detectable in only 35 % of these patients plasma. Whilst

mutant plasma DNA was detectable in 5 patients with stage II disease, the authors of this study concluded that the poor sensitivity precludes its use as a test for detecting treatable disease (279).

Plasma K-ras mutations have been investigated in various other tumours including lung (280;281) and colorectal cancer (282). Results have varied according to tumour site, investigating group and specific differences in methods employed, but in no cases has test performance been sufficient to allow clinical application. This is likely to be in part due to lack of sensitivity inherent in methods, and the fact that neither K-ras mutations nor release of mutant DNA into the blood stream is ubiquitous in any given tumour type.

The results of investigation of circulating K-ras mutations in colorectal cancer are discussed further in section 1.5.3.

c) Methylation

Promoter hypermethylation has been recognosed to play an important role in carcinogenesis, and is a common event in a range of cancers. Methylation of genes seen in neoplastic tissue is rarely observed in normal tissue (283). Similarly to other plasma DNA based potential biomarkers no single marker will be informative for the totality of a given cancer type. However since methylation is a widespread phenomenon, and not specific to a given gene, a panel of markers based on the principle of aberrant methylation, may allow greater informativity for a series of patients than any one genetic marker in isolation. Additionally there is evidence that methylation of each gene appears to segregate independently (284). Furthermore it is likely that analysis of the pattern of methylation across a panel of genes will be informative as to the site of origin of the tumour, which clearly has impact on the practicalities of assessing asymptomatic individuals' plasmas.

Methylated DNA may also be preferentially present in plasma, since methylation alters chromatin structure, with a more compacted nucleosome that may be less accessible to the action of DNAse action in the circulation (284). Nucleosomes have been detected in serum of cancer patients(285), and these studies are now at the forefront of the field. Additionally methylated sequences have been reported at proportionally higher levels in the circulation (286).Therefore the ability to detect methylated plasma DNA is an attractive proposition across various medical disciplines and this is reflected by the variety of studies highlighted below:

A study of 22 hepatocellular carcinomas detected p16 methylation in 73% of tumour specimens and in 81% of the matched pre-treatment plasma samples from those with methylation positive tumours. Importantly those with detectable methylated plasma DNA included 7 patients with stage T1 or T2 disease, and no methylation was found in 38 patients with chronic liver disease or in 10 healthy controls. This suggests that this form of analysis is both sensitive and specific with the ability to detect potentially curable hepaticellular cancer (75).

Similarly in a study of non-small cell lung cancer, 15 of 22 tumours showed aberrant methylation at one of a panel of 4 genes, and 11 of these had the corresponding changes detectable in matched serum samples (287). This group also investigated promoter hypermethylation using the same panel of markers in 95 head and neck cancer patients. They found that 55 % had aberrant methylation in the primary tumour and 40% of these had the same changes detectable in serum DNA (288). A small study of breast cancer patients showed p16 methylation in 23 % of breast cancer cases, with the same change detectable in plasma of 63% of these (289) A study of h MLH1 methylation in plasma DNA of ovarian cancer patients enrolled in a chemotherapy trial demonstrated that the rate of methylation was increased at relapse, with 25% of 138 patients developing plasma DNA methylation that was

not detectable in matched pre-therapy samples. This increase was mirrored by increased microsatellite instability, and poorer prognosis (hazard ratio 1.99: 95% CI; 1.2-3.3), and was postulated to be due to selection of tumour clones resistant to the effect of chemptherapy on account of dysfunction of mismatch repair (74). A study of cervical cancer analysed methylation status in plasma DNA of a panel of 5 genes where methylation was associated with cervical cancer (290). Methylation of one of the panel was detectable in 87% of 93 cases, with the same methylation present in the limited number of matched tissue samples. Methylation of the gene MYOD1 was detected more frequently in plasma in patients with late stage disease, and therefore unsurprisingly was associated with poorer survival. A further study of 93 cervical and 122 breast tumour patients used a high throughput methylation assay and determined that plasma posivity was associated with poorer outcome in cervical cancer, and that a marker of APC methylation was an independent marker of outcome in breast cancer patients (291). A similar approach looked for hypermethylation at a panel of 6 markers in urine from renal cancer patients showing 88% sensitivity and 100% specificity in a cohort of 50 cases (including early stage disease) althought there were no controls (292). Whilst several of the results described above are impressive, less promising results have also been reported, for example P16 promoter methylation has been reported as detectable in 82% of 38 oesophageal squamous cell carcinomas, but with the same abnormality detectable in only 23% of matched plasmas, and each of these were in late stage disease (293)

Overall methylation is an attractive target for the analysis of tumour derived DNA in plasma, particularly since a panel of markers can be expanded using the same principle at different loci in order to try to improve assay performance.

Recently there has been a relative increase in the field of papers assessing circulating DNA methylation status, showing some promising results. Small panels of methylation markers have been shown to be of use in discriminating benign from malignant abnormal chest CT

findings (294). Additionally technological developments have increased the possibilities of this approach including the ability to interrogate the status of the entire 'DNA methylome' (295), and the ability to simplify to technical process of assessing multiple methylation markers, such as a panel of 37 methylation specific assays within a single reaction (296). Microarray mediated methylation analysis has also been performed on plasma DNA from pancreatic cancer patients, showing sensitivity and specificity of 90% (297). The results of recent studies of methylation in colorectal cancer are presented in section 1.5.3c.

d) Loss of Heterozygosity

Another attractive target for the analysis of plasma DNA is the near ubiquitous phenomenon of LOH. LOH derives from genetic instabilty resulting in losses or gains of defined chromosomal regions, has been termed a defining molecular signature of most human cancers (51), and is characterised molecularly by allelic imbalance (AI) (52). As such a panel of LOH markers may be able to give informativity for the great majority of cancers and furthermore the pattern of LOH in plasma may have inference as to the site of primary tumour. The rationale for detectability would be that a tumour with LOH/AI would contribute relatively more of the allele that had not been lost into the circulation hence resulting in AI in the plasma.

This rationale has been investigated in many different tumour types and body fluids as described below. The predominant method used for analysis has been microsatellite markers that have been variously quantified by autoradiography to fluorescent electrophoresis. Examples of results from studies in various cancers are given below.

e) Microsatellite Analysis

i) Urological

A study of clear cell renal cancer, where LOH of regions of chromosome 3 is a common event (298), examined 4 microsatellite markers on chromosome 3p in 40 patients. Microsatellite alterations were detectable in 65% of plasma DNA samples, with microsatellite instability accounting for only one case. There were no alterations in healthy controls, and the changes in plasma DNA of cancer patients were not significantly associated with disease stage in this small sample (299). A later study of 60 patients, the majority of whom had early stage disease, utilised 9 microsatelllite markers on 6 chromosomes. They detected changes in serum DNA in 74% of cases, plasma analysis being called positive only if there was a matching alteration in tumour DNA. The sensitivity was increased to 87% by increasing the number of microsatellites analysed to 20. Microsatellite alterations consistent with LOH were observed in 15% of 20 normal controls, giving a specificity of this analysis of 85%. There was a significant association of plasma positivity to advancing disease stage (300) .

An initial study in transitional cell carcinoma (TCC) of the renal tract using microsatellite analysis suggested sensitivity of 84.5% and specificity of 100% in a small sample (301). In a follow up study of 61 cancers and 20 controls, using a panel of 17 microsatellite markers, 80.3% of cancer cases had the same alterations in serum DNA to those found in the primary tumour. However 20% of controls had microsatellite alterations detected in serum DNA, giving a specificity of 80% (302). Whilst plasma positivity was not associated with tumour stage, it was significantly associated with higher tumour grade.

These studies do not define what level of change was required to be seen to be called an alteration. It seems likely that the high sensitivity is at the expense of specificity as small changes in allelic ratio are being called that may simply represent PCR / analysis artefact. Indeed recent study of 40 transitional cell cancer patients and 20 controls, using a panel of 16 microsatellite markers, found plasma LOH frequency of 25% in the cancer cases and 14% of

controls, a difference that was not statistically significant. This was despite demonstrating LOH in tumour tissue in 78% of cases (303). The authors suggest that microsatellite analysis of plasma DNA in transitional cell cancer should be interpreted cautiously, and that some of the differences may reflect utilisation of plasma compared to serum DNA.

ii) Lung

A 1996 study performed microsatellite analysis on plasma DNA from 21 patients with small cell lung cancer patients (304). 76% of primary tumours exhibited LOH using 3 markers, with changes detected in plasma of 71%. In one case the alteration observed in plasma was not observed in the tumour, which may reflect clonal heterogeneity or a lack of specificity of this form of analysis. However the authors defined LOH as a change in band intensity of 50% or greater suggesting fairly stringent calling of microsatellite alterations. A similar study of 87 non-small cell lung cancer cases, using 2 markers showed alterations in 56% of tumours and in 61% of these patients plasmas (305). Importantly there were detectable plasma DNA abnormalities in 43% of patients with stage 1 or small tumours. However 5 patients had changes in their plasma not seen in the primary tumour and there was no association with tumour stage. A later study of both non-small cell (23 cases) and small cell (11cases) lung cancers, used 12 microsatellite markers and determined that 83% of primary tumours and 85% of matched plasma samples demonstrated LOH, although the pattern of the alteration was seen to alter between tumour and plasma DNA (306). There was also a trend to plasma positivity to advancing disease stage, but there were a proportion of patients with localised disease that demonstrated plasma DNA alterations. A larger study of 86 cancers and 120 patients with benign disease demonstrated evidence of LOH in plasma in 60% of cancer cases, but also in 40% of patients with benign disease although 7% of controls were subsequently diagnosed with malignant disease (307).

iii) Breast

An early study of 17 breast carcinomas, showed few cases of LOH or shift (2 and 3 respectively) in the primary tumours, and only one case had concordant microsatellite patterns in matched plasma. They noted that microsatellites over 200 bp were hard to amplify, and suggested that this was due to plasma DNA being fragmented (308). A subsequent study of 62 breast cancer patients analysed 6 microsatellites, p53 mutations and aberrant methylation of p16, identifying a genetic abnormality in the primary tumour in 90% of cases. They observed a similar mutation in 66% of the cases paired plasma DNAs, with plasma positivity associated with clinical parameters indicative of poor prognosis (309). Anker and Stroun's lab generated higher sensitivity, rising with the use of tetranucleotide repeats to detecting changes in 81% of 23 primary tumours and 48% of matched plasma samples including some small tumours (310) .

A larger study of 71 cases and 9 controls, using only 2 microsatellite markers found plasma DNA LOH in 31.3% and microsatellite instability in 11.6% that corresponded to those found in the primary tumour. They were able to detect tumour cells in blood by immunocytochemistry in only one patient, but of 10 patients with plasma DNA alterations 5 had tumour cells by immuno-cytochemistry, and 6 had elevated mRNA for tumour markers within bone marrow samples. This may suggest that plasma positivity is a reflection of clinically indetectable tumour load (311).

A more recent study analysed a small study population with a large number of microsatellite markers. They were able to detect microsatellite abnormalities in 40% of serially taken samples. 80% of these changes were concordant with those seen in the primary tumour, but the volume of allelic loss was small (0.05 compared to 0.52 in tumour) and was inconsistent

in serially collected samples. This would suggest that in attempting to improve the sensitivity, the reproducibility of this analysis made it uninformative (312).

iv) Melanoma

LOH is a common phenomenon in malignant melanoma. A study of 10 microsatellite markers in 76 melanoma patients, 19 of whom had early stage disease clinically, 52.6% of cases had matched alterations in their plasma and tumour samples. However the positivity rates were 29% and 25% for stages 1 and 2, and plasma positivity was associated with later stage disease (313).

To investigate if plasma DNA LOH might have a role in predicting response to systemic therapy for advanced malignant melanoma, the same group monitored LOH in plasma DNA in 41 patients with metastatic melanoma (314). The overall response rate to targeted treatment with standard chemotherapy agents plus interleukin and interferon, was 56%, including 32% undergoing complete response. However those patients with LOH detectable in plasma had a response rate of 17%, significantly worse than those without detectable changes in plasma DNA (72% response rate). As such plasma positivity was the only significant independent predictor of disease progression.

v) Head and Neck

An early study of 21 patients with head and neck squamous cell carcinoma examined 12 markers of microsatellites in matched plasma and tumour specimens. One or more of the microsatellites had a matching alteration in plasma to that in the tumour tissue in 29% of cases. All of those with plasma positivity had advanced disease. However this demonstrated the ability to detect tumour derived DNA in plasma by microsatellite analysis (315). A larger study of 117 head and neck cancer patients found microsatellite alterations at a single marker

in 55% of the primary tumours, but in only 1 case was there corresponding change in plasma DNA. They concluded that <2% of total plasma DNA was derived from the tumour (316). A further study that included 40 controls determined both sensitivity and specificity to low at around 30% (317), whilst conversely a large study indicated a sensitivity of 100% for informative cases, although was unable to generate specificity levels as did not include controls (318)

Clearly from the results described above there is variation in assay performance between tumour types as well as according to the precise methods and panel of markers used for analysis. The results of studies in colorectal cancer are discussed further below. There are a number of technical considerations that are likely to have bearing on results and their validity which are considered further below and in the discussion in chapter 6 that relates to this work on microsatellite analysis of plasma DNA in colorectal cancer.

f) Digital SNP analysis:

Amore recent approach to determining allelic status has been described (319), that allows counting of individual alleles in multiple replicates, and has been termed digital single nucleotide polymorphism (SNP) analysis. This approach has some inherent advantages over technical difficulties experienced with microsatellite analysis that are discussed further below and in chapter 8 on allele counting experiments.

Digital SNP analysis has been investigated using ovarian cancer as a paradigm (52), initially being applied to defining allelic ratio in malignant ascitic fluid, demonstrating the ability of this technique to detect the same allelic distortion in ascites and tumour samples in 95% of known malignant ascites (320)

They analysed 122 cancers (54 ovarian, only 11 gastrointestinal), 44 healthy controls and 164 individuals with non-neoplastic diseases for plasma [DNA], then subset analysis of 54 ovarian cancer cases with 31 individuals with benign disease and plasma [DNA] higher than 50ng/ml. The highest sensitivity and specificity was at an allelic proportion of 0.6, where specificity was 100% and sensitivity 93% (50 out of 54), using a panel of 4 informative markers. Whilst the majority of ovarian cancer cases had late stage disease, digital SNP analysis had sensitivity of 87% for stage I and II disease. In 15 of 17 available matched tumour samples, the same AI was detectable. Additionally when estimating the contribution of tumour DNA to the total plasma DNA using mathematical modelling, the majority of cases contributed 0.48 (0.43-0.53) although the total range was 0.26-0.89.

Criticisms of this study include the fact that patient recruitment was retrospective and the patients had already been diagnosed by conventional methods, and therefore does not necessarily indicate improvement over methods already available. Additionally LOH has been demonstrated in benign diseases such as endometriosis, therefore patients with these conditions should be included in the control group to see if the methodology is able to discriminate (321).

A further elegant adaptation of this approach, called BEAMing, has been more recently described (322), that allows counting of multiple individual alleles in large numbers by binding each one to magnetic beads and then counting them by flow cytometry. This approach has been employed to quantifying APC gene copy numbers in plasma DNA from colorectal cancer patients (323) and is described further below in the section on colorectal cancer.

Another adaptation of this approach has been employed in the assessment of EGFR mutations in lung cancer. A microfluidic digital PCR system was employed to assess a panel

of 2 markers, with the ability to perform 9,180 PCRs at nanolitre quantities, and claimed to be able to detect a single mutant DNA molecule. This study demonstrated a sensitivity of 92%, with specificity of 100%, for analysis of plasma from those patients with the appropriate mutation in the primary tumour (324). An interesting outcome from another study of EGFR status in lung cancer demonstrated that analysis of plasma DNA could be used as a surrogate for analysis of primary lung tumours, where there was an insufficiently sized clinical sample from the primary tumour, showing that assessment of circulating DNA could infer the EGFR status of the tumour and the response to EGFR inhibitors (325).

g) p53

P53 mutations are difficult to identify efficiently due to the multiple sites in the gene at which mutation has been shown to occur resulting in gene inactivation. Sequencing of large tracts of the gene are feasible, if labour intensive, such as exons 5-8, as performed in tumour and paired plasma DNAs from 25 patients with either breast or small cell lung cancer (326). This study identified p53 mutations in 6 primary tumours and in the matched plasma of 50% of these, giving a sensitivity of 12% in this small group.

An exception to the diffuse nature of p53 mutation is of codon 249 of exon 7 in hepatocellular cancer patients caused by aflatoxin metabolites. Several studies have examined the detectability of this mutation in plasma DNA. An early study using RFLP assay, with results confirmed by direct sequencing, demonstrated the mutation in 36% of the cancer cases plasma from West African patients, but not in European patients. However the same mutation was detected in plasma DNA in 15% of patients with cirrhosis and 6% of normal controls,

indicating little likelihood for clinical utility (327). Subsequent studies have demonstrated higher rates of mutation in plasma DNA of affected cases, e.g. 46.7% of cases from China. Whilst there were no changes in control plasmas in this study their controls were healthy and taken from a Western population, so of less relevance to the utility of the test (328). A larger study

of 158 cases from South Africa found a plasma mutation rate of 18%, indicating a suboptimal sensitivity (329). The most promising results came from a further study population in the Gambia where 35% of 29 primary tumours showed the mutation, with the same mutation detectable in 88.5% of matched plasma samples (330).

One approach to more diffuse p53 mutation detection is to screen exons 5-8 by temperature gradient gel electrophoresis. These methods have been employed in the analysis of gastrointestinal malignancies both generically (331) and in colorectal cancer in isolation (332). Promising results have been obtained for GI malignancy in general, with a sensitivity of 78.6% prior to treatment, and also as part of a panel of markers in colorectal cancer as described in the following section. However methods are somewhat involved with multiple steps required in analysis that require repetition for subdivisions of the examined gene, perhaps resulting in limited practical applicability.

1.5.3 Colorectal Cancer

Colorectal cancer is an ideal paradigm disease for plasma DNA as a biomarker for reasons already discussed in this chapter. These include the high incidence and mortality of the disease as well as the extensively investigated mutational events in tumour initiation and progression. Additionally current non-invasive detection and screening methods are suboptimal, although there is a priori evidence that early detection is worthwhile in terms of mortality and disease incidence.To this end there is a body of work assessing plasma DNA utility as a biomarker, predominantly assessing K-ras status but also with analysis at other genetic loci.

a) K-Ras

K-ras mutation has been extensively studied in colorectal cancer and hence was an early target for assessment of mutant plasma DNA. An early study of K-ras mutations in 14 patients with colorectal cancer used sequence specific primers (PASA-PCR), with confirmation of mutations by cloning and sequencing, and additional mutational analysis using RFLP PCR. The sensitivity of the PASA-PCR was down to detection of 1 mutant gene copy in 10,000 wild-type copies, with DNA from SW-480 cancer cell lines used as a positive control. Extraction of plasma DNA was with phenol-chloroform and quantification with spectrophotometry. Normal and tumour DNA underwent 35 cycles of PCR with 45 cycles for plasma DNA. Using PASA-PCR K-ras mutations were detected in 7 (50%) of primary tumours, 6 of these being at the second base of codon 12. Six of the 7 cases (86%) with a ras mutation in the primary tumour had identical mutations detected in paired plasma samples. Whilst 3 cases had metastatic disease, 1 patient with each of Duke's stages A,B and C also

had K-ras mutations detectable in plasma. There were no mutations detected in plasma of 6 healthy controls, suggesting that the results were not false positives. This preliminary study showed feasibility of K-ras mutation detection in colorectal cancer patients' plasma DNA, with apparent high sensitivity and specificity.

A larger study examined K-ras mutations in plasma of 240 patients undergoing colonoscopy for colorectal symptoms (333). They employed a RFLP assay for codon 12 mutations, with visualisation by gel electrophoresis, a method found to have sensitivity of 1 mutant copy in 10^5 in their hands, although not specifically in the context of plasma DNA (but rather mutant copies added to placental normal DNA). Mutations in tumour were confirmed by dot-blot hybridisation. Of 240 patients 8 had colorectal cancer and 62 had adenomatous polyps alone, with half of these having only one polyp. There were therefore 170 controls without neoplasia at colonoscopy. K-ras mutations were present in 43% of primary neoplasms. K-ras mutations were also present in colonic biopsies in 5 of 65 controls. All 5 patients with ras mutations within colorectal cancer had changes detectable in plasma. Of 25 patients with ras mutations in their polyp, 20 had changes detectable in plasma (80%). A total of 28 of 105 controls had mutant ras in plasma DNA, including those with mutant ras in non-neoplastic biopsies. This study showed a high sensitivity for mutant ras analysis of plasma DNA, including in patients with pre-malignant lesions, but a poor specificity suggestive of some false positive results. However ras mutations can be observed in non-malignant tissue, and also in a common phenomenon in non-colorectal cancer that may present with symptoms mimicking those of colorectal cancer.

The utility of detecting mutant K-ras in plasma DNA of colorectal cancer cases in relation to follow-up and prognosis has also been assessed. A study of 58 patients with a full range of disease stage underwent plasma DNA analysis for K-ras mutations and aberrant p16 promoter methylation (68). They used mutation specific primers for K-ras mutations and methylation

specific PCR for p16 methylation. 38% and 53% had ras mutations and aberrant methylation respectively in their primary tumours. Sensitivity of detecting changes in plasma DNA was 45% for K-ras and 68% for aberrant p16 methylation. Plasma positivity was significantly associated with poorer prognosis, $p<0.03$ by log rank test when stratified for tumour stage, and was an independent prognostic factor.

Similarly a prospective study of 94 cases of colorectal neoplasia examined K-ras mutation plasma positivity and its relation to prognosis (282). They followed a total of 94 patients for three years, with blood samples at 1 week and 1 month post-operatively and then at three monthly intervals. The control group was 20 patients who underwent normal colonoscopy. Analysis was of serum DNA for K-ras mutations using a semi-nested mutant enrichment PCR technique and enzymatic digestion. K-ras mutation was detected in 53% of tumour samples and 41% of plasma samples of a cohort undergoing pre-operative testing only, including in 4 of 7 cases with adenomas with K-ras mutations in the polyp., and indeed there was no significant association between plasma positivity and disease stage. Of the cohort undergoing longitudinal follow up, 64% had K-ras mutations in their primary tumour, and there was no association between the primary tumour K-ras status and prognosis. 16/60 (27%) of patients with mutant K-ras in their tumour became persistently positive for serum K-ras mutation during follow-up and 10 (63%) of these developed recurrent disease, compared to 1/44 (2%) that developed recurrence but remained serum K-ras negative. Therefore the odds ratio of recurrence given a positive serum K-ras was 71.6 (7.7-663.9, $p\leq0.001$). Plasma positivity was an independent predictor of recurrence by cox regression analysis with a relative hazard of 6.37 (2.3-18.0, $p\leq0.001$), higher than that of Dukes stage. Overall detection of post-operative serum mutant K-ras was 52.6% sensitive and 92% specific for disease recurrence, rising to 91% sensitive and 88% specific when analysing

those with mutant K-ras in their primary tumour. These results would suggest that K-ras mutation status in plasma DNA may be of prognostic significance, but also demonstrates that mutant plasma DNA is detectable in patients with early stage and pre-malignant disease, when amenable to surgical cure.

A significant deficiency with plasma DNA K-ras analysis in colorectal cancer is that strategically it can only possibly detect the minority of tumours. Given the relatively low prevalence of K-ras mutations in colorectal cancers (40-50%), even an assay with sensitivity of over 80% could only detect upto 40% of the totality of colorectal cancer (334). However this approach remains the most extensively studied with respect to colorectal cancer and is likely to form part of a panel of markers should plasma DNA in CRC reach the point of clinical utility.

b) Microsatellite Analysis

In contrast to K-ras assessment, microsatellite analysis provides potential for strategic informativity for the great majority of colorectal cancer, however remains little investigated. An initial study of microsatellite alterations in serum DNA of colorectal cancer patients was performed in 44 cases. They also performed p53 mutational analysis and mismatch ligation assay for K-ras mutations (335).The microsatellite markers used were: Chromosome 18q: DS18S55, DS18S58, DS18S61 and DS18S69; Chromosome 17p: CHRNB1 and D17S786; Chromosome 8p: D8S133 and D8S254.

The primers were radioactively labelled, and the PCR products analysed by autoradiography. LOH was scored if there was >30% decrease in allelic frequency.

Either LOH or microsatellite instability was detected in 80% of primary tumours, but was observed in none of the matched serum DNA samples. The authors suggested that this

negative result might be the result of filtering of mutant DNA by the liver, or a lack of sensitivity of the type of analysis. Additionally serum DNA was used as opposed to plasma, and there is evidence of increased release of normal DNA from blood cells during clotting, that might mask the relatively small allelic ratio differences in DNA derived from the tumour. Autoradiography is an older technique for microsatellite analysis, whereas fluorescently labelled primers using commercially available platforms allow more accurate quantification of allele frequency. These findings had led observers to suggest that 'Microsatellite alterations seem of no interest since their frequency is low or absent in colorectal cancer patients' plasma and their detection in plasma could be subject to artefacts' (68).

However a subsequent study employed fluorescent microsatelite analysis to paired tumour and plasma samples from 27 colorectal cancer cases and 10 healthy controls. They employed 9 microsatellites across 7 chromosomal regions (336). They demonstrated at least one microsatellite alteration in 96% of microdissected primary tumours, with alteration frequency at individual markers ranging from 19 to 59%.

Serum DNA was extracted using QIAgen commercial kits. PCR cycling was for only 30 cycles. LOH was scored when there was an alteration in peak intensity of >50%.

Microsatellite alterations were detected in 59% of plasma DNAs from the cancer cases, with robust ability to generate products at all markers studied. LOH was detectable in 26% of serum samples and microsatellite shifts in 48% of samples. There was no association between plasma positivity and advanced disease stage. When these figures were compared to the changes observed in the primary tumour, LOH was detectable in plasma of 35% of the patients with the change in their primary tumour, compared to 93% for microsatellite instability, with this difference being statistically significant. However microsatellite shifts were detected only in the plasma DNA and not the primary tumour for 3 patients. Providing evidence against these observations being due to artefact, no changes were seen in the plasma

DNA from healthy controls. The authors' rationale for the increased sensitivity of microsatellite shifts compared to LOH is that the methodology favours the identification of new alleles as a new peak over the discrimination of subtle variations in peak intensity which might be associated with LOH.

An additional reason for the relative inability to detect LOH in plasma of colorectal cancer cases is the presence of genetic heterogeneity. The increased amount of one allele released into circulation by tumour clones might be 'cancelled out' by DNA from a different clone that exhibits LOH of the other allele (alternate AI). Therefore despite AI in particular portions of the tumour, plasma DNA might remain in allelic balance. The multi-step theory of colorectal carcinogenesis would expect low tumour heterogeneity as each mutation results in a proliferative advantage for that clone. This would be expected to be the case particularly for early mutational events, such as inactivation of the APC gene. There is evidence however that tumoral heterogeneity exists in colorectal cancer albeit in advanced stage disease.

A study to investigate genetic heterogeneity was performed with relation to plasma DNA (337). This study investigated 24 lung cancer cases and 26 colrectal cancer cases with documented liver metastases, using 33 dinucleotide repeat microsatellite markers across the majority of chromosomes for the colorectal cases. An alteration in peak intensity was classed as LOH. Whilst this study demonstrated the presence of alternate AI in the plasma of 25% of lung carcinomas, the colorectal cohort had analysis of primary tumour to liver metastasis only (not of plasma DNA). However alternate AI was observed in 54% of liver metastasis compared to primary tumour, suggesting a substantial degree of heterogeneity in advanced colorectal cancer. Notably alternate AI was not observed in relation to chromosome 5q in colorectal cancer, possibly a reflection of this being an early mutational event. As such alternate AI in metastasis may be the result of polyclonal extension of cells that are already malignant hence the predominant clone in the primary tumour may not be the clone

responsible for metastasis. Alternatively incongruous results may be a reflection of artefact with methods employed a possibility discussed further in the discussion in chapter 6.

c) Methylation

A 2001 study of hMLH1 promoter methylation in serum DNA analysed 19 CRC cases (338) using methylation specific PCR. Their assay was tested on cell lines with methylated and unmethylated hMLH1 promoters, and derived a sensitivity of 1 methlayted copy in 100 unmethylated promoter copies . 10 of the CRC cases had microsatellite instability, and 9 of these exhibited promoter methylation in the primary tumour, compared to none of those that were microsatellite stable. 3 of the MSI cases, all with late stage disease, had detectable methylated serum DNA, giving a sensitivity of 33% (given knowledge of those that were MSI), and a specificity of 100%.

A further study of 52 cancers, 34 adenomas and 10 controls demonstrated p16 hypermethylation in 38% of cancer cases and 70% of these had matching changes in plasma, with no changes found in the tumour negative, adenoma or control plasmas (339).

Subsequentlymethylation status in serum DNA from104 colorectal cancer cases was demonstrated to associated with tumour size and stage and significantly poorer prognosis, using a panel of 3 methylation markers (340). A larger study using 3 different methylation markers gave sensitivity of50-70% and specificity of 65-85% for inference of the primary tumour by analysis of plasma DNA (341).

More recently methylation analysis of SEPT-9 in plasma yielded sensitivity of around 70% and specificity of approximately 85% in 2 separate populations (342). Simulataneously there have been technological advances which have allowed simple analysis of many more methylation markers, and with higher technical sensitivity. Vogelstein's group described a

variation of their beaming technique for methylation analysis, able to detect 1 methylated copy in 5000 unmethylated copies, detecting 59% of colorectal cancer cases, hence being four times more sensitive than CEA (343). In a large study of early colorectal cancer cases, polyps and controls, 10 genes were assessed for methylation in tumour and plasma, and a 4 marker panel generated which performed with positive and negative predictive values of around 90% (344).

The application of methylation based approaches in colorectal cancer have progressed significantly in recent years, and are now generating performance figures that rival those which are close to clinical utility. In combination with the strategic informativity of this approach and the technological advances in methylation detection, it seems likely that methylation analysis will have a role, in colorectal cancer particularly, should circulating DNA translate to implementation in clinical settings.

d) Panels of markers

Little work has been done with panels of markers in colorectal cancer with the exception of multiple microsatellite markers, and now methylation panels. A few studies have examined more than one genetic locus, such as a study of P53 (exons 5-8 were amplified and directly sequenced) and K-ras mutations (mismatch ligation assay) (335). One small study of 20 colorectal cancers analysed multiple genetic alterations (332). Analysis was performed of the APC gene mutation cluster region (MCR) between codons 1286 and 1513 of exon 15 and p53 mutation analysis of exons 5-8 was performed by dHPLC scanning analysis. K-ras and BRAF mutations were investigated using mutant allele specific amplification (MASA). This study was able to detect alterations at one or more of the above regions in all 20 CRC cases (including 2 Dukes A cancers). Clearly a sensitivity of 100% is impressive however no

controls were included for analysis to allow assessment of specificity. Additionally the analysis as described for each of the loci for each case would be extremely labour intensive and costly and hence of limited practical applicability. A further study examined the use of a 5 marker panel using quantitative real time PCR, generating an area under the curve in ROC analysis of 0.88. That said specificity remained suboptimal at 65-75%(345). It seems likely however that application of plasma DNA biomarkers to CRC will constitute a panel of markers, as has been suggested at previous international meetings in the field (Stroun, personal communication). By this approach it is likely that each of the different genetic pathways thought responsible for colorectal cancer could be represented by specific markers and hence improve the strategic informativity and sensitivity of these approaches.

e) Quantification

Until recently there has been little data in the literature regarding quantification of plasma DNA in colorectal cancer. Diehl's paper regarding APC mutations in plasma of colorectal cancer patients gives some information on plasma DNA quantity, although this was not the primary aim of the study and draws no conclusions as to plasma [DNA] per se (323).
Two similarly sized studies demonstrated a significant difference in quantity of circulating DNA between cancer cases and controls (346;347). The slightly larger study (75 cases and 75 controls) quantified serum DNA, again using real-time PCR (347), and correlated the results with carcinoembryonic antigen (CEA) levels. Median serum DNA concentration was 5 times greater in cancer cases compared to controls ($p<0.001$), and whilst there was a higher mean concentration in patients with metastasis at time of treatment, there was not an association of higher levels with increasing disease stage in patients without metastasis. Analysis of test accuracy using ROC curves demonstrated that serum DNA concentration performed marginally better than CEA concentration, with a quoted sensitivity of 81.3% at a specificity

of 73.3%. Combining both serum DNA and CEA concentration improved sensitivity to 88% without a reduction in specificity. These findings led the authors to conclude that this approach represents a potentially useful tool for the diagnosis of early stage colorectal cancer. A subsequent studyagain described a significant difference in plasma DNA quantity between cases (n=70) and controls (n=20), but surprisingly reported no overlap in plasma DNA concentrations between groups and hence 'perfect discrimination' in inferring the presence of colorectal cancer from quantification of plasma DNA(348).

One further study assessed the utility of plasma DNA concentration in determining prognosis in and monitoring the response to chemotherapy treatment of patients with stage IV colorectal cancer (349). They reported a significantly poorer survival for those patients with markedly elevated levels plasma DNA concentrations, but observed fluctuating levels at differing points of treatment in the small subset of their patients with follow up data.

1.5.4 Biology and Technical Considerations

Much remains to be elucidated regarding the biology of circulating DNA. The source of circulating DNA remains unclear, despite investigators searching for 30 years (350). The majority of commentators suggest thatrelease from apoptotic or necrotic cells is the fundamental process(351), alongside impaired enzymatic and cellular DNA clearance (336).Evidence to support this hypothesis includes the ability to detect cancer derived mutations and the classical ladder pattern of degradation of DNA from the circulation as seen from apoptotic cells. It also appears that macrophages are central to the process since mice lacking in macrophages have an impaired response to stimuli that would otherwise result in increased plasma DNA levels (352).

However living cells can spontaneously secrete DNA (350;350;350;350;353), and benign and malignant disease processes may effect the balance of synthesis and clearance.Alterations in plasma DNA are detectable in patients with benign diseases and premalignant lesions. Examples of this include raised levels in myocardial infarction and diabetes (354), as well as in benign naevi influencing the diagnostic accuracy for melanoma (265) suggesting that the process of altering plasma DNA may not be due to the presence of cancer per se. There is evidence of increased endogenous DNA from a mouse model, whereby mice bearing human tumours, had increased plasma DNA resulting from both exogenous and endogenous sources (355).

Furthermore it has been suggested that cell free DNA may have biological activity(356), with some evidence for the ability to transform cultured cells (357).

Whatever the origin, it is clear from the evidence described earlier in this chapter that not all tumours result in mutant DNA being detectable in the bloodstream. The inability to detect mutant DNA in circulating body fluids may be due to not all tumours releasing DNA, limited stability of DNA in the circulation, or normal DNA may interfere with assay sensitivity (246). Non-neoplastic tissue DNA released from normal cells can mask tumour derived DNA since potentially small quantities from tumour cells may be overwhelmed by the larger quantities of normal DNA. There are wide ranging estimates of the relative contribution of tumour derived DNA to the totality of plasma DNA. Estimates have been reported from 5-48% of total DNA (52;358) to a more recent study using BEAMing technique estimated the contribution of tumour DNA to between 0.001-1.75% for early stage CRC, upto 27.4% for metastatic disease (323).

This fundamental issue remains to be conclusively resolved and is likely to prove difficult to do so as it seems likely to vary according to the tumour type studied as well as the precise details of collection, extraction and quantification which remain non standardised.

The exact nature of plasma / serum DNA remains debated. Plasma DNA is degraded to a variable extent, potentially artificially enriching for smaller alleles (359)

Analysis of plasma EBV DNA from nasopharyngeal cancer and lymphoma patients, suggested that the fragments are short, with 87% of them being shorter than 181 bp, and also that they are indeed free, rather than associated with virions (360). Further evidence of the fragmentary nature of circulating DNA has been obtained from cloning, with the majority of cloned sequences being of around 200bp. However sequencing of the cloned DNA demonstrated heterogeneous sequences including large repeats (361)

The integrity of free DNA within plasma is subject to decrease by the action of DNAse, resulting in smaller fragments in which the sequence that confers neoplastic characteristics is no longer recognisable. The presence of an inhibitor of DNAse activity has been documented in the sera of cancer patients (253), however degradation is certainly a problem, with DNAse resulting in decreased DNA yields(362). That said, DNA based markers have advantage because of the inherent stability of DNA when compared to RNA and some proteins, and there is some evidence to suggest that plasma DNA in cancer patients has higher integrity (363).

Mutant DNA clearance from the circulation has been most extensively studied in head and neck cancer, following both radiation therapy and surgery, showing first order kinetics and a faster clearance following surgery (2.3 hours vs 3.8 days) (246;364). Evidence from investigation of foetal DNA in maternal plasma also indicates rapid clearance (365-367). With respect to colorectal neoplasia it has been suggested that the half life of mutant DNA is less than 1 week (282). The mechanism of DNA clearance also remains to be conclusively

determined, although from studies of renal dialysis patients it does not appear to be cleared by the kidneys (368).

Fastidious collection and processing within specific time frames appears to be the best solution to counter the problem of DNA breakdown or clearance, and the storage of processed samples should be uniform and repeated freeze thawing avoided. Since plasma DNA may be low molecular weight short amplicon markers and specific extraction kits may be better, with a study showing higher proportion of mutant to wild-type ras sequences in urine from CRC patients with the low molecular weight extraction protocol; but this is in urine and hence may have been filtered to some degree (358). However doing the same comparison of extraction on plasma gave detectability in 1/6 for high molecular weight extraction compared to 5/6 for low molecular weight. They suggest improving mutation detection by selectively amplifying the mutant by various means. Similarly it has been shown that >80% of circulating EBV sequences are shorter than 181bp (360). Additionally it has been demonstrated that circulating DNA from control subjects is no more degraded than that from cancer patients (369), with a further study of lung cancer patients demonstrating no difference in circulating DNA integrity between cancer patients and controls (370).

Not only is the quantity and quality of template an issue for work on plasma DNA, but what template there is may be replicated less efficiently than standard preparations of DNA. Each fraction of blood has been shown to be highly inhibitory to standardised PCR (371), and size exclusion chromatography suggested the inhibitor within plasma was IgG. Further experiments suggested that IgG was a potent inhibitor of a range of polymerases. Whilst pre-treatment of IgG at 95 degrees obviated the inhibitory effect, heating of the IgG and target DNA together to 95 degrees blocked amplification, suggesting hot-start PCR is not the solution.

Early studies in the field highlighted the quandry of using DNA extracted from plasma or serum. Suggestions that free DNA in serum is a result of release from leucocytes during clotting, were supported by a study using 4 assay methods on plasma and serum from normal subjects (372). They were unable to detect DNA from plasma where serum DNA was detectable from the same individuals, and advocated the use of plasma where free DNA detected would be the result of pathological processes. A study of patients with colorectal cancer metastasis, matched with healthy controls, demonstrated a differing relationship between concentrations of plasma compared to serum DNA (373), and concluded that plasma DNA levels better represent in vivo levels of circulating DNA. A further paper demonstrated increasing levels of detectable DNA in serum over plasma, with higher levels still in serum samples left for a period of time before processing. However there remains no consensus regarding the use of plasma versus serum and no standardised method for processing and extraction (374)despite increasing calls in the field for standardisation.

A potential biological difficulty with the assessment of plasma DNA in gastrointestinal tumours is that it has been suggested that first pass metabolism by the liver may result in clearance of tumour derived DNA. This has been suggested as a particular issue for colorectal cancer. One study examined detectability in portal venous blood at time of operation compared to that in peripheral blood, and reported higher detectability in portal blood overall (375). However there are a number of explanations for these results, including tumour manipulation during surgery, and the detection of LOH was not significantly different between the two sources of blood. Additionally tumour derived DNA has been repeatedly demonstrated in the blood of CRC patients and it is counterintuitative that the liver would clear some types of mutant DNA but not others (e.g. K-ras).

Given the uncertainties regarding the biology of plasma DNA, and the implications for performance when applied as clinically useful tests, there is need for considerable further work in this regard. Encouragingly there are increasing reports of biological models to try to delineate these issues, including mouse models with xenograft tumours (376)

A considerable further hurdle for the field of plasma DNA as a biomarker is that studies to date are of patients already diagnosed with tumours. The ultimate aim of these biomarkers is to provide non-invasive diagnostic or screening tests and hence extrapolation of described assay performances to asymptomatic populations as indication of performance as a screening test must be interpreted with caution.

1.6 Other Approaches To Circulating Molecular Markers

1.6.1 Mitochondrial DNA:

An alternative approach to the analysis of genomic DNA in the circulation is the assessment of mitochondrial DNA (mDNA), mutation of which has been implicated in neoplasia (377;378). Potential advantages of this approach is that mutational rates in mDNA are high on account of a high level of reactive oxygen species generation, and an apparent mutational hot spot within the D-loop of mDNA. An attractive paradigm disease for the study of the presence of mutant mDNA in plasma is melanoma on account of the association with UV exposure in carcinogenesis. A study of 12 melanoma patients demonstrated m DNA alterations in 42% of tumour samples from metastases, and in 40% of the paired plasmas from the positive tumour cases, giving an overall sensitivity of 17% (379). Whilst these preliminary results are suboptimal this approach warrants further investigation.

1.6.2 RNA

An alternative approach to analysis of circulating DNA is the more recent development of detection of plasma RNA. An inherent disadvantage of this approach is the instability of RNA (380), although it has been shown to be robustly detectable in plasma of cancer patients by several investigators, particularly when comparing endogenous to exogenous RNA (381), and may be protected from degradation to some degree in plasma (382). Plasma RNA detection has developed predominantly since the advent of real time reverse transcriptase PCR which allows quantitation of specific RNA sequences. The presence of human telomerase reverse transcriptase (hTERT) as a marker of telomerase expression has been reported in a small number of colorectal cancer patients using a quantitative real time PCR assay (383)

Thyroglobulin mRNA has also been reported at significantly higher levels in thyroid cancer patients than controls (384). Similarly prostate specific membrane antigen mRNA has been detected in prostate cancer patients although with suboptimal sensitivity and specificity in comparison with benign prostate disease.

1.6.3 Proteomics

Investigation of abnormal proteins in the circulation of cancer patients is another potential biomarker that holds considerable promise. The field of proteomics has undergone rapid expansion in recent years since the more widespread availability of advanced detection hardware such as surface enhanced laser desorption ionisation (SELDI) and matrix assisted laser desorption ionisation (MALDI) time of flight (TOF) mass spectrophotometers. An advantage of this approach is that the proteins that are abnormally expressed do not necessarily need to be identified. The protein expression patterns are however extremely complex and require sophisticated analysis hardware and attendant software. Impressive initial results have been reported in ovarian cancer (including stage 1 ovarian cancer which is amenable to surgical cure) with 100% sensitivity and 95% specificity (385). Many groups and institutions are employing similar approaches in many diseases with a view to determining disease specific protein profiles.

For these and other approaches, continuing technological advances hold promise for improving the utility and accuracy of mutation detection with particular reference to circulating biomarkers such as the single base primer extension method termed shifted termination assay (STA) that allows primer extension only in the presence of a given mutation. A large panel of markers or potential mutations may be analysed in high throughput methods utilising a 96 well microplate (386). Additionaly the BEAMing

technique holds promise for the detection for high volumes of individual alleles in a relatively inexpensive way.

Whilst there is much that remains to be clarified in the field of circulating nucleic acids, there is much that makes the field attractive for further investigation. Work in this field not only has the potential to delineate biological processes but also to be truly translational research with the generation of clinically useful tests. The added benefit that these tests would be essentially non-invasive, and hence likely to be embraced by at risk populations, make their potential application to disease screening a tantalising target.

1.7 Biomarker Classification

Given the proliferation of translational studies in disease detection and response monitoring, there has been an increasing focusin recent years on robust means of biomarker classification and development. Several leading clinical research bodies have issued guidelines in this regard including Cancer Research UK and the National Cancer institute in the US. In the last few years it has become accepted that biomarkers may classified on a number of levels (http://clincancerres.aacrjournals.org/content/16/6/1745.)

Firstly biomarkers may be classified according to their intended purpose into

a) Pharmacodynamic; aimed to provide evidence that there is a direct pharmacological effect of a drug

b) Prognostic: providing evidence about the patient's overall disease outcome independent of any specific intervention.

c) Predictive: determining the probability of benefit or toxicity from a specific intervention

d) Surrogate: reflecting subsets of biomarkers that are intended to serve as a substitute for a clinically meaningful end point.

Secondly clinical biomarkers may be classified according to their envisaged clinical role i.e. early detection, diagnostic or prognostic and how that new biomarker might integrate with currently available diagnostic methods, which are often held as the 'gold standard' prior to introduction of a further test. As such new tests might be considered as substitute (replacing current modalities), triage (to be used as an initial test to identify those who might benefit from further more invasive investigation) or add-on (able to provide some additional information for a subset of patients in their management)(387). A particular biomarkers performance characteristics reflect its potential application, with the ideal characteristics varying by class, e.g. a triage test can have a low specificity but should be cheap and easy to perform.

Thirdly within biomarker development there is a general distinction between biomarker validation and qualification. Biomarker validation refers to the extent of the process by which biomarker assay characteristics are tested, including test sensitivity, specificity and accuracy, whereas qualification is the extent of the process by which a biomarker is linked to a clinical significance. Therefore the majority of the work described in this thesis refers to biomarker validation, with the clinical correlation and assessment of prognosis described in chapter 3 having relevance to biomarker qualification.

Finally biomarkers can be classified according to their delineated applicability into known, probable or exploratory. Most biomarkers in early development constitute exploratory biomarkers, as is the case with the work described herein.

1.8 Hypothesis

The hypothesis of the work presented in this thesis was that it is possible to detect DNA extracted from plasma, and to differentiate between samples with induced alterations in that DNA, and subsequently to differentiate between colorectal cancer cases and controls based on analysis of plasma DNA.

Specific Aims

1) Optimise DNA extraction methods applicable to small volumes of plasma
2) Quantify extracted plasma DNA and investigate a relationship between plasma DNA concentration in disease states and survival
3) Develop novel assays to detect tumour related DNA alterations with respect to mutations in TGFBR2 and K-ras genes.
4) Assess the ability to detect relative proportions of alleles in plasma DNA with a view to detecting tumour derived LOH in clinical samples (i.e. fluid tissue biopsy)
5) Assess whether the performance characteristics of tests developed might have applicability within themore recently defined classes of biomarkers.

CHAPTER 2

MATERIALS AND METHODS

2.1 Study Subjects

In order to test hypotheses on clinical samples a prospective cohort of control subjects and cases with colorectal neoplasia was ascertained.Local research and ethics committee (LREC) and appropriate managerial approvals were obtained; reference number LREC/2002/5/6. Copies of LREC patient information sheet and consent forms are provided in appendix 1.

2.1.1 Controls

The control group were derived from two sources

1)Healthy volunteers.

8apparently healthy volunteers consented to blood sampling, with their samples processed, quantified, assessed for plasma LOH and genotyped for a chromosome 5 SNP as described below.

2) Patients undergoing a normal colonoscopy.

These supercontrols were identified from elective colonoscopy lists.Patients undergoing investigation for rectal bleeding or a positive family history of colorectal neoplasia were approached, with blood collected prior to colonoscopy. Patients were included as controls following a negative colonoscopy to the caecum (n=23).

2.1.2 Cases

a) Ascertainment

Colorectal cancer cases were identified from planned elective lists at a single institution (Western General Hospital (WGH), Edinburgh), under the care of 7 consultants. Patients were approached more than 24 hours prior to surgery to allow due consideration by trial participants. Inclusion criteria were patients aged 18-89, with histologically proven colorectal adenocarcinoma (n=87) or undergoing resection of benign colorectal neoplasia (n=7). Exclusion criteria were: previous diagnosis of extra-colonic malignancy or metachronous colorectal cancer, pre-operative chemotherapy or radiotherapy, learning or memory difficulties or psychiatric illness.

Patient demographics, including site of disease, are provided in appendix 2. Summary demographics are presented in table 2.1 below.

Table 2.1

	Right colon	Transverse	Left colon	Rectum
Number of cases	28	5	24	38

Table 2.1 legend: summary table of locations of neoplasms.

The mean age was 63 with a range of 22-88

There were 62 males and 65 females.

b) Stage

Disease stage was ascertained directly from pathology records.

Classification was performed according to AJCC staging, details of which are provided in appendix 2., with summary data in table 2.2.

Table 2.2

	Controls	Adenomas	Stage I	Stage II	Stage III	Stage IV	Unknown
Number of cases	24	8	14	29	26	15	1

Table 2.2 legend: summary table of disease stage in prospective series of patients.

c) Treatment

Treatment modality intent for cases is given in table 2.3

	Curative resection	Palliative resection	Local resection	Endoscopic resection only
Number of cases	71	12	1	9

Patients who had preoperative adjuvant treatment were specifically excluded, as from the literature it was unclear what effects these treatments might have on plasma DNA in pre-operative blood samples.

d) Survival

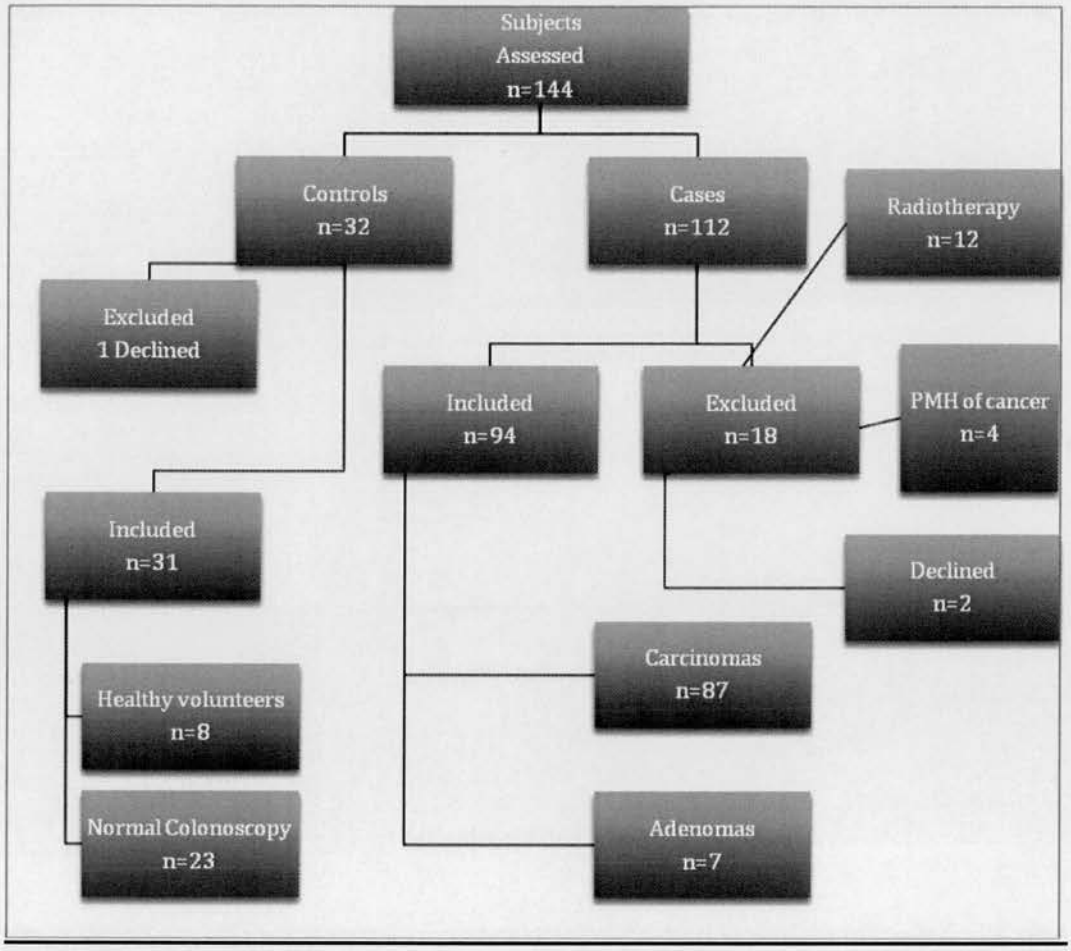
Survival was determined directly from clinical records. Date of death was also ascertained from Scottish central records. Survival data were not available for 5 patients and these were excluded from analysis. Patients were censored at the date last known to be alive. Censoring is a form of missing data problem which is common in survival analysis. 'Right censoring' occurs when death occurs at some point after a prescribed time, and applies when patients are

alive when lost to follow up or at the end of the study period, and applies in this analysis. Start time refers to the date of entry into the study- for these investigations at the point of surgical cancer treatment.

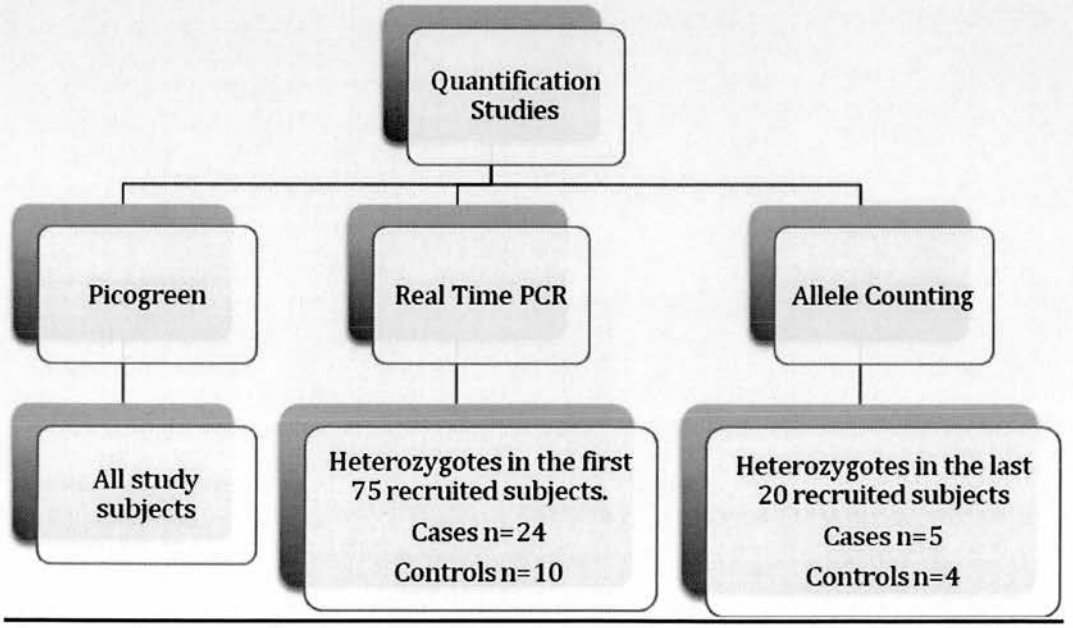
All cause mortality was subjected to survival analysis, performed with Kaplan-Meier curves, with significance of differences between groups assessed by Wilcoxon test using Statsdirect software. This approach is commonly employed in aanalyse the survival ofpatients in a clinical trial, and to isolate the effects of treatment from the effects of other variables. It is also known as proportional hazards regression analysis. Briefly, the procedure models or regressesthe survival times (or more specifically, the so-called hazard function) on the explanatory variables (http://www.medicine.ox.ac.uk/bandolier/painres/download/whatis/cox_model.pdf). Calibration refers to how close the predicted risks are to the actual observed risks using this model.

The CONSORT flow diagram of study subjects is given on the following page

Flow diagram of study subjects



Quantification Studies



2.2 Clinical Samples

2.2.1 Blood sampling

Blood samples were taken from subjects' arm with use of a tourniquet. Equal volumes of blood (10 mls) were taken in both EDTA tubes and Lithium-heparin tubes for the majority of the cohort (n=103). For the remaining 24 patients a larger volume of blood (40mls) was collected in EDTA tubes only, for the purpose of allele counting experiments described in chapter 7.

Following sampling, collection tubes were placed directly on ice, and remained on ice until further plasma processing, which was performed within 2 hours of collection.

Separation of blood fractions was performed using centrifugation on ficoll hypaque gradient. Blood was added onto an equal volume of ficoll hypaque. Centrifugation was then performed at 2500 rpm for 10 minutes. Following this the plasma (top layer) was carefully pipetted off, placed in sealable eppindorphs and stored at –80 oC until DNA extraction. The white blood cells in buffy coat were also pipetted off and stored separately and used as the source of patient's normal DNA.

2.2.2 Extraction

a) Normal DNA extraction

Normal DNA was derived from buffy coat, which was defrosted on ice. The sample was centrifuged at 2,000 rpm for 10 minutes, then excess plasma carefully removed with a pipette (leaving approximately 50ul of plasma on top of pellet). The sample was washed with 1ml of phosphate buffered saline (PBS), then centrifuged at 2,000 rpm for 10 minutes, and the PBS removed. A further wash was performed with 1ml of PBS and centrifuged at 2,000 rpm for

10 minutes. The cell pellet was resuspended in 200ul of PBS. Resuspended buffy coat DNA was extracted using Qiagen blood mini kit protocol, as described for plasma DNA extraction.

b) Tumour DNA extraction

Matched tumour samples were identified for all cases where available.

i) Blocks

The majority (n=85) of tumour samples were obtained as fixed blocks directly from the histopathology department with a corresponding H&E slide to allow idenification of tumour tissue. Tumour blocks were not microdissected. Approximately 25mg of tumour tissue was dissected from the block and submitted to processing.

Tumour DNA was extracted as per the protocol for the Qiagen tissue protocol as follows: 25mg of tissue was cut up into small pieces, placed into a 1.5 ml centrifugation tube and 180ul buffer ATL added. 20ul of proteinase K was added, mixed by vortexing and incubated at 56 ^{o}C overnight in a shaking waterbath. The sample was then briefly centrifuged to remove drops from the inside of the lid. 200ul of buffer AL was added, mixed by pulse vortexing for 15 seconds and incubated at 70 ^{o}C for 10 minutes. 200ul ethanol was added and mixed by pulse vortexing for 15 seconds. This mixture was then applied to a QIAamp spin column and centrifuged at 6000g for 1 minute and the filtrate discarded. 500ul of buffer AW1 then added to column and centrifuged at 6000g for one minute, with the filtrate collected in a clean collection tube. 500ul of buffer AW2 was then added and column centrifuged at 20,000 g for 3 minutes, following which the filtrate was discarded. 200ul of buffer AE was added to the spin column and incubated at room temperature for 10 minutes, then the column was centrifuged at 6000g for 1 minute. This final step was repeated with further 200 ul of buffer AE giving total elution volume of 400ul.

ii) Slides

Six patients did not have tumour blocks available on account of disease recurrence (n=3), no resection (n=1, asystole on induction), or block not received from histopathology as routine (n=2). For these patients slides were obtained from the pathology department with corresponding H&E slides. For those patients with disease recurrence, slides from the original resection were used in 2 cases, with tumour tissue from liver biopsy used for the remaining case. The patient not undergoing surgery had tumour tissue sampled from slides from the diagnostic endoscopic biopsies. The remaining 2 cases had slides from the resection blocks processed. Tumour tissue was identified from the corresponding H&E slides and slides scraped to collect tumour sample. Tumour samples were then processed using the Qiagen tissue protocol as described above.

iii) Fresh Tumour

In a small subset of cases (n=3) the tumour tissue was collected unfixed directly from the operating theatre. Thesecases were also enrolled in a separate study within the research group necessitating collection of RNA and hence unfixed samples. Specimens were opened and a portion of tumour tissue sampled. Tumour samples were placed in universal containers and put directly onto dry ice. Samples were taken directly to the laboratory for processing. Samples were collected fresh to allow tumour RNA and protein analysis by other members of the group and were therefore processed to allow extraction of DNA, RNA and protein using a trireagent protocol as follows:

25 mg of tissue was added to 500ul of TRI REAGENT solution and homogenised, then left to stand at room temperature for 5 minutes. 100ul of chloroform was added, mixed by shaking for 15 seconds and then allowed to stand at room temperature for 15 minutes. The resulting mixture was centrifuged at 12 000 g for 15 minutes at 4 ^{o}C, resulting in 3 phases, with DNA

contained in the interphase. The aqueous phase overlying the interphase was carefully removed and stored for later RNA extraction. 150ul of 100% ethanol was added to the interphase and organic phases, mixed by inversion and allowed to stand for 2-3 minutes at room temperature. Sample then centrifuged at 2 000 g for 5 minutes at 4 °C. The resulting supernatant was removed and stored for later protein extraction. The DNA pellet was washed twice in 500ul of 0.1M sodium citrate, 10% ethanol solution. During each wash the DNA pellet was allowed to stand for 30 minutes, then centrifuged at 2 000 g for 5 minutes at 4 °C. DNA pellet then dried at room temperature for 1 hour, then dissolved in 400 ul of 8mM NaOH.

c) Plasma DNA extraction

Plasma DNA extraction was submitted to experiments to identify optimal methods for extraction and processing and are described in Chapter 3.

2.3 Real time PCR

Real-time PCR utilises specific hardware platforms that allow cycle-by-cycle visualisation of PCR product through detection of fluorescent signal in real time. For these purposes training was undertaken for the ABI 7900 HT platform (Applied Biosystems) (appendix 2).
Applied biosystems support different specific reaction chemistries, this project used SYBR Green and Taqman probes (probes being either TAMRA labelled or minor groove binding (MGB) probes).
SYBR Green technology is based on the generation of a fluorescent signal on binding of the dye to double-stranded DNA. As such a signal is generated related to the quantity of PCR product present at the end of each cycle of the reaction. Signal production occurs with

binding to any double stranded DNA and hence is not sequence specific. The discriminatory value of these assays is therefore dependant on the specificity of the primers employed.

Taqman probes employ fluorescently labelled sequence specific probes, with varying fluorescence (reporter) for different sequences analysed by the assay. Fluorescent labels are bound to quenchers that prevent signal generation whilst the probe is intact. The action of Taq polymerase cleaves the reporter from the quencher resulting in signal production. Detection of different fluorescent wavelength amplitudes allows analysis of proportions of different sequences within a reaction. (388).Quenchers produced by Applied Biosystems include minor groove binding non-fluorescent quenchers (MGB-NFQ). The advantage of MGB-NFQ is that probes are stabilised and hence may be shorter in length, hence a single base mismatch has greater impact and allows greater specificity(389). Therefore MGB-NFQ probes were used for the majority of this project (Figure 2.1).

Figure 2.1:

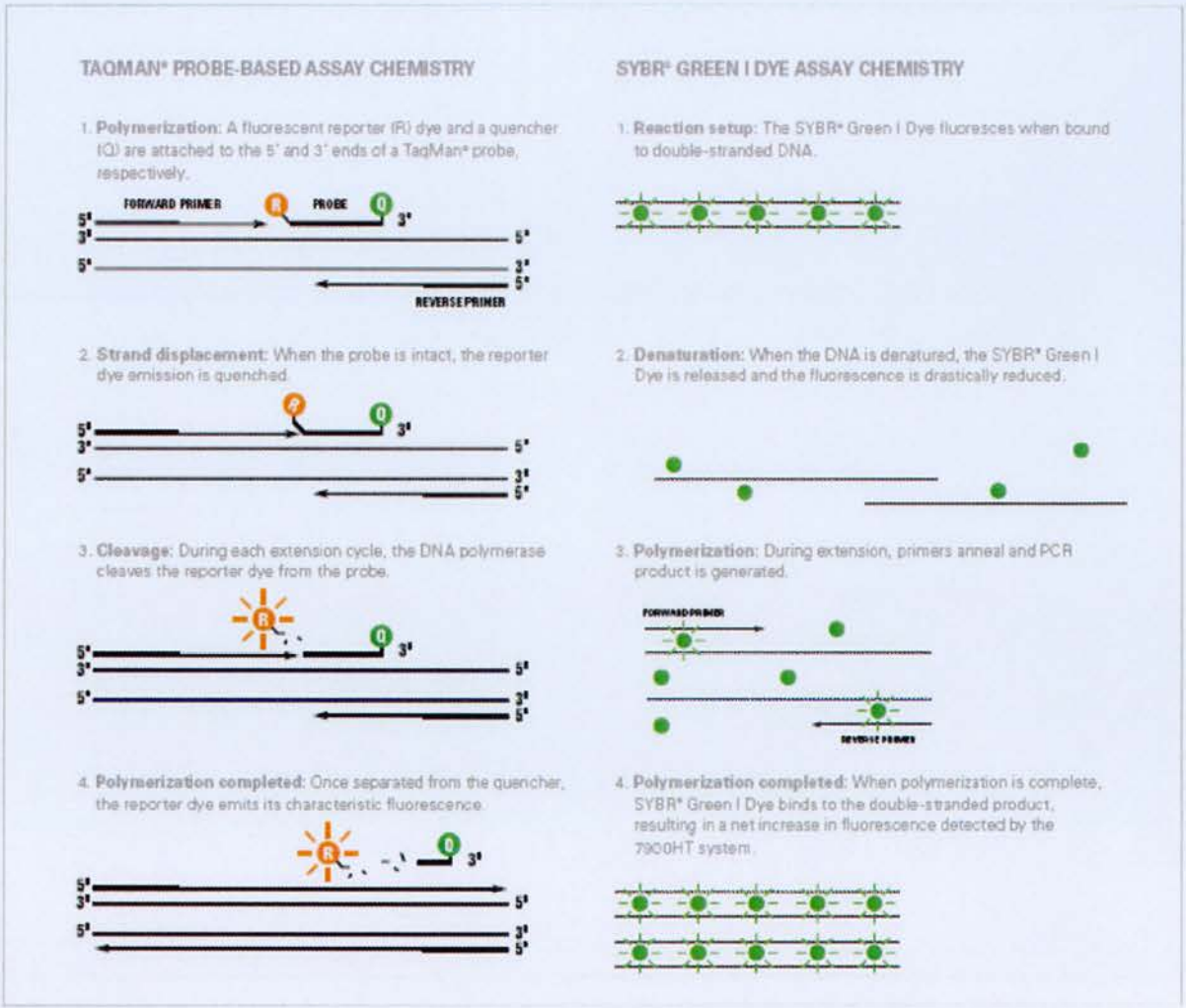


Figure 2.1 legend: Summary diagrammatic representation of Taqman and SYBR Green assay chemistries.

From http://www3.appliedbiosystems.com/AB_Home/applicationstechnologies/Real-TimePCR/TaqManvsSYBRGreenChemistries/index.htm

Manufacturer requirement guidelines for Taqman MGB/NFQ primer and probe design were employed throughtout this project (http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_042505.pdf)

2.4 Plasma DNA Quantification

2.4.1 Spectrophotometry

Extracted plasma DNA samples were diluted in dH2O by a factor of 20 (5ul added to 95ul of d H_2O). Spectrophotometer GeneQuant *pro* (Biochrom, Cambridge, UK), allowed to calibrate for 5 minutes, and used according to manufacturer's instructions including calibration performed with d H_2O alone to give zero reading. Readings were performed on each sample in triplicate.

Plasma DNA concentration calculated according to the formula:

Absorption 260 x 50 (conversion factor) x 20 (dilution factor) in ng /ul.

Each plasma DNA extracted from 200ul eluted in 100ul of elution buffer.

Therefore total DNA per sample =absorption x50x20x100 ng.

Therefore [plasma DNA]= absorption x50x20x100 x5 ng per ml of plasma.

2.4.2 Real time PCR

Plasma DNA concentration was determined using real-time PCR employing an assay individually designed assay for detection of differing alleles at a SNP within exon 15 of the APC gene as described in section 2.7. Reagent concentrations and reaction conditions were identical to those described in that section. In order to perform absolute quantification of DNA samples a standard DNA concentration curve was generated using serial dilutions of commercially available DNA standard at stock concentration of 10ng / ul.

Dilutions were made to:

1 in 20	=	0.5ng/ul
1 in 100	=	0.1ng/ul
1 in 200	=	0.005 ng/ul
1 in 250	=	0.004 ng/ul
1 in 400	=	0.0025 ng/ul

Calculation of [plasma DNA] was performed as follows:

Total extracted plasma DNA (in 100ul) = reading x20

Plasma DNA concentration (from 200ul) = total plasma DNA x 5 in ng/ml.

2.4.3 Picogreen ®

Picogreen ® ds DNA quantitation reagent is a fluorescent nucleic acid stain for quantiating double stranded DNA (ds DNA). Quantification was performed according to manufacturer's instructions (390). TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5) was used to dilute the Picogreen ® ds DNA quantitation reagent, and the working solution used on the day of preparation. A DNA standard curve was generated using dilutions of the lamda DNA standard provided at 100ug/ml between 0 and 10ng of total DNA. 10 ul of extracted plasma DNA samples were incubated in Picogreen ® ds DNA quantitation reagent for 5 minutes at room temperature. Sample fluorescence was measured using a Victor 2 spectrofluorometer (Perkin Elmer).

Analysis of test performance was performed using receiver operator curves (ROC). ROC curves are a commonly employed method of assessing test accuracy, with a perfect test have and area under the curve of 1. A ROC curve is a plot of a test's false positive rate versus its sensitivity (plotted on the y axis). However patient management is more complex than is allowed with a decision threshold that classifies the test into positive or negative, particularly

when a quantitative test gives a continuous output of variables, necessitating the adminstration of an often arbitrary cut-off point. Additonally different classes of biomarkers may be best served by different performance charactersitics in terms of sensitivity and specificity as outlined in biomarkers section of the introduction.

2.4.4 Dilution / Allele counting

Allele counting and dilution was performed as described in and section 2.8 and chapter 7, using the APC SNP assay. Plasma DNA concentration was estimated by calculation as follows:

1 genome equivalent ~ 6.6.pg. Dilution to concentration whereby 1 ul gives an allele in every other well approximates to 3.3pg / ul. For the allele counting cohort plasma DNA extracted from 2 mls of plasma and eluted in 400 uls of elution buffer.

Therefore plasma DNA concentration equals dilution factor x 33 x 400 / 2000

2.5 Tumour specific mutation analysis

2.5.1 Positive controls from cell lines

a) Microsatellite Instability

The colon cancer cell line LoVo has been demonstrated to have biallelic alterations in the poly A tract of TGF-βRII gene (391), and confirmed by our group (392) and was hence used as a positive control for these experiments. LoVo DNA previously extracted from cultured cells, and suspended in TE, was quantified using spectophotometry.

b) Kirsten ras mutation

DNA was extracted from 2 colorectal cancer cell lines Colo 320 and SW 480 which were used as positive controls. Colo 320 has been characterised as having GGT -> GAT (glycine to aspartate) mutation at codon 12, whilst SW 480 has GGT->GTT (glycine to valine) mutation at codon 12(393).

2.5.2 Positive controls from patient samples

a) Microsatellite Instability

Matched tumour and plasma was analysed from archived material constituting 10 patients with metastasis (Stage IV), enrolled in previous genetic studies within the group (MD numbers 1092,1801,1808, 1816, 1854, 1964, 1996, 2009, 2164, 2221). Microsatellite status was available for these patients from previous data using Bethseda marker set, and categorising 9 cases to be microsatellite stable and one to have MSI-H (MD 1092). These cases were selected since without curative resection, tumour derived DNA might be expected to be present at timepoints after surgery and since the prevalenceof MSI increases with advancing disease stage, and hence might be expected to be a selected sample whereby tumour derived DNA might be most detectable.

a) Kirsten ras mutation

Additional positive controls were derived from archived samples of patient tumours within the group from previous studies, whose K-ras status had previously been determined by sequencing as having the mutation GGT to GAT at codon 12
(n=2: ref AM 17& 35).

2.5.3 Cell culture

Cells were raised from storage in liquid nitrogen. Cells were defrosted in RPMI medium with 50 mls of 10% foetal calf serum and 5mls of penicillin / streptomycin, then incubated for 48 hours (Gibco BRL, Paisley, UK). Cells were then inspected and seen to be adherent and confluent, and then split. Cell splitting involved transfer of the medium into a centrifugation tube. The adherent cells remaining in the flask were washed with 1 ml of phosphate buffered saline (PBS) that was then discarded. 1 ml of trypsin/versene (1:1) was then added to the adherent cells and left for 5 minutes. The flask was then tapped to release some adherent cells. 0.5 mls of solution was then added to the centrifugation tube. The remaining 0.5 mls had 10 mls of fresh medium added and then was reincubated. The sample in the centrifugation tube that had been separated off was centrifuged at 1200 rpm for 8 minutes, the pellet washed in PBS and then re-centrifuged. The pellet was then resuspended in culture medium, transferred into a small flask and incubated.

DNA was extracted from cultured cells using QIAgen DNA extraction kit as per protocol with 400ul of proteases added to 2 mls of PBS suspended cells and final elution in a total of 600 ul elution buffer. DNA was quantified by spectrophotometry.

2.5.4 Sequencing

Sequencing using sequence specific primers designed to the appropiate gene as described separately in the materials and methods section relating to each chapter.

The constituents for the initial PCR reactions were 2ul 10x buffer, 1ul 50mM $MgCl_2$, 2ul 2 mM dNTP, I unit of Platinum Taq DNA polymerase and 0.4ul each of 20uM forward and reverse primers (Invitrogen, UK) and 1ul of DNA template. PCR conditions were an initial

denaturing step of 95°C for 3minutes, followed by 35 cycles of 60 seconds at 95°C, 60 seconds at 57 °C, and 90 seconds at 72 °C, with final 5 minutes at 72 °C.

PCR products were cleaned using Exonuclease I (EXO I) and Shrimp Alkaline Phosphatase (SAP) (Amersham Biosciences) with 3ul of stock EXO/SAP added to 5ul PCR product. Cycling was performed as 37°C for 15 minutes followed by 80°C for 15 minutes. The sequencing reaction was performed using 4ul of EXO/SAP treated PCR product with 2.5ul Big Dye terminator, 1.3ul of forward and reverse primers and 5.8ul dH_20. The sequencing PCR program consisted of 25 cycles of 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes.

DNA was then precipitated by adding 55ul of 95% ethanol/ 10% volume NaOAc (pH 5.2), to each well, and leaving on bench for 30 minutes. Samples centrifuged at 2000 g for 30 minutes at 20°C and supernatant removed, including inversion of plates on paper towels and pulse centrifugation. 150ul of 70% ethanol then added and pulse centrifugation repeated. Pellets were stored at –20°C until sequenced. Sequencing was performed on ABI 3700 platform with accompanying sequencing analysis software.

2.5.5 TGFB RFLP assay

Analysis was performed on a poly A tract of the TGFBRII gene as a paradigm for the analysis of microsatllite sequences in plasma from microsatellite unstable colorectal cancer patients. A restriction digest based assay was adapted from Mironov (394) specifically for the detection of 1bp deletions in the poly$(A)_{10}$ tract of the TGFBRII gene(392).

The poly A tract (highlighted) of the TGF-βRII gene is a 10 base pair adenosine repeat (A_{10}) at codons 125-128 of its 565 codon open reading frame:

gcaactgcag catcacctcc atctgtgaga agccacagga agtctgtgtg gctgtatgga

gaaagaatga cgagaacata acactagaga cagtttgcca tgaccccaag ctcccctacc

atgactttat tctggaagat gctgcttctc caaagtgcat tatgaaggaa aaaaaaaagc

ctggtgagac tttcttcatg tgttcctgta gctctgatga gtgcaatgac aacatcatct

tctcagaaga atataacacc agcaatcctg acttgttgct agtcatattt caagtgacag

The assay is based on the introduction of a Hinf1 restriction enzyme cleavage site in the presence of a 1 bp deletion within the polyA tract (Figure 2.1). Reaction conditions as described below result in the generation of a 141 bp wild type product and / or a 118bp mutant product, as illustrated in figure 2.2

Strategy for detecting mutations within the poly $(A)_{10}$ tract of TGFBR2

TGFBR2 exon 3

690 737

CCA AAG TGC ATT ATG AAG G**AA AAA AAA AA**G CCT GGT GAG ACT TTC TTC

Pro Lys Cys Ile Met Lys Glu Lys Lys Lys Pro Gly Glu Thr Phe Phe

1. Wild type sequence -no *hinf1* restriction site present in the PCR product

2. A 1bp deletion generates a *hinf1* restriction site

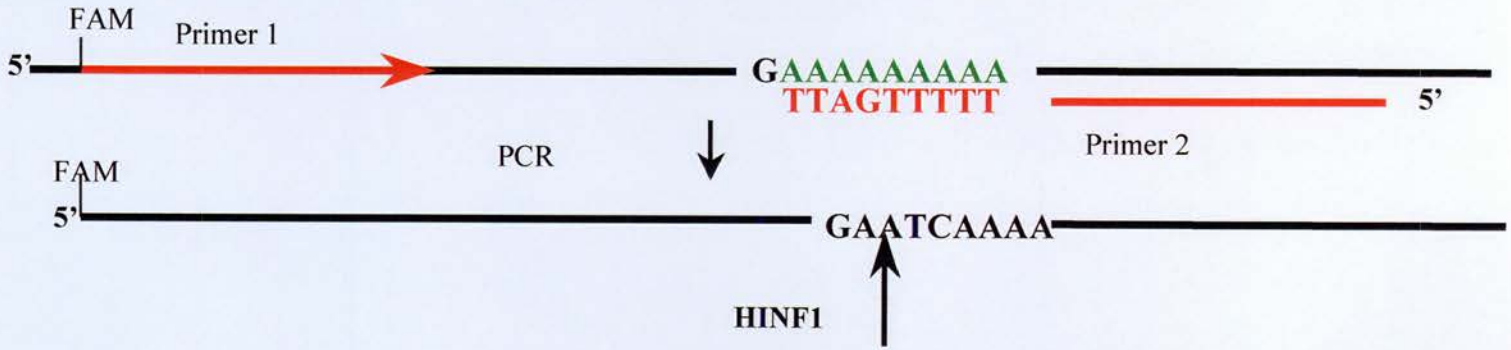


Figure 2.3:

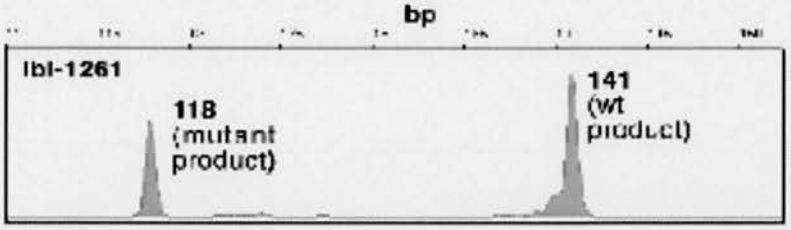


Figure 2.3 legend: Demonstration of wildtype and mutant product from cancer cell line. (392)

5ul of PCR product was digested at 37°c for 16 hours in a total volume of 15 ul with 5 units Hinf1 restriction enzyme and 1x buffer H (Boehringer Mannheim). Digested and undigested PCR products were analysed on an ABI 310 automated genetic analyser (Applied Biosystems) and Genescan 2.1 software. 3ul Hinf digestion product was added to 0.5ul size standard and 10.5 ul of formamide. Samples then denatured at 95° for 5 minutes, then placed on ice for 5 minutes. Sample ABI 310 injection times were 5 seconds for PCR products and 10 seconds for digestion products.

a) Determination of Sensitivity

Serial dilutions of LoVo DNA were added to control plasma to yield spiked concentrations of 100pg, 500pg, 1ng, 10ng and 50ng per ml of plasma. Plasma DNA was then extracted using QIAgen extraction kit as described in chapter 3.

b) Primers and Reaction Conditions

i) Primers

Assay primers were as follows. The forward primer was initially labelled with FAM and subsequently with HEX.

Forward (fluorescent labelled): CAC TCT AGG AGA AAG AAT GAC GReverse:

GAA AGT CTC ACC AGG CTT TTT GAT T

ii) Reaction Conditions

Reaction constituents were as follows:

5ul each ofPCR buffer II, MgCl and dNTPs, 0.25ul of taq polymerase, 0.5ul of forward and reverse primers, 5ul template and 29.25 ul of dH_2O. Following initial difficulties the taq polymerase was substituted with high fidelity Taq (Expand High Fidelity PCR System-Roche), and was used in all subsequent reactions.

Initial PCR reagents and cycling were as follows:

95°C for 3 mins followed by 35 cycles of 95 ° for 1 minute, 50 ° for 1 minute and 72 ° for 1 minute, followed by 72° for 5 minutes then 32 ° for 15 minutes. These conditions were utilised for assay validation on positive and negative control genomic DNA as well as in the analysis of tumour DNA from cases.

For analysis of plasma DNA, including determination of assay sensitivity in spiked plasma samples, PCR cycling conditions required optimisation. Annealing temperature was serially altered at inceasing cycle numbers. Performance was optimised at 45 cycles of the above cycling with an annealing temperature of 58 oC.

iii) Nested PCR

Given the limited performance of the assay with plasma DNA, the assay was adapted using a nested PCR approach.

Primary PCR was perfomed using primers for exon 3 of TGFBRII, and secondary PCR with the assay primers described above.

Exon 3 primers:

Forward CCT CGC TTC CAA TGA ATC TC

Reverse TTG GCA CAG ATC TCA GGT CC

Optimisation was performed by serial alteration of primer concentration, annealing temperature and cycle number in both primary and secondary, to a maximum of 55 cycles in total. The minimum cycling at which product was reproducibly detectable was as follows:

Primary PCR 15 cycles at 58 oC annealing.

Secondary PCR 35 cycles at 58 oC annealing

iv) Analysis

5ul of PCR product was digested at 37°c for 16 hours in a total volume of 15 ul with 5 units Hinf1 restriction enzyme and 1x buffer H (Boehringer Mannheim). Digested and undigested PCR products were analysed on an ABI 310 automated genetic analyser (Applied Biosystems) and Genescan 2.1 software. 3ul Hinf digestion product was added to 0.5ul size standard and 10.5 ul of formamide. Samples then denatured at 95° for 5 minutes, then placed on ice for 5 minutes. Sample ABI 310 injection times were 5 seconds for PCR products and 10 seconds for digestion products.

2.5.6 TGFB Real Time PCR analysis

Fluorescently labelled MGB oligonucleotide probes were designed to the poly A tract in accordance with manufacturers guidelines (Applied Biosystems)

Wt CTC CAA AGT GCA TTA TGA AGG AAA AAA AAA AGC CT

35 bases. Tm 69.3. GC 34.3%. 6 G's, 6 C's.

1bp deletion TCT CCA AAG TGC ATT ATG AAG GAA AAA AAA AGC C

34 bases. Tm 69.5. GC 35.3. 6 G's, 6 C's.

Primers: Forward TAC CAT GAC TTT ATT CTG GAA GAT GCT

Length 27 bases, GC 37%, Tm 59.5, 3'GC 2/5

Reverse CTA CAG GAA CAC ATG AAG AAA GTC TCA

Length 27 bases, GC 40.7%, Tm 58.9, 3'GC 2/5

2.5.7 K-ras assay:

Sequence derived from nucleotide accession number L00045.1 (locus map 12p12.1) showing codon 12 at base 129 coding GGT, being the normal sequence:

```
1   gtactggtgg agtatttgat agtgtattaa ccttatgtgt gacatgttct aatatagtca
61  cattttcatt atttttatta taaggcctgc tgaaaatgac tgaatataaa cttgtggtag
121 ttggagctgg tggcgtaggc aagagtgcct tgacgataca gctaattcag aatcattttg
181 tggacgaata tgatccaaca atagaggtaa atcttgtttt aatatgcata ttactggtgc
241 aggaccattc tttgatacag ataaaggttt ctctgaccat tttcatgagt
```

The specific mutation investigated was alteration GGT -> GAT (glycine to aspartate), which has been reported as the most frequent mutation of codon 12 in colorectal cancer (395).

a) Sequencing

Sequencing of k-ras was performed as described in section 2.5.4 using the following primers:

Forward	TTT ATT ATA AGG CCT GCT GAA AAT GA	(Length 26,Tm58)
Reverse	CAA AGA ATG GTC CTG CAC CAG	(Length 21,Tm59)
Amplicon	length 181 bp	

b) Real-time PCR

i) SYBR Green

Sequence specific primers were designed to wild type (wt) and aspartate mutant sequences for use in real time PCR, according to manufacturer's guidelines, using the manufacturer's primer design software (Primer Express, Applied Biosystems).

Wt ras forward: ATA TAA ACT TGT GGT AGT TGG AGC TGG

Length 27, Tm 59

Aspartate mutant: GAA TAT AAA CTT GTG GTA GTT GGA GCT GA

Length 29, Tm 59.4

Common reverse TGG ATC ATA TTC GTC CAC AAA ATG

Length 24, Tm 59.6

Each primer diluted with dH_20 to 100 pmol/ul

These primers were subsequently modified, with an induced base mismatch adjacent to the mutation site to reduce annealing stability to increase primer specificity for wild type and mutant sequences. These modified primers were termed wobble primers and the sequences are given below:

Wt ras wobble forward: ATA TAA ACT TGT GGT AGT TGG AGC AGG

Aspartate mutant wobble: GAA TAT AAA CTT GTG GTA GTT GGA GCA GA

Common reverse: TGG ATC ATA TTC GTC CAC AAA ATG

Diluted 100 pmol/ul

For each of the above primer pairs, the primer concentration was optimised using a SYBR green primer optimisation grid, given below with concentrations in nM:

50 Forward 50 Reverse	50 Forward 300 Reverse	50 Forward 900 Reverse
300 Forward 50 Reverse	300 Forward 300 Reverse	300 Forward 900 Reverse
900 Forward 50 Reverse	900 Forward 300 Reverse	900 Forward 900 Reverse

This identified primer concentrations of 300 nM forward and 900 nM reverse primers as giving the lowest cycle number to cross threshold (Ct) for the same templates whilst not giving false positive readings with non-template controls.

This resulted in the following reaction constituents for SYBR green reactions:

12.5 ul SYBR green mastermix, 0.75 ul forward primer, 2.25 ul reverse primer, 4.5 ul dH_20, 5 ul template DNA.

Reaction Conditions were

Step 1; 95°C for 10:00 minutes

Step 2; 50 cycles of 95 °C for 0:15 then 60°C for 1:00

ii) Taqman MGB Probes

Specific Taqman MGB primer and probe pairs were designed according to manufacturer's instructions, generating the following sequences:

Wild type probe (antisense strand)

CCT ACG CCA **C**CA GCT (15 bases, Tm 69.1)

Aspartate mutant probe (antisense strand)

CCT ACG CCA **T**CA GC (14 bases, Tm 68.8)

Forward primer (antisense)

TAG CTG TAT CGT CAA GGC ACT CTT (24 bases, Tm 58.3)

Reverse primer (sense)

AAA ATG ACT GAA TAT AAA CTT GTG GTA GTT G (31 bases, Tm 58.6)

These primers and probes were tested on positive and negative controls as detailed above.

The real time PCR mixture consisted of 12.5ul of TaqMan Universal PCR Mastermix, 0.75ul of both forward and reverse primers, 0.625ul of both FAM and VIC labelled probes, 1ul of template and dh_20 to a final volume of 25ul.

Reaction conditions were:

Step 1; 95 °C for 10:00 minutes

Step 2; 50 cycles of 95 °C for 0:15 then 60 °C for 1:00

Real Time PCR Analysis of K-ras codon 12 using SYBR Green

K-ras exon 1 (from accession no. L00045.1)

```
101                     121
T GAA TAT AAA CTT GTG GTA GTT GGA GCT GGT GGC GTA GGC AAG AGT GCC TTG ACG ATA CA    Wild type
                                   GAT                                             Aspartate mutant
```

1. Specific Primers to Mutant and Wild-type

Wild type Forward Primer

ATA TAA ACT TGT GGT AGT TGG AGC TGG

5' ——— GA ATA TAA ACT TGT GGT AGT TGG AGC TGGT GGC GTA GGC AAG AGT GCC TTG ACG ATA CA

GAT (aspartate mutant)

GA ATA TAA ACT TGT GGT AGT TGG AGC TGT

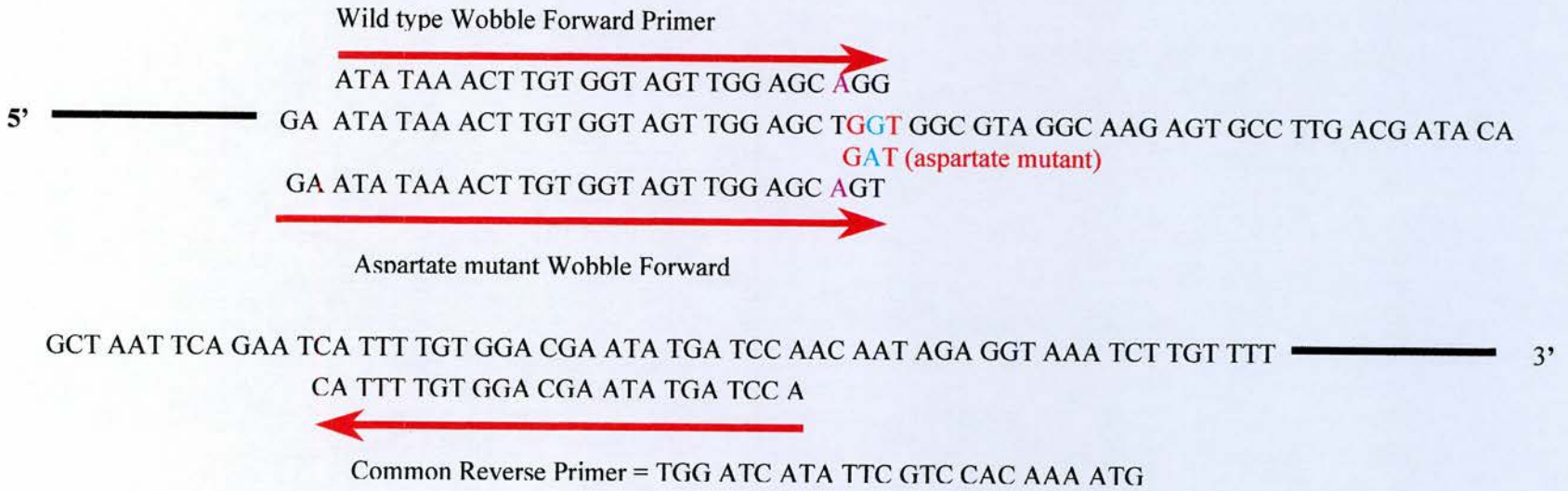
Aspartate mutant Forward Primer

GCT AAT TCA GAA TCA TTT TGT GGA CGA ATA TGA TCC AAC AAT AGA GGT AAA TCT TGT TTT ——— 3'

CA TTT TGT GGA CGA ATA TGA TCC A

Common Reverse Primer = TGG ATC ATA TTC GTC CAC AAA ATG

2. Primers Designed with Base Mismatch (Wobble)



2.6 MicrosatelliteFluorescent PCR Analysis

Fluorescent microsatellite analysis was performed on matching normal, tumour and plasma DNA from 85 cases and 24 controls at 3 microsatellite markers. The microsatellite markers chosen were D5S346, D17S250 & D18S58 on the basis of their being highly polymorphic, located in chromosomal regions important in colorectal cancer and frequently used in determining microsatellite status in biological samples.

2.6.1 Microsatellite markers

a) D5S346

Located at 5q22.2, within the region of chromosome 5q, related to the APC gene, commonly deleted in colorectal cancer (396). D5S346 is one of five Bethseda markers for determining microsatellite status. Estimations of frequency of microsatellite alterations in colorectal cancer at D5S346 are around 50% (397). Primers were as follows:

Forward (HEX labelled): ACT CAC TCT AGT GAT AAA TCG GG

Reverse (unlabelled): AGC AGA TAA GAC AAG TAT TAC TAG TT

Amplicon length ~ 113 bp Sequence from NT_034772.5

AATGTATTTGTGCACATGTACATATGGAAATGTTACTGTCTGACTACAACATGCATCATGCTCATGGGGAGGGAG

CAGGGGAAGGTTGTATGTGTCATTTATAACTTCTGTACAGTAAGACCACCTGCCAAAAGCTGGAGGAACCATTGT

GCTGGTGTGGTCTACTAAATAATACTTTAGGAAATACGTGATTAATATGCAAGTGAACAAAGTGAGAAATGAAAT

CGAATGGAGATTGGCCTGGTTGTTTCCCTAGTATATGGCATATGAATACCAGGATAGCTTTATAAAGCAGTTAGT

TAGTTACTCACTCTAGTGATAAATCGGGAAATTTACACACACACACACACACACACACACAGAGTAACCTGTA

ACTCTCAATTCCCTGAAAAACTAGTAATACTGTCTTATCTGCTATAAACTTTACATATTTGTCTATTGTCAAGAT

GCTACAATGGAAACCATTTCTGGTTTTATCTTCAAAGCGGAGAAACATGTTGATTTAGTCTTCTTTCCCAATCTT

CTTTTTTAAACCAGTTTAAGGAACTTCTGAAGATTTGTCCACCTCTGATTACATGTATGTTCTTGTTTGTATCAT

b) D17S250

Located at 17q12, related to the phosphatase and tensin homologue (PTEN) tumour suppressor gene. D17S250 is one of five Bethsada markers for determining microsatellite

status. Estimations of frequency of microsatellite alterations in colorectal cancer at D17S250 are around 50% (397). Primers were as follows:

Forward (TET labelled) GGAAGAATCAAATAGACAAT

Reverse (unlabelled) GCTGGCCATATATATATTTAAACC

Amplicon length ~ 151 bp

Sequence from: ref|NT_010755.15|Hs17_10912:876300-877000 Homo sapiens chromosome 17 genomic contig

TGGCCAGGCTGGTCTCAAACTCCTGACCTCAGGTGATCCACCCACCTCAGCCTCCGAAAGTGCTGGGATTACAGG
CATGAGCCACTCAGCTGGCCATATATATATTTAAACCATTTGAAAGTGTGTGTGTGTGTGTGTGTGTGTGTGT
GTGTGTGTGTTTGAAACCATTTGAAAGTTTATGTATGTGTATATATATATATAAACACACACATATTTTTATTGT
CTATTTGATTCTTCCTTTTTATGCTTACTTTTTTCTTCTCTCTTCTTTTTGATCAATTACTTTTCATCACTTCAT
TTTTTTCTTATTTTTCCCCCTCTACTAGTTTGGAAGCTAAATATTCTATTTCTCATCTTTTAGTGACTGACCTAA
AAATTTATTGTGCATGCTTGACTTACCAAAATCCACAGTAAATCTTTATCCTTTTACCTTCTCCCATACACCACA
ATGGCCTTAGAACACTTGAACACCAATCGCTCACCTTCTGACTTCTATGCTACGGGCTGTGTATTTTATTCTGTC
TTGGTTTATTCCATCAAGGTTTCATTATGCACAGACAGAGGAGGAGCTCAGTCCTTGTGGCAGCTGCTTTCCTAA
TGAAGACTGGGGCCCTGCTCAGTCCTTCCCACTCCCTCTTGCTGAGGATGTATGTCCCCACCCTTTTCTTGATGA
ATGTGAGGGCCAGATCTCCCGTGGAT

c) D18S58

Located at18q22. Related to the Deleted in colon cancer (DCC) gene, with estimated frequency of microsatellite alterations in colorectal cancer of 58% (398) –73% ((101).

Forward GCTCCCGGCTGGTTTT

Reverse GCAGGAAATCGCAGGAACTT

Amplicon 144-160 (bp),

Sequence derived from: NW_927129.1|HsCraAADB02_620:6945258-6945658

GCCTAAGCCACATTATATCTTCTTCCTGGAGGAGGTTCATTCCCCACTGGGGTGATTTCCAAGAAGAAAGAAGGC
CTCAAAAATAAATTCAAGTTTCTATCCCTTAGGAGGCAGGAAATCGCAGGAACTTTAAATTTACAACGTTTAAAT
ATTATCTCTTCATTAAGGACGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGCGCGCGCGCGCATATATG
TACCATTTATATATAAATAAAACCAGCCGGGAGCAGTGGCACTTTGGGAGGCTGAGGGGGGCGGATCACCTGAAG
TCAGGAGTTCGAGACCAGCCTGGTCACCATGGTGAAACCCTGTCTCTACTAAAAATAAAATAAAATAAATAAAAA
TACATAAAAAAATTAAAAAAATTAGC

2.6.2 Reaction Conditions

a) Constituents

The constituents for the PCR reactions were 2ul 10x buffer , 1ul 50mM $MgCl_2$, 2ul 2 mM dNTP, I unit of Platinum Taq DNA polymerase and 0.4ul each of 20uM forward and reverse primers (Invitrogen, UK). For tumour and normal samples 1ul of DNA template was added, and for plasma samples 2.4ul of plasma DNA was added. Distilled water was added to give a total reaction volume of 30ul. All reactions included a negative, no template control well.

b) PCR Cycling

PCR conditions were an initial denaturing step of 95° for 3minutes, followed by 35 cycles of 60 seconds at 95 °C, 60 seconds at 57 °C, and 90 seconds at 72 °C, with final 5 minutes at 72 °C. A 55 °C annealing temperature was used for amplification of D17S250 marker in plasma samples.

c) Analysis

PCR products were processed for analysis by adding 0.25-5.0ul (tumour and normal) or 5.0-6.0 ul (plasma) to 1ul of GeneScan 400HD size standard (Applied Biosystems) and denatured Hi-Di formamide to a total volume of 14ul.

Samples were denatured at 95 °C for 3 minutes, cooled on ice and then analysed using ABI 3730 platform and GeneMapper version 3.7 software (Applied biosystems).

Maximal peak heights were recorded, corresponding to differing alleles. Where a single allele was observed this was taken to represent homozygosity at the given marker and hence non-informativity. A ratio of peak heights from differing alleles was calculated from normal, tumour and plasma DNA and compared to determine the presence of LOH in tumour and / or plasma samples.

There is no consensus in the literature as to what level of change in allelic ratio may be classified as LOH, with studies using values between 30-70% alteration. Analysis was performed at 50% change in allelic ratio.

2.7 APC gene 15n Real time PCR Assay

2.7.1 Assay Design

Previous work within this group had identified a polymorphism within exon 15 of the APC gene that was frequently heterozygous in colorectal cancer patients. This '15n' polymorphism constitutes a base change CCA to CCG between bases 5886 and 5940 on accession number M74088. The assay was designed according to the guidelines given by the primer and probe manufacturer (Applied Biosystems), and performed using primer express software provided with the real-time PCR platform, ABI7900HT (Applied Biosystems). The resultant primer and probe sequences are given below.

The assay is presented diagrammatically in figure 2.5.

a) Primers and Probes

APC 15n sequence:

APC exon 15 at codon 1961. Base 5883 of accession number M74088.

Polymorphism is CCA – CCG

```
5701
accagccaca cagaactaac ctccaaccaa caatcagcta ataagacaca agctattgca
aagcagccaa taaatcgagg tcagcctaaa cccatacttc agaaacaatc cacttttccc
cagtcatcca aagacatacc agacagaggg gcagcaactg atgaaaagtt acagaatttt
gctattgaaa atactccagt ttgcttttct cataattcct ctctgagttc tctcagtgac
attgaccaag aaaacaacaa taaagaaaat gaacctatca aagagactga gccccctgac
tcacagggag aaccaagtaa acctcaagca tcaggctatg ctcctaaatc atttcatgtt
gaagataccc cagtttgttt ctcaagaaac agttctctca gttctcttag tattgactct 6121
```

A Taqman MGB assay was designed according to manufacturer instructions. The probes generated were:

CCA Allele; 6-FAM-AAATACTCCAGTTTGCT-MGB.

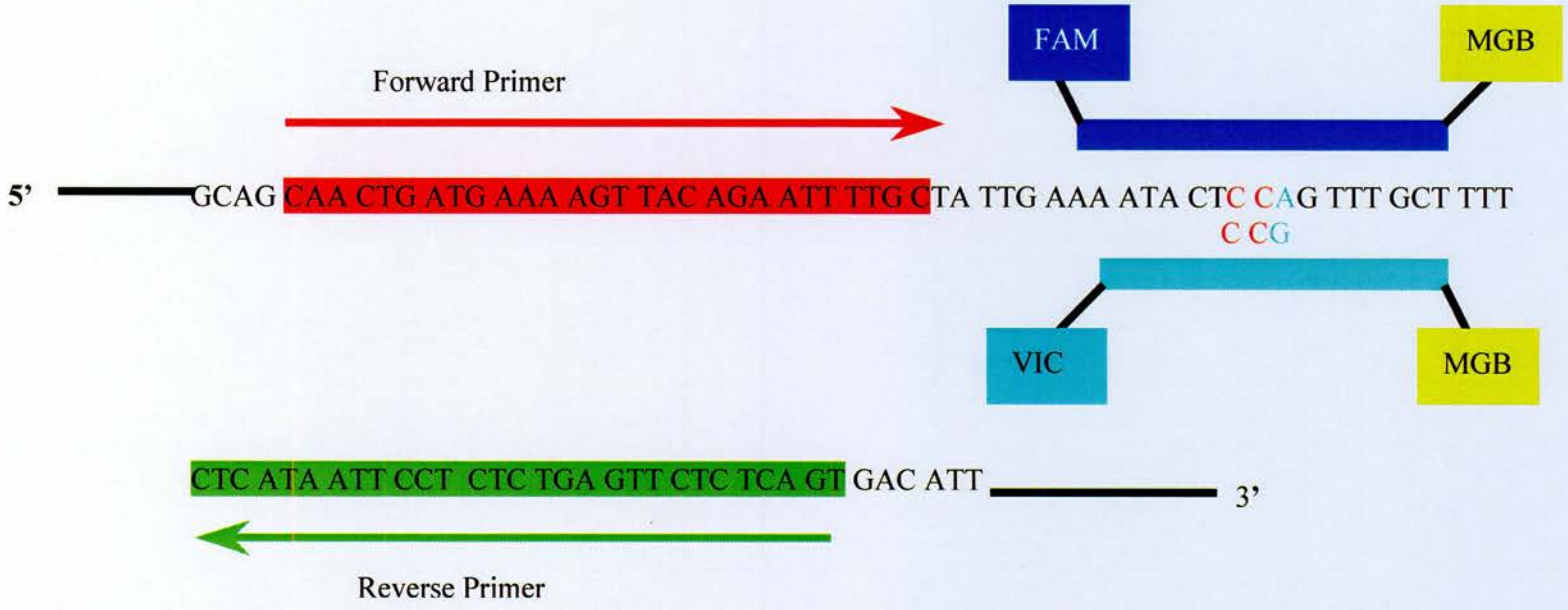
CCG Allele; VIC-AATACTCCGGTTTGCT-MGB

The corresponding primer pair was:

Forward: CAACTGATGAAAAGTTACAGAATTTTGC

Reverse: ACTGAGAGAACTCAGAGAGGAATTATGAG

Real Time PCR Analysis of Chromosome 5 '15n' Polymorphism



b) Reaction constituents

The real time PCR mixture consisted of 12.5ul of TaqMan Universal PCR Mastermix, 0.75ul of both forward and reverse primers, 0.625ul of both FAM and VIC labelled probes, 1ul of template and dH_20 to a final volume of 25ul.

Real time PCR was performed on the ABI 7900 HT (Applied Biosystems) with cycle-by-cycle fluorescent product visualisation. Cycling conditions were 95° for 10 minutes, followed by 40 cycles of 95° for 15 seconds and 62° for 1 minute.

c) Assay Validation

Assay validation was performed on buffy coat DNA from 45 cases from the prospective cohort. Confirmation of DNA sequence at the 15n SNP was confirmed by sequencing of 10 of these cases' normal DNA using 15n primers, with sequencing performed as described in section 2.5.4.Sequenced cases were numbers MD 2535, 2536, 2537, 2538, 2540, 2541, 2543, 2544, 2565 and 2566.

The primers used for the sequencing reaction were:

Forward: 5' AAA GAC ATA CCA GAC AGA GGG

Reverse: 5' CTT TTT TGG CAT TGC GGA GCT

2.7.2 Determining the Limit of Resolution Allelic Imbalance Detection in Plasma Using Taqman Assay

AI was experimentally introduced into control plasma by adding serial dilutions of known quantity DNA homozygous for the CCA (FAM) allele. Control plasma was from patient ID 72. Homozygous DNA for the CCA allele was extracted from buffy coat from case 2536 (2536 N) as previously described (section 5.2.2).

Quantification of 2536 N DNA was performed using spectrophotometry.

Serial dilutions of 2536 N DNA were performed and added to 1ml of control plasma DNA. Concentrations of added CCA homozygous DNA were 100 ng / ml of control plasma, 10ng/ml, 5ng /ml, 3 ng/ml.

Plasma DNA was extracted using the QIAgen protocol, as described in chapter 3, from 200ul of plasma in a total of 100ul of elution buffer. Spiking, extraction and analysis were performed in triplicate.

2.7.3 Analysis of Tumour Samples

Comparison was made between the 15n assay and conventional fluorescence electrophoresis in determining LOH in tumour samples. An unselected subset of the prospective cohort was studied (n= 45). This cohort was analysed since these constituted the recruited patients at the point at which this portion of assay development was undertaken.

a) Microsatellite analysis

i) Primers

Fluorescence microsatellite analysis was performed for matched tumour and normal samples using the markers D5S346 and D5S82. These markers were chosen on the basis of their location within the frequently deleted region of chromosome 5p, and hence their relation to the APC gene and its 15n SNP. Primers for D5S346 were as described in section 2.6.1 a).

D5S82

Forward primer (FAM labelled)	ATCAGAGTATCAGAATTTCT
Reverse primer (unlabelled)	CCCAATTGTATAGATTTAGAAGTC
Amplicon	169 bp

Sequence from accession M76582:

1 CCCAATTGTA TAGATTTAGA AGTCATTTTA CACACACACA CACACACACA CACAATTAAT

61 ATGATATGTG TGTGCATGTA TAATTTTTAT CTCAATATTT TAGATGTGAA CAGGGAAAAG

121 TCATAATCCG TTGGCCCTCA CTTCATTTGA AAGAAATTCT GATACTCTGA T

ii) Reaction constituents

PCR reaction mixture consisted of 5ul of 10x PCR buffer, 5ul of MgCl, 5ul of dNTPs 1 ul of each primer (at 100ng/ul concentration), 0.25ul of Taq polymerase, 2ul of template and dH_20 upto a volume of 50ul. PCR thermal cycling was performed on PTC-225 thermal cycler (MJ Research). An initial denaturing step of 95°c for 5 minutes, followed by 35 cycles of 95 °c for 60seconds, 55 °c for 60 seconds, 72 °c for 90 seconds, with a final step of 72 °c for 5 minutes.

iii) Analysis

PCR products were processed for analysis by adding 1ul product to 1ul of GeneScan 350 Tamra size standard (Applied Biosystems) and denatured Hi-Di formamide to a total volume of 14ul. Samples were denatured at 95 ° for 3 minutes, cooled on ice and then analysed using ABI 310 platform (Applied Biosystems) with analysis of Genescan 2.1 software (Applied Biosystems). Where a single allele was observed this was taken to represent homozygosity at the given marker and hence non-informativity. A ratio of peak heights from differing alleles was calculated as described in section 2.6.2 c).

Levels of allelic loss were classified into 3 groups

1) Clear-cut LOH: where allelic ratio was altered >50% in tumour compared to normal

2) Ambiguous LOH: alteration in allelic ratio between 20-50%

3) No LOH: alteration of allelic ratio <20%.

b) 15n assay

Analysis of the same 45 cases tumour and normal samples, as well as buffy coat DNA from 12 controls was performed using the 15n assay. Reaction constituents and conditions were as described for assay validation.

The FAM and VIC fluorescence readings for each sample were recorded numerically. An allelic ratio was generated by dividing the FAM fluorescence by the VIC fluorescence reading. Allelic ratios were compared between control, normal and tumour DNA with reference to the results of fluorescence electrophoresis.

2.7.4 Analysis of Plasma DNA

Real time PCR analysis using the 15n assay was performed on matched pre-operative plasma samples for the same cohort of cases (n=45) as those with tumour DNA analysis described above. Additionally plasma DNA 8 controls were analysed.

The primers and probes were as described, with reactions being performed in a final volume of 25ul. Reaction constituents were therefore 12.5ul of TaqMan Universal PCR Mastermix, 0.75ul of both forward and reverse primers and 0.625ul of both FAM and VIC labelled probes. Initially 5ul of plasma DNA template was used then experiments repeated using 10ul of DNA template, with the remainder of the reaction mixture made up with dH_20.

Real time PCR was performed on the ABI 7900 HT (Applied Biosystems) with cycle-by-cycle fluorescent product visualisation. Cycling conditions were 95° for 10 minutes, followed by 50 cycles of 95° for 15 seconds and 62° for 1 minute.

2.8 Dilutional PCR

The 15n assay was adapted to count large numbers of individual alleles. Dilutional small pool PCR was performed using serial dilutions, similar to previously described digital PCR.

2.8.1 Assay validation

Assay validation was performed by allele counting normal DNA from a heterozygous control subject (case number69) in triplicate.

2.8.2 Study Subjects

A subset of the prospective cohort was analysed where a larger volume of plasma had been taken to allow sufficient template for multiple reactions. This subset constituted 13 cases and 7 controls (all with negative colonoscopies), and are presented in table 2.3. Patient allelic status was determined using the 15n assay applied to DNA from buffy coat, under conditions as described above. 4 heterozygous (informative) controls were identified, and 4 heterozygous cases.

Table 2.3 Allele counting study group characteristics

Case number	Case / Control	Stage	15n status
5004	Case	II	CCA
5005	Case	III	CCG
5006	Case	III	CCA
5007	Case	IV	CCA
5008	Case	III	CCG
5009	Case	II	CCA
5011	Case	III	HETERO
5012	Case	Adenoma	CCA
5504	Control	n/a	HETERO
5505	Control	n/a	HETERO
5506	Control	n/a	CCA
5507	Case	III	CCA
5508	Case	III	HETERO
5509	Case	II	HETERO
6335	Control	n/a	HETERO
6336	Control	n/a	HETERO
6337	Case	I	CCA
6338	Control	n/a	CCG
6339	Control	n/a	CCG
6341	Case	I	HETERO

A further informative case was analysed (2530- Stage IV) where there were sufficient quantities of plasma DNA for analysis. Prior results for this patient described in chapter 6 had determined that this patient's tumour had clear cut LOH of a region of chromosome 5, confirmed with 15n assay analysis.

2.8.3 Assay conditions

For plasma DNA analysis 15n assay primers and probes were as described in section 2.7. PCR reaction mixture and cycling were also unchanged, except for only 1ul of (diluted) template was added with a corresponding increase in water (total volume 12.5ul). 384 well plates were constituted using a Biomek automated robot (Beckman Coulter). The majority of PCR cycling of plates was performed on an ABI 7900 (Applied Biosystems), with a proportion were performed using PTC-225 thermal cycler (MJ Research), with analysis performed on ABI 7900 using plate reading configuration.

Plasma DNA dilutions were based on the results of quantification using picogreen (chapter 3). Test plates were made with dilutions around the level suggested by these results, using 48 wells of a 384 well plate per dilution. Confirmed dilutions were chosen when 24 out of 48 wells registered no product, and remaining wells contained generated only FAM or VIC fluorescence without evidence of heterozygotes. Multiple plates for each plasma DNA sample were performed at these dilutions. Plates were included for analysis when >192 wells were recorded as empty, and <20 wells registered as heterozygotes. Individual CCA and CCG alleles were counted and recorded manually. Replicates were performed until >1000 individual alleles were recorded. Allele counting of plasma DNA from cases was performed blinded, i.e. without prior knowledge of the allelic status of those patients' tumours, with the exception of case 2530 (the test case).

2.8.4 Analysis

The numbers of observed plasma DNA alleles was compared to the number of expected alleles for a heterozygote with allelic balance (i.e. 1:1, or 500:500). Statistical analysis was performed using the X^2 test.

Subsequent to analysis of plasma DNA, allele counting was performed on matched tumour samples. Methods were as described for plasma DNA analysis, with the exception of fewer alleles were counted (>100).

CHAPTER 3

EXTRACTION AND QUANTIFICATION OF PLASMA DNA

3.1 Introduction

The published literature on circulating DNA describes a broad range of published methods that have been used in identifying tumour derived DNA in the circulation. Studies have commonly analysed either serum or plasma DNA that has been extracted using various generic and commercially available extraction reagents and protocols. Whilst the positive results from these different methods suggest a degree of flexibility in the approach employed, it also raises difficulties of comparing results between studies using different methods particularly where contradictory results have been reported. Although discussion occurred in this respect at an early international meeting (CNAPSI-circulating nucleic acids in plasma and serum I) with methods published (399), to date there is no consensus on standard methods employed by researchers in the field, despite subsequent meetings and remains a source of much debate within the field (400), although there is evidence that new commercially extration kits may give higher yields (401).

Authors have suggested that the process of blood clotting results in the release of normal DNA from cells involved in initiation and propagation of the clot, and hence may result in the 'dilution' of tumour derived DNA in a further pool of normal DNA. In this project it was therefore elected to analyse plasma DNA and this was performed throughout. There are however potential problems with the use of plasma DNA, since the chemicals employed to prevent clotting have been suggested to have an effect on the PCR process. To this end duplicate samples were collected from all patients (except those recruited for allele counting

experiments, on account of the relatively large volume required for this process) in both EDTA and Lithium Heparin tubes.

The absolute quantity of plasma DNA from patients and controls has been correlated with clinical states both in benign and malignant conditions, as discussed in section 1.5.1and 1.5.3 e. Furthermore quantity of template added to qualitative reactions is likely to have an impact on assay performance.

This chapter describes an assessment of methods of plasma DNA extraction and the results of a number of assays to quantify total plasma DNA. The best performing assay for quantification of plasma DNA was then correlated with the presence of disease as well as disease stage and survival.

3.1.1 Quantification of plasma DNA:

a) Methods for quantifying DNA

Spectrophotometry is a commonly used method for quantifying and assessing the purity of DNA. The principle is absorption of different wavelengths of light by differing substances including DNA and protein. As such the quantity and purity of DNA can be estimated according to the equation given below in materials and methods, based on absorbance of light at 260nm (A_{260}). This approach is however relatively insensitive, is unable to discriminate RNA from DNA and is affected by nucleotides and single stranded DNA.

Real time PCR allows cycle-by-cycle visualisation of template amplification. Inclusion of template standards of known concentration allows generation of a standard curve and hence a direct measure of target DNA template quantity.

Picogreen® ds DNA quantitation reagent is an ultrasensitive fluorescent nucleic acid stain for quantitating double stranded DNA (dsDNA). Level of detectability has been suggested to a level of 25pg/ml of ds DNA (390). Additionally the technique for determining concentration

is simple and can be performed quickly on multiple samples with the use of fluorescence plate readers. Picogreen has been previously used to determine plasma DNA concentration in patients with a variety of cancers (52;402;403)

b) Rationale for quantifying plasma DNA

The rationale for wishing to quantify DNA included the technical implications of quantity in assay development for detecting tumour derived DNA in plasma. Additionally there is some evidence that quantity of plasma DNA may have utility in the detection and prognosis of disease states, including in colorectal cancer as discussed in chapter 1 (1.5.1 & 1.5.3 e)), although there was little data in the literature at the outset of this project.

3.2 Materials and Methods

3.2.1 Plasma DNA Extraction

Several methods of extracting plasma DNA were investigated with protocols for each of the extraction methods used detailed below.

a) Phenol chlororform Extraction / CNAPS I protocol

Variations from published protocol are indicated in brackets (404):

i) DNA Extraction

1.5mls (200ul) of plasma was added to 1.5mls (200 ul) of 1X SDS proteinase K solution and mixed well. The sample was digested overnight in water bath at 55 oC. The digested sample was then added to equal volume, 3mls (400ul) of water saturated phenol/chlororform (not performed in vacutainer brand SST tubes with gel barrier). The sample was vortexed for 30 seconds, followed by centrifugation for 10 minutes at 2500 rpm. The upper layer was taken off and phenol / chloroform treatment repeated as above.

ii) DNA Precipitation

The upper layer from repeat phenol / chloroform treatment was taken and 3ul (20mg/ml) of glycogen added (Boehringer Mannheim), in addition to 1ml of 7.5M ammonium acetate and 8ml of 100% ethanol . The sample was mixed by inverting several times (sample placed in –80 freezer for 60 minutes). The sample was then centrifuged at 6000rpm (4500g) for 60 minutes (at 20 oC). The supernatant was then carefully poured off, keeping the pellet at the bottom of the tube. 10 mls 70% ethanol was added and further centrifugation performed at 6000 rpm (4500g) for 10 minutes. The ethanol was then discarded, and the sample left open for residual ethanol to evaporate. 200ul of LoTE buffer was added and left overnight at room temperature to resuspend pellet. Samples were stored at –20 oC.

b) Nucleon Protocol

Variations from published protocol are indicated in brackets:

5 mls of whole blood (200ul plasma) were added to 40 mls (1.6mls) of reagent A. the sample was mixed for 4 minutes at room temperature, then centrifuged at 1300g for 4 minutes. The supernatant was discarded without disturbing cell pellet. 2ml of reagent B was then added and vortexed to resuspend the cell pellet. (RNAse step omitted). 500ul of Sodium Perchlorate was added and rotary mixed/shaken at room temperature for 15 minutes. Tubes were then incubated in shaking water bath at 65 oC for 25 minutes. 2 mls of choloroform, which has been stored at –20 oC, was then added and the sample rotary mixed/shaken at room temperature for 10 minutes and then centrifuged at 800g for 1 minute. 300ul of Nucleon Silica Suspension (shaken vigorously before adding) was added and then centrifuged at 1400g for 3 minutes. Holding the tube vertically and without disturbing the Nucleon Silica Suspension layer, only the DNA containing phase above the nucleon layer was transfered into a fresh centrifuge tube. The sample was centrifuged briefly at 1300g to pellet any residual Nucleon silica and supernatant carefully decanted to a fresh tube. Aqueous DNA containing phase was added to two equal volumes of cold ethanol at 4 oC. The sample inverted gently to precipitate DNA.

c) Qiagen Protocol

200ul of plasma was added to 20ul proteinase K and 200 ul buffer AL and mixed by pulse vortexing for 15 seconds. The sample was incubated at 56 oC for >10 minutes, then briefly centrifuged. 200ul pure ethanol was added and mixed by pulse vortexing. This mixture was applied to QIAamp spin column and centrifuged at 8000rpm for 1 minute. 500ul of buffer AW1 was then added to spin column and centrifuged at 8000 rpm for 1 minute. Next 500ul of

buffer AW2 was added to spin column and centrifuged at 14000 rpm for 3 minutes. 50ul of buffer AE was then added to spin column and left to stand at room temperature for 30 minutes. The column was centrifuged at 8000 rpm for 1 minute. A further 50ul of buffer AE was added to spin column and left to stand at room temperature for 30 minutes. Column centrifuged at 8000 rpm for 1 minute. This extraction was performed for all cases and controls.

Additionally for cases and controls utilised in allele counting experiments a further large volume extraction using the QIAamp maxi kit was performed extracting DNA from 8 mls of plasma with elution in 300ul of elution buffer.

d) Analysed Samples

Negative controls were performed with an aliquot of water, of the same volume as plasma subjected to extraction, undergoing each extraction protocol.

Positive controls were provided by LoVo DNA derived from cell culture as described in chapter 2

Normal subjects plasma was derived from 3 healthy control subjects (MD 68, 69 and 74).

Extraction and analysis was performed on plasma alone, and of serial dilutions of added LoVo DNA (0ng, 50ng, 100ng and 1microgram).

e) Assessment

Assessment of extracted DNA was performed by

i) PCR and visualisation by gel electrophoresis as described in chapter 2.

ii) PCR with microsatellite markers and fluorescent microsatellite analysis as described in chapter 2.

iii) Quantification: described below

3.2.2 Quantification of Plasma DNA from Cases and Controls

a) Real time PCR subset

Plasma DNA from a subset of the prospective cohort was quantified by real time PCR. This subset comprisedcolorectal cancer cases (n=24) and controls (n=10) heterozygous for a APC gene SNP, derived from the first 75 subjects recruited from the prospective cohort.

b) Picogreen

Plasma DNA was quantified for the whole prospective cohort of cases and controls.

c) Small pool PCR

Allele counting was performed for a total of 5 cases and 4 controls, being those cases where sufficient quantity of plasma DNA was availableto perform the high number of replicate samples required.

Plasma DNA Quantification was performed by spectrophotometry, real time PCR, picogreen assay and by allele counting as described in chapter 2.

3.3 Results

3.3.1 Extraction Protocols

Initial experiments aimed to compare 3 extraction methods for plasma DNA, for ease of use and reproducibility both of the extraction process and analysis of extracted DNA.

a) Phenol Chloroform extraction

Phenol chloroform extraction was performed as per the protocol published following CNAPS I international conference (405), and as described in materials and methods with variations from the published protocol indicated. Briefly, plasma DNA was extracted from 200ul of plasma by double treatment with an equal volume of phenol choloroform, and precipitation with ethanol.

Performing this extraction procedure however resulted only in a cloudy solution, without an obvious interface between layers. Repeating phenol cholorform treatment a third time resulted in semi-solid white matter on which it was not possible to perform further processing. Precipitation of the cloudy solution with ethanol did not result in the generation of a visible pellet, however reconstitution was performed with LoTE. Spectrophotometry of the resultant solution gave very low values, not consistent with the presence of appreciable quantities of DNA, and PCR using standard microsatellite markers using the resuspended solution as template failed to yield any demonstrable PCR product, by gel elctrophoresis, or by fluorescent PCR analysis. Replication of this process in triplicate from further control samples and samples with added serial dilutions of exogenous (LoVo) DNA resulted in the same results.

b) Nucleon Extraction Kit

Plasma DNA extraction was attempted using a Nucleon extraction kit, as described in materials and methods. Attempts at extraction of plasma DNA, gave a large amount of crystalline precipitant that was not possible to dissolve in TE. Further analysis was attempted with the solution above the crystalline precipitant in which resuspension was attempted, including leaving the solution on the precipitant for >24 hours following repeated vortexing. However the 'resuspension' solution failed to yield any appreciable signal on spectrophotometry, and failed to generate PCR products when used as template for standard microsatellite marker PCR.

c) Qiagen Extraction Kit

Plasma DNA from 200 ul control plasma was repeatedly extracted using the QIAgen QIAAMP mini body fluid protocol as described in materials and methods. This commercially available kit is designed for use with small volume samples, and allowed reproducible sample processing. Precipitants remained contained within the extraction column, resulting in a predictable volume of clear elutant at the end of the process. Quantification of the extracted DNA is described further below. Extraction in triplicate on test samples allowed consistent generation of PCR products

Given the difficulties with the other extraction methosds detailed above, it was elected to employ the QIAgen extraction protocol for the remainder of the project.

3.3.2 Quantification

a) Spectrophotometry

Spectrophotometric quantification of plasma DNA was performed in triplicate on samples as described in section 3.2.. Extraction was performed from 200ul of plasma using the QIAgen protocol, eluted in 100 ul of elution buffer.

Plasma DNA concentration was calculated for all samples, in micro gram quantities. This was the case for each control and illustrated by the results in table 3.1 for control 1 (case number 68). Additionally inter-sample variation was wide indicating the inaccuracy of the technique when applied to low copy number template DNA. Finally results were incongruous by giving smaller concentrations for plasma samples where large quantities (microgram) of cell line DNA had been spiked into control plasma, compared to the control plasma alone (table 3.2).

Table 3.1

Control 1 (MD68)	Absorption	Ratio	PlasmaDNAconcentration ug/ml
Extraction1	0.039	1.311	19.7
Extraction2	0.103	1.375	51.3
Extraction3	0.019	2.397	9.3
Total			26.8
			95%CI =-9.1 to 62.7

Table 3.1 legend: Spectrophotometry results (average of 3 readings) from triplicate extractions of control plasma.

Table 3.2

	Absorption (average of 3 readings)	Ratio	Concentration ug/ml
Controlplasma	0.018	2.172	9
Controlplasma+50ngLovo	0.019	2.098	9.5
Controlplasma+ 100ngLoVo	0.018	1.813	9
Controlplasma+ 1ugLovo	0.017	1.652	8.5

Table 3.2 legend: Spectrophotometry results from control plasma spiked with exogenous DNA.

b) Real-Time PCR

Quantification of plasma DNA was performed utilising a real-time PCR Taqman assay designed to a SNP on chromosome 5, as described in chapter 2. Standard DNA from Applied Biosystems was serially diluted to provide concentration standards. Amplification was performed using standard reagent concentrations and 50 replication cycles.

i) Test set

Real time PCR quantification was initially tested on control plasma that had serial dilutions of exogenous CCA allele artificially added as described in sections 2.7.2 & 6.3.3. Triplicate quantification results are presented in table 3.3.

Table 3.3 Real time PCR quantification results from spiking of control plasma with known quantities of normal DNA homozygous for the CCA (FAM) allele.

Sample	Quantity 1	Quantity 2	Quantity 3	Mean
Control (68)	16.4	40.5	7.2	21.4
C+ 3 ng CCA	28.7	22.0	34.3	28.3
C+ 5ng CCA	19.3	11.8	29,5	20.2
C+ 10ng CCA	28.6	24.8	27.3	26.9
C+ 100ng CCA	41.8	102.6	75.1	73.2

Table 3.3 legend: Triplicate results and mean quantities from real time PCR quantification of control plasma spiked with known quantities of normal DNA from a CCA allele heterozygote.

ii) Plasma from cases and controls

The assay was then assessed on a subset of the total cohort of prospectively collected colorectal cancer cases and controls, namely cases (n=24) and controls (n=10) heterozygous for the 15n SNP. Results are presented in table 3.4 for cases and table 3.5 for controls. Examples of standard curves are given in Figures 3.1 and 3.2.

Table 3.4:

Case number	Mean[FAM]	[plasmaDNA]	Case number	Mean[FAM]	[plasmaDNA]
2461	2.335ng	467ng/ml	2605	0.932	186.4ng/ml
2488	0.0845ng	16.9ng/ml	2609	0.195	39.0ng/ml
2490	1.655ng	331ng/ml	2645	3.685	737.0ng/ml
2494	0.912ng	182.4ng/ml	2661	0.730	146.0ng/ml
2530	9.043ng	1808.6ng/ml	2662	1.106	221.2ng/ml
2532	1.518ng	303.6ng/ml	2679	0.391	78.2ng/ml
2537	0.884ng	176.8ng/ml	2683	0.556	111.2ng/ml
2539	1.545ng	309.0ng/ml	2684	0.589	117.8ng/ml
2541	0.788ng	157.6ng/ml	2699	0.112	22.4ng/ml
2567	1.455	291.0ng/ml	2700	0.414	82.8ng/ml
2568	1.398	279.6ng/ml	2710	0.694	138.8ng/ml
2597	25.485	5097.0ng/ml	2714	0.215	43.0ng/ml

Table 3.4 legend: plasma DNA quantities from 24 cases analysed by real time PCR

Table 3.5:

Control no.	Mean FAM	Plasma [DNA]	Control no.	Mean FAM	Plasma [DNA]
2535	0.132	26.4ng/ml	2726	0.136	27.2ng/ml
2542	2.487	497.4ng/ml	69	0.089	17.8ng/ml
2543	1.089	217.8ng/ml	71	0.241	48.2ng/ml
2721	0.237	47.4ng/ml	72	0.385	77.0ng/ml
2725	0.499	99.8ng/ml	75	0.149	29.8ng/ml

Table 3.5legend: plasma DNA quantities from 10 controls Spectrophotometry results from 10 controls analysed by quantitative real time PCR.

Mean [plasmaDNA] was 108.88 ng/ml for controls and 472.68 ng /ml for cases. There was a non-significant difference between groups (p= 0.110, t-test (type 3, 2 tailed)).

Figure 3.1

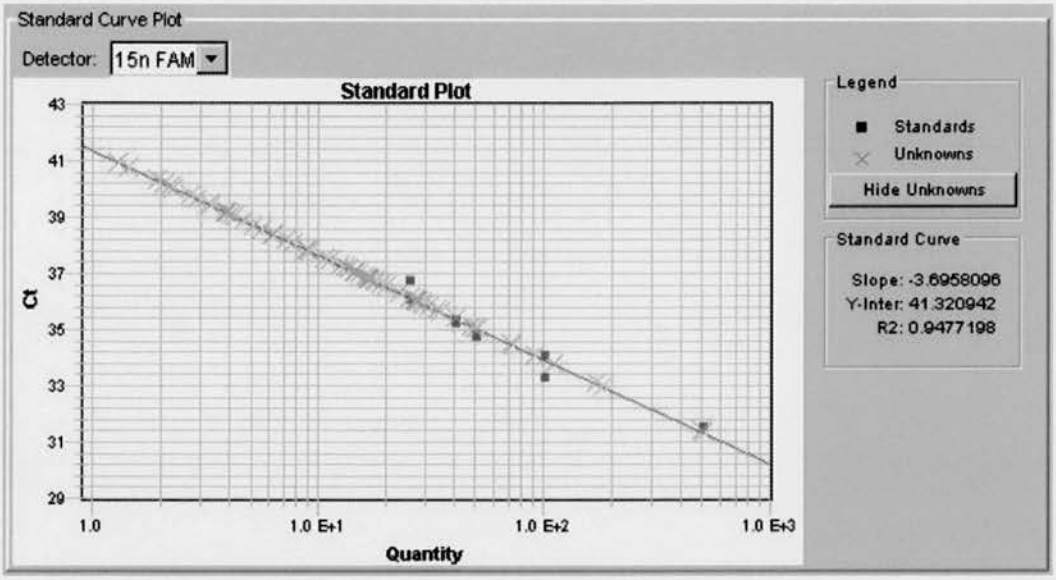


Figure 3.1 legend:

Real time PCR quantification of plasma DNA from cases and controls (light blue crosses). Standard dilutions of known concentration are indicated by blue squares, generating a satisfactory standard curve line.

Generation of accurate quantities from plasma DNA is dependent on a reliable standard curve. The standard DNA used in these experiments was at stock concentration of 10ng/ul, and hence was diluted by up to a factor of 400 times in order to generate a standard curve down to the concentrations observed in plasma DNA. Dilution to this level is prone to inaccuracy due to pipetting very small quantities into large volumes of water. Therefore a suitable standard curve was not always generated, as illustrated in figure 3.2, and resulted in inability to replicate consistent results in triplicate.

Figure 3.2:

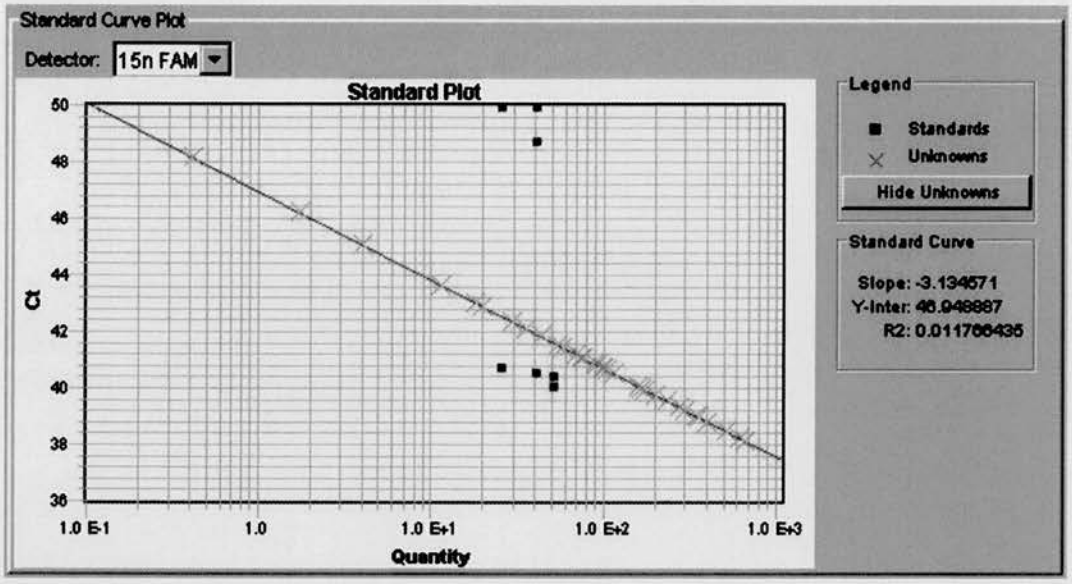


Fig 3.2 legend:

Real time PCR results for quantifying control DNA spiked with DNA from cultured cell lines. Blue squares indicate dilutions of standard concentration DNA, which are widely spread and do not generate a straight line between them.

c) Picogreen

i) Test set

Picogreen quantification was performed on control plasma spiked with known quantity of DNA, including samples spiked with LoVo DNA (as used for spectrophotometry assessment) and those samples spiked with normal DNA homozygous for the CCA allele at the 15n SNP as for the assessment of real time PCR quantification. The results from these assay assessment experiments are presented in table 3.6.

Table 3.6

Sample	Mean of triplicate readings (ng/ml)	Sample	Mean of triplicate readings (ng/ml)
Control	21.1	Control	22.5
C + 50 ng LoVo	59.7	C+3ng CCA	23.2
C+100 ng LoVo	115.3	C+5ng CCA	26.8
C+ 1ug LoVo	976.9	C+ 10ng CCA	30.7
		C+ 100ng CCA	113.8

Table 3.6 legend: Mean quantities by picogreen analysis of control plasma with added exogenous DNA

ii) Plasma from cases and controls

Quantification using this picogreen method was performed for all of the prospectively collected cohort, namely 87 adenocarcinomas and 7 adenomas. A total of 30 control patients were analysed. One of these control subjects was subsequently shown to have a low grade B cell lyphoma and was excluded from further analysis.

Linear standard DNA curves were generated, with similar results generated for replicate samples. An example of a linear standard curve is presented in figure 3.3.

Figure 3.3

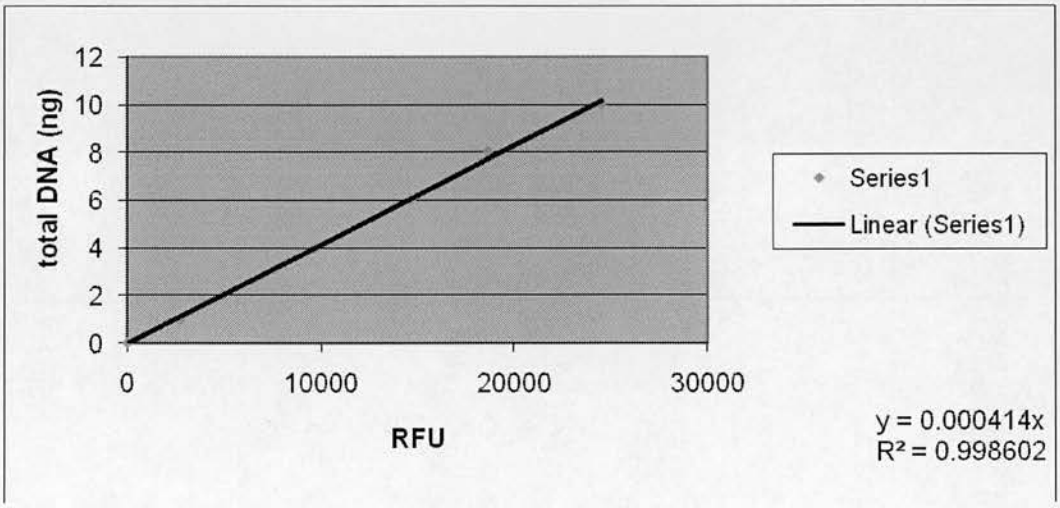


Figure 3.3 legend: Standard curve for picogreen quantification. The x axis demonstrates units of fluorescence, and the y axis quantity of DNA in ng. The blue diamonds indicate serial dilutions of known quantity standard DNA.

Mean and range values for each pathological stage, polyps and controls are presented in table 3.7.

Table 3.7

	Stage I	Stage II	Stage III	Stage IV	Polyps	Controls	Unknown
Number	15	30	26	15	7	29	1
Mean[plasma DNA] ng/ml (and range)	498 (3.2 – 4143)	576 (0 – 6790)	170 (8 – 530)	511 (59 – 2455)	113 (56 – 269)	103 (0 - 318)	

Table 3.7 legend: Plasma DNA quantity and number of cases by disease stage, with quantity range presented in brackets.

The raw triplicate sample quantity data for cases, controls and polyps are presented in figure 3.4.

Figure 3.4:

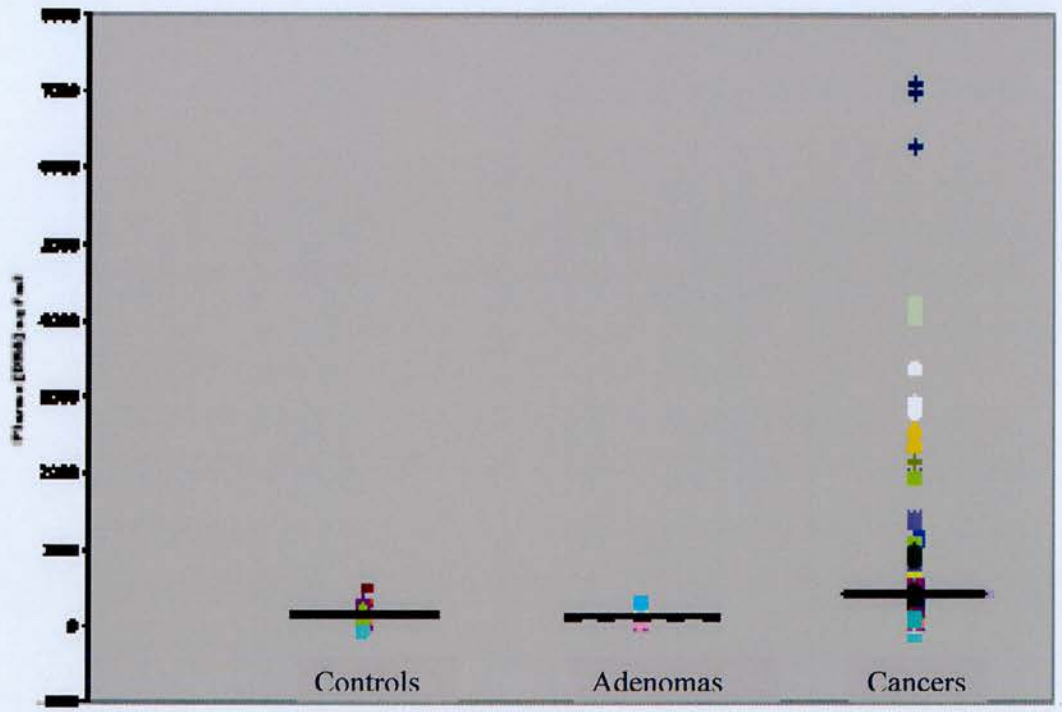


Figure 3.4 Legend: Graphic representation of raw triplicate data from cancer cases, adeomas and controls. The horizontal black bar indicates the mean value for each group.

There was a significant difference in mean plasma [DNA] between cancer cases and controls (p=0.002). There was no significant difference between adenoma cases and controls (p=0.611). There was no association of higher plasma [DNA] with increasing disease stage, with no significant difference between early cancers (Dukes A&B) and late stage cancer (Dukes C&D) (p=0.078).

The receiver operator curve for cancer is presented in Figure 3.5.

Figure 3.5:

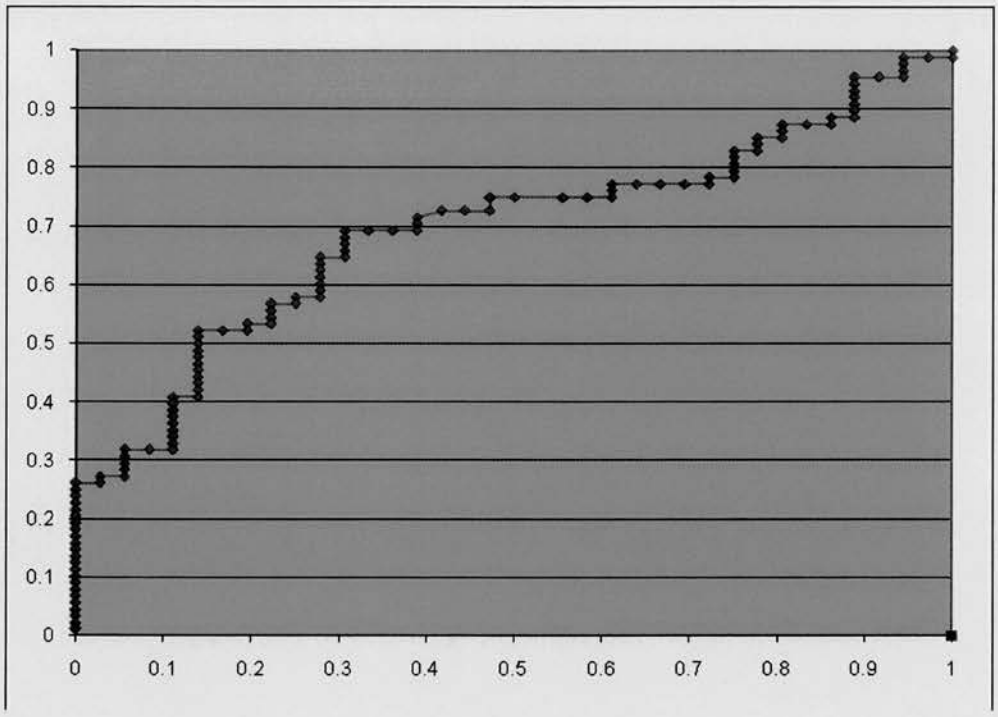


Figure 3.5 legend: ROC curve for cancer

The area under the ROC curve for cancer was 0.696. Therefore given specificity set at set at 100%, sensitivity for cancer was 26.1%. With specificity set at 90%, sensitivity was 31.8%.

d) Dilution

Further data on plasma DNA quantity was generated in allele counting experiments described in chapter 7. Whilst these experiments were designed to examine allelic ratios within plasma DNA, the methods necessitated dilution of plasma DNA to visualise a single copy within a reaction well, hence alsoproviding direct evidence of plasma DNA concentration in the analysed samples. The test samples of control plasma spiked with exogenous concentrations of DNA was not performed by allele counting given the high degree of reagent costs.
Allele counting was performed for a total of 5 cases and 4 controls.
Extrapolation of the degree of dilution required to give a single DNA copy in every other well was performed and the results demonstrated in table 3.8:

Table 3.8

Cases	Dilution	plasma [DNA] from dilution	plasma [DNA] by Picogreen
2530 (QiaAMP mini)	1 : 200	3300	2455
5011	1 : 9	59	56
5508	1 : 20	132	184
5509	1 : 55	363	482
6341	1 : 28	185	285
Controls			
5504	1 : 10	66	82
5505	1 : 11	73	127
6335	1 : 10	66	78
6336	1 : 9	59	28

Table 3.8 legend: Dilution required to generate one copy per well for allele counting experiments, with calculated plasma DNA concentration and quantity by picogreen analysis given in the last column.

3.2.3 Survival

Survival analysis of cancer cases was performed. Mean survival was 1417 days. The survival curve for all cases is presented in figure 3.6.

Figure 3.6:

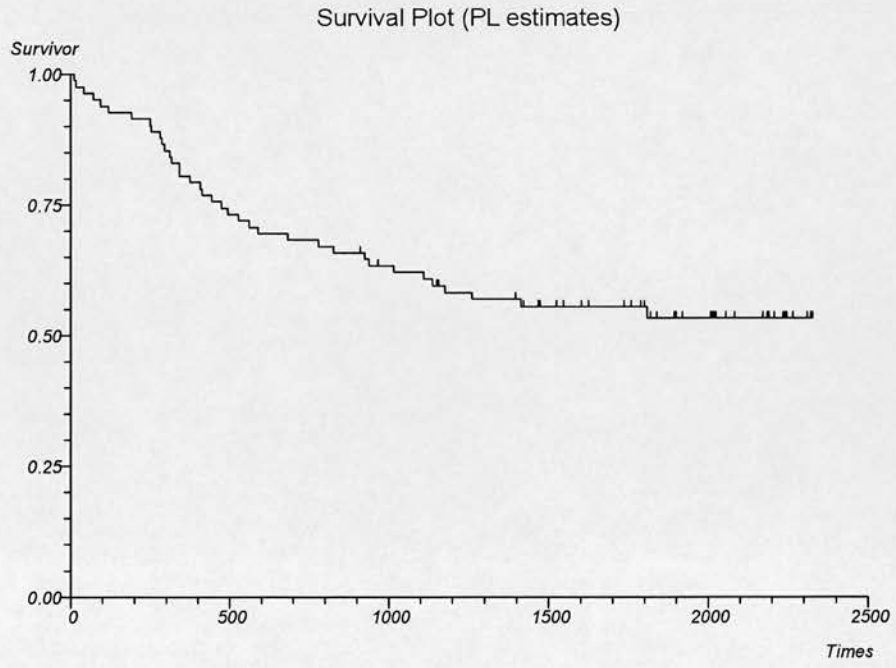


Figure 3.6 legend: Kaplan- Meier survival curve for all cancer cases.

Survival analysis was then performed to compare the survival of cancer cases with a plasma DNA concentration of below 250ng/ml to those with a higher concentration as determined by picogreen quantification. The survival curves for these 2 groups is presented in figure 3.7. The hazard ratio for death for those with plasma DNA concentration lower than 250ng/ml was 0.72 (95% confidence interval 0.36-1.46), with a Chi-square for equivalence of death rates of 0.91 (p=0.34).

Figure 3.7:

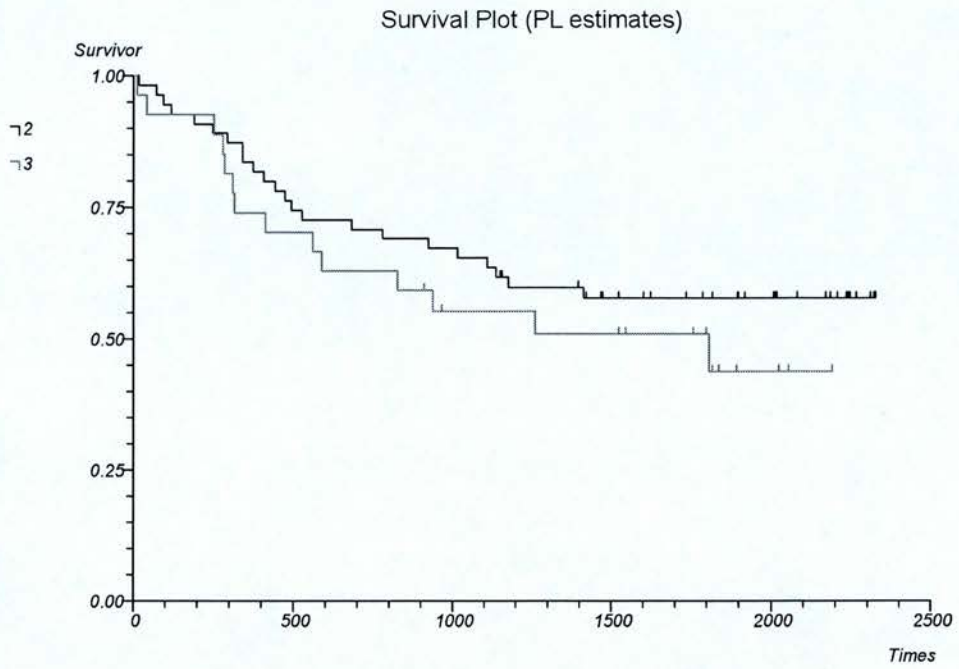


Figure 3.7 legend: Kaplan- Meier survival curves for those with plasma DNA concentration of under 250ng/ml (shown in black), compared to those with plasma DNA concentration of over 250ng/ml (shown in red).

Further survival analysis was performed of those cases with early stage cancer (Dukes A and B) to assess for an impact of plasma DNA concentration on survival in cases where survival is expected to be good. These analyses are shown in figure 3.8. The hazard ratio for death for those cases with Dukes A or B, and with plasma DNA concentration lower than 250ng/ml was 0.59 (95% confidence interval 0.17 – 2.05), with a Chi squared equivalence of death rates of 0.81 (p=0.37).

Figure 3.8:

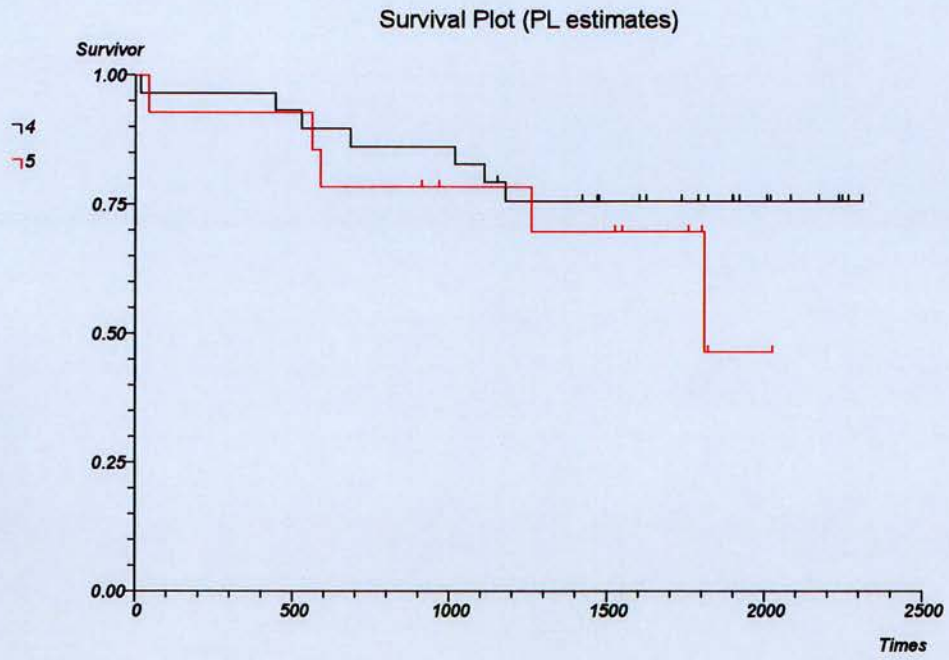


Figure 3.8 legend: Kaplan- Meier survival curves cancer cases with Dukes stage A or B, showing those with plasma DNA concentration of under 250ng/ml (in black), compared to those with plasma DNA concentration of over 250ng/ml (in red).

3.4 Discussion

3.4.1 Extraction

Experiments to investigate extraction methods were performed on small volumes of control plasma DNA. This approach provides a stringent assessment of the reproducibility of the methods due to small volumes and likely low plasma DNA concentrations. It is however necessary for the approach to be valid under these conditions to allow interpretation of data from samples with very low copy number DNA and where small volumes of plasma are available. It is likely that archived samples constitute a limited stored biological resource, and additionally that tests on prospectively collected cohorts may be more acceptable when a smaller volume blood sample is required.

Phenol chloroform extraction of plasma DNA was unsuccessful. It is likely that this was on account of the changes made from the published protocol, namely reducing the quantity of plasma extracted from 1.5ml to 200ul. With the reduced volumes the precise collection of different solution layers became technically difficult, and would also likely result in significant inter-sample human error. The difficulties encountered with the nucleon extraction kit likely reflected a similar problem. Additionally the nucleon kit is a protocol for extraction of DNA from whole blood of larger volumes, and hence further testing of this extraction method was not performed.

Extraction using the Qiagen QIAAmp commercial extraction kit proved easy to use with several different kits designed for use with differing volumes of plasma, mini (upto 200 ul), midi(upto 2 mls) and maxi(upto 10mls). There remained difficulties in the early part of this study with both quantitative and qualitative assessment of extracted plasma DNA, as described above, when the decision was being taken as to which extraction method to employ through the remainder of the study. However the protocol proved easy to use, with samples giving predictable volume of elutant and consistently amplifiable and detectable template by

PCR using microsatellite markers. In view of this and the large number of published studies employing this method of plasma DNA extraction, it was elected to standardise extraction using this method. Subsequent to the completion of the laboratory work for this project there has been ongoing debate in the literature as to optimal extraction methods., with authors suggesting that isolation of nucleic acids from body fluids remains deficient in robust methods (406). There are now many available commercial kits including anion exchangers and magnetic beads (407)(su YH). Board et al described the qiagen kit as the most efficient of 4 commercial kits(408) however others have demonstrated deficiencies in both efficiency(409) and selectivity of size fragments (410).

3.3.2 Quantification methods

Quantification of plasma DNA has inherent difficulties due to the small concentrations, particularly from normal controls. As such methods for determining plasma DNA concentration are required to be robust. In this work, several methods of determining plasma DNA concentration were utilised. The classical method of DNA quantification using spectrophotometry proved unreliable, due to the inherent variability in results on repeated testing of the same samples. This was likely partly a reflection of the small quantities of DNA and also a relative lack of DNA purity, an unavoidable factor when dealing with the complex milieu of human body fluids. As such further quantification of plasma DNA using spectrophotometry was not performed.

Real-time PCR affords cycle by cycle visualisation of template amplification, and is able to determine relative quantities of DNA within a template. The inclusion of standards of known concentration allows the generation of a standard curve and hence the calculation of absolute DNA quantities. This approach has been used with respect to circulating DNA, both for DNA and RNA templates (260).

However there are inherent problems with absolute quantification using real-time PCR, in that calculations are dependent on accurate standards or an efficient assay reaction to remain valid. In the results presented in this chapter plasma DNA concentrations were generated using real-time PCR absolute quantification, for cases and controls. There was notan association between higher concentrations in cancer cases compared to controls. These results however need to be interpreted with caution since the assay reaction was not of optimal efficiency to allow reliable calculation of absolute quantities without inclusion of known concentration standards with each reaction plate. Additionally results were seen to vary considerably, using the same standard DNA dilutions to generate the standard curve, as illustrated when quantifying plasma DNA in spiked samples. Finally a relatively small number of cases and controls were assessed by this method hence reducing the potential to detect a significant difference between cases and controls.

Fluorescence DNA detection, using a fluorescent dye that generates signal when bound to DNA, is a simplified method for quantifying DNA. Estimates of sensitivity for picogreen detection are as low as 25 pg/ml (411), and this approach has been used to quantify plasma DNA in clinical samples(362;412).

3.3.3 Quantification of plasma DNA in Prospective Cohort of Cases and Conrols

This study evaluated a prospective cohort of colorectal cancer cases and controls as to whether plasma DNA concentration was a marker of malignancy, from triplicate data derived using picogreen. A significantly higher mean concentration was demonstrated in cancer cases. Additionally using ROC analysis the area under the curve for cancer was 0.68.

Therefore using a cut off of 250 ng/ml, [plasma DNA] alone gave specificity of 93% with a sensitivity for cancer of 31.8 %. None of the patients with adenomas in this series had high plasma [DNA], although the numbers were small.

The performance of plasma [DNA] as a test to detect colorectal cancer is suboptimal, which is unsurprising since there is much that remains to be elucidated regarding the biology of tumour associated free circulating DNA. The majority of studies, whilst identifying a clear association of increased levels in cancer patients, have identified both cancer cases with low plasma [DNA] (comprimising sensitivity) and controls with raised plasma [DNA] (compromising specificity). Reduced specificity is a particular concern when assessing a test with potential utility for screening in asymptomatic populations, since false positive results require further expensive and potentially hazardous investigation. Therefore in proof of principle studies, controls must be carefully selected to exclude occult neoplasms of other organs that might also cause a raised plasma [DNA]. To this end controls were ascertained from normal colonoscopies from patients with a history of rectal bleeding or a mild to moderate genetic risk of colorectal cancer, as well as from healthy asymptomatic volunteers. Despite the careful selection of controls one was subsequently shown to have lymphoma and hence were excluded from the control cohort, although interestingly had a markedly raised [plasma DNA] (1142 ng/ml). Even given this exclusion and rigorous, uniform collection and processing of samples some control subjects had high levels of plasma [DNA] detected. These findings are in keeping with results of other studies that suggest raised levels are not specific to cancer. However in our study population very high (> 325 ng/ml) plasma [DNA] was specific to cancer. Importantly these high levels were seen in cases including earliest stage cancer (Dukes A).

The figures for test accuracy demonstrate that plasma [DNA] is unlikely to be of clinical utility in isolation. However current non-invasive tests such as FOBT have a sensitivity of

only around 50% (134), and constitute the basis for population screening. FOBT is based on different biological principles than plasma [DNA] and therefore the tests might prove complimentary. Setting specificity of plasma [DNA] at the same level reported for FOBT (around 90-100% (124;125;413), sensitivity from our data is 26.1-31.5%. Potentially therefore addition of plasma [DNA] assessment to FOBT might detect a quarter of the cancers missed due to the poor sensitivity of FOBT, although our results were not obtained from asymptomatic individuals. This hypothesis warrants further investigation, particularly in regard to the possible effect of the presence of benign colorectal and non colorectal conditions on plasma [DNA] that are prevalent in screened populations.

The relationship of plasma DNA concentration to survival is an important consideration for its utility as a clinical test. If there is potential to detect early stage disease that is curable by surgery, then the survival of cases with raised plasma DNA concentration might be equivalent to those with lower levels. In this cohort there was no significant difference in survival between those with plasma DNA levels above and below 250ng/ml. However there is a tendancy in many cancers for those with highest concentrations of circulating DNA to be associated with metastatic disease, therefore a subset analysis of cases with early stage disease was performed, again with no significant difference between those with plasma DNA levels above and below 250ng/ml. This would suggest that plasma DNA concentration has the ability to detect cases with truly early stage disease, and not simply those with more advanced disease that is not detectable by current staging modalities.

Conversely an increased hazard ratio of death with increased plasma DNA levels might suggest clinical understaging of disease. There was a non-significant trend to poorer survival of cases with high plasma DNA concentration both in the cohort as a whole and in those with earliest stage disease. The non-significance of this finding may be a reflection of insufficient sample size, particularly in respect to those with earlier stage cancers. Investigation on this

finding on larger cohorts of colorectal cancer patients would be worthwhile. Should this prove to be a significant association there might be potential utility for this biomarker in detecting patients who might benefit from further treatment. One potential application might be the identification of Dukes B cancer cases with a poorer prognosis who might therefore be considered for chemotherapy. Another potential application is highlighted by one case of particular interest, of a patient with a polyp cancer who had a markedly elevated plasma DNA prior to polypectomy. This patient went on to develop a late disseminated recurrence and died from the disease after 1808 days. Polyp cancers cause difficulties in management when deciding whether a patient should go onto colonic resection after complete endoscopic removal. Current pathological guidance is based on Kichuchi (414) or Haggitt (415) levels, but these methods have limitations. Radiological staging of colorectal cancer has suggested high sensitivity for distant metastasis but only around 85 % for lymphadenopathy in relation to rectal cancer (416). Therefore understaging of disease using these methods is a clinical problem. This is an increasingly common dilemma since the onset of FOBT screening for colorectal cancer over 15% of screen detected cancers are polyp cancers (417). A biomarker with the ability to infer the presence of residual disease would be of great benefit.

CHAPTER 4

DEVELOPMENT OF ASSAYS TO DETECT TUMOUR SPECIFIC MUTATIONS IN PLASMA

4.1 Introduction

Having investigated total DNA quantity in plasma and its associations in chapter 3, I next looked to develop assays aimed at detecting tumour specific mutations in plasma, namely a TGFB poly A tract length variation and a mutational hotspot in codon 12 of the k-ras gene.

4.1.1 TGFB

TGF-β is an important growth factor implicated in carcinogenesis as discussed in section 1.2.6 e. TheTGF-β receptor II is integral to signal transduction, and contains a polyA tract (A_{10}), mutation which has been demonstrated to display tract instability in 90% of MSI colorectal cancer (110). The ability to detect tumour-derived mutations of the polyA tract in plasma might act as a marker of MSI colorectal cancer.

Methods have been described that allow detection of a one base pair deletion within the polyA tract, employing a RFLPassay, demonstrated diagrammatically on page**(394). This assay, confirmed by work within this group, is sensitive with ability to detect one mutant copy within a population of 10^3 normal copies (392;394). This assay was therefore assessed for its ability to detect mutant sequences in plasma DNA. Additionally the use of highly specific real-time PCR probes (Taqman) might allow for mutation detection without post-amplification processing, hence simplifying analysis and reducing the opportunity for contamination of products prior to analysis. High throughput systems are available for realtime PCR analysis, which might allow mutation detection on a large scale as would be required for population based applications of an assay, should polyA tract mutations in plasma be proven to be a reliable biomarker.

4.1.2 Kirsten ras gene

The Kirsten-ras gene has been repeatedly shown to be an important gene in carcinogenesis, as discussed in chapter 1. The gene is one of the most extensively investigated of those that have been shown to be mutated in colorectal cancer, with implications in colorectal cancer progression and prognosis.

An attractive element to studying the K-ras gene is that mutations are common within a mutational 'hotspot' at codons 12 and 13. Mutational frequency has been reported to be highest at codon 12 with the commonest mutation being GGT->GAT (glycine to aspartate) in 37.5% and GGT->GTT (glycine to valine) in 31.3% of one series (395). Hence mutation analysis can be performed by targeting assays to these sites. This has been repeatedly performed using a small panel of RFLP assays both in tumour tissue and a variety of body fluids. As such K-ras is the most extensively studied tumour derived mutation in circulating DNA. A number of small studies have demonstrated detectability of tumour derived K-ras mutations in plasma/serum of colorectal cancer cases as discussed in chapter 1. A larger study demonstrated a sensitivity of 76% (in those cases harbouring a k-ras mutation) along with an association of persisting plasma positivity of the assay with disease recurrence (282). A particular difficulty of RFLP methods to assess K-ras status is the need for multiple steps in performing the assay. This results in assessment being relatively laborious and time consuming, and increases the possibility of error by contamination.

An alternative approach is to employ real-time PCR technology that enables single tube reactions, along with the potential to automate the process on a large scale. This chapter describes experiments designed to develop real-time PCR methods for the assessment of K-ras mutations for application to plasma DNA.

4.2 Materials and methods

4.2.1 TGFB RFLP assay

Analysis was performed on a poly A tract of the TGFBRII gene as a paradigm for the analysis of microsatllite sequences in plasma from microsatellite unstable colorectal cancer patients as described in chapter 2. Positive controls, determination of sensitivity, study population, primers and reaction conditions.and analysis, as well as consideration ofTGFB Real Time PCR analysis,were performed as described in chapter 2.

4.2.3 K-ras

Mutation detection was performed at the mutational hotspot at exon 1, codon 12 of k-ras gene, using real time PCR as described in chapter 2. Positive controls, including cell culture and sequencingand real-time PCR, with both SYBR Green and Taqman probes were performed also as described in chapter 2.

4.3 Results

4.3.1 TGF RFLP

a) Assay Validation

Initial reactions revealed a product peak at 141 base pairs in both negative and positive control samples, but also a peak at approximately 118 base pairs, consistent with the position of the mutant digestion product in all samples including the no template control (Fig 4.1).

Fig 4.1

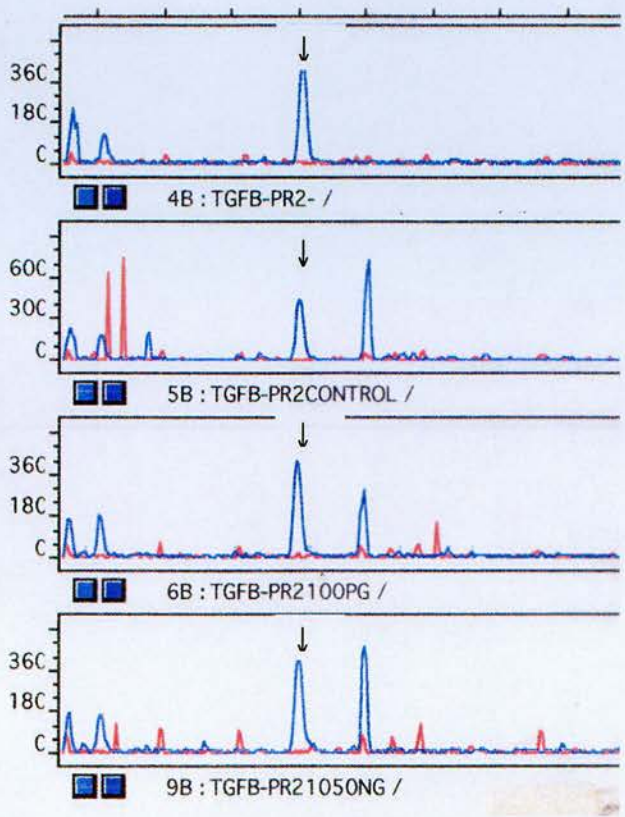


Figure 4.1 legend: electrophoretogram demonstrating the presence of a peak present in all samples around 118-120 base pairs.

These results suggested the possibility of contamination of samples with the mutant digest product. Therefore all reaction reagents, except primers were sequentially changed, but gave similar results with the peak at 118 remaining in all samples. Primers were reordered and reaction conditions replicated, however results remained similar. The peak at 118 base pairs was visible both prior to and after product digestion (Fig 4.2).

Fig 4.2:

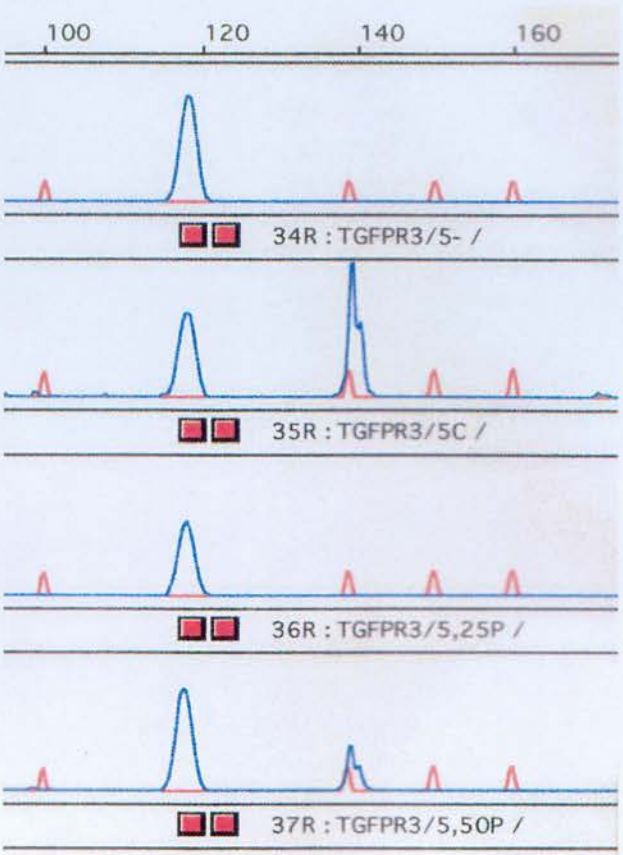


Figure 4.2 legend: electrophoretogram demonstrating the presence of a peak present in all samples around 118-120 base pairs.

To further determine whether the abnormal peak was due to contamination with Hinf digestion product or an artefact of the digestion process, all reagents except primers where

utilised to amplify a different marker, D17S250, and the products subjected to Hinf digestion. Using these conditions no peak was apparent at 118 base pairs (Fig 4.3).

Fig 4.3:

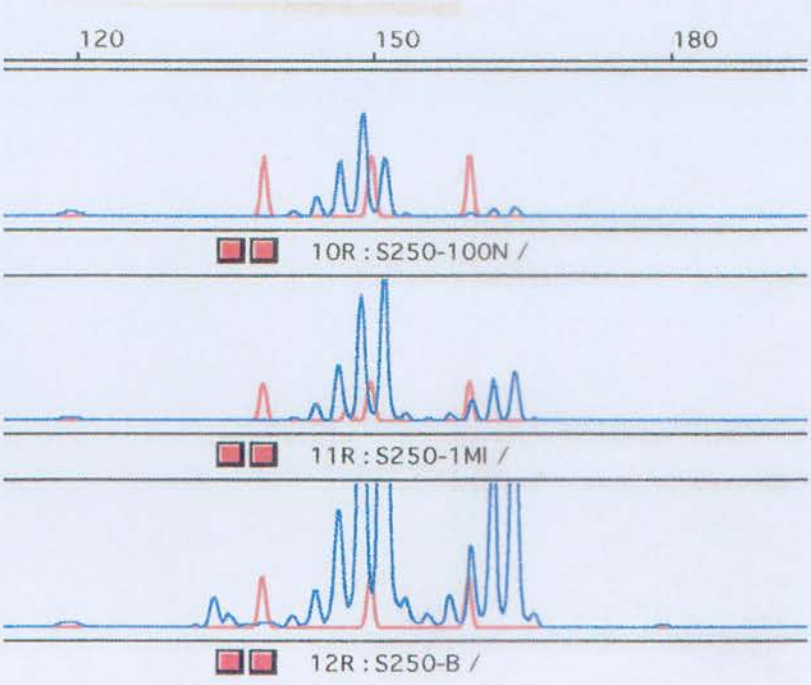


figure 4.3 legend: electrophoretogram demonstrating results of D17S250 PCR, with absence of a product peak between 118-120 bp.

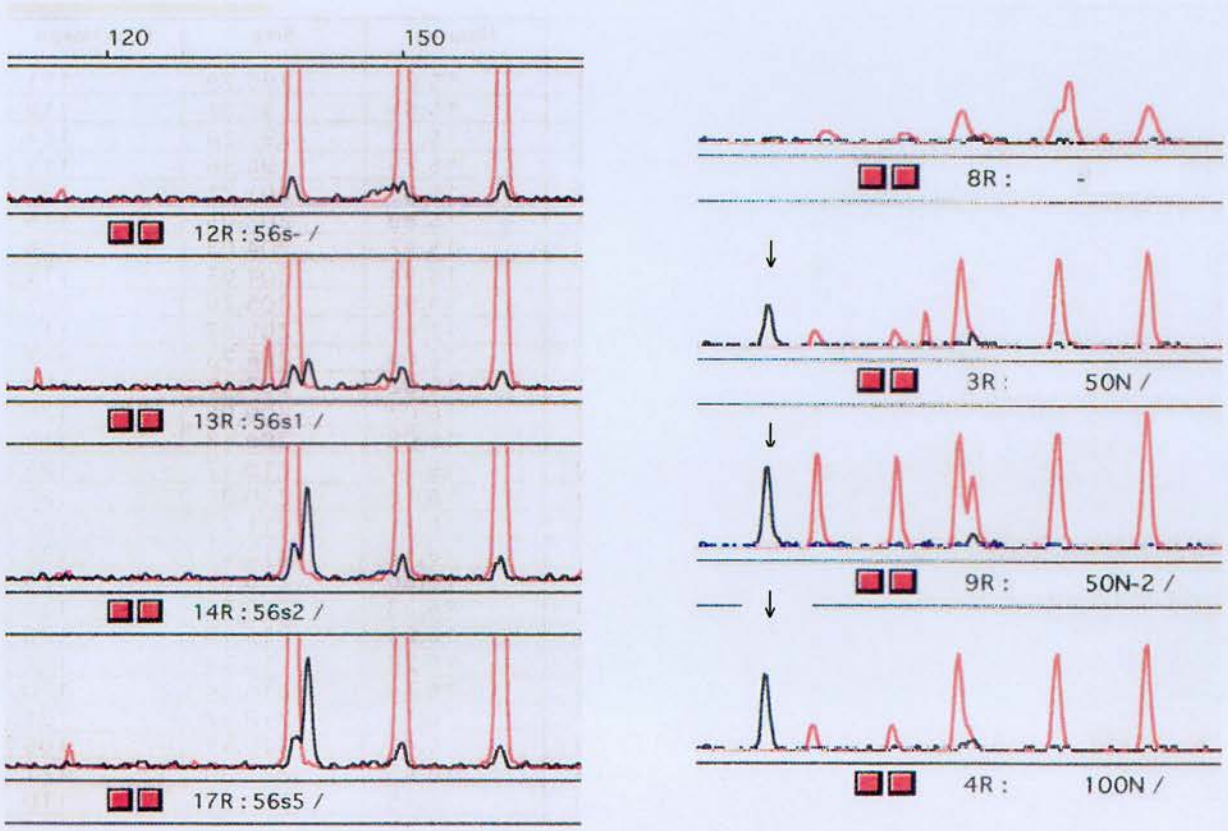
To address if the abnormal peak resulted from residual constituents of plasma, not removed during the extraction process, each of three extraction methods (chapter 3) were performed and compared. Qiagen extraction process was also repeated on previously extracted plasma DNA in an attempt to improve purity. Additionally fresh plasma samples were obtained and re-extracted in triplicate. Alternate stocks of LoVo DNA were used for spiking plasma, also in triplicate. Two sequential reagent changes for each of 3 different primer sets and 3 changes in stock of Hinf and related buffers were performed. Consultation with the primer manufacturing company (MWG) was carried out to check for a constitutive part of the primer

manufacture process that might result in the generation of the peak at 118 bp. Analysis by them concluded that only the designed oligonucleotides were present. Primers were also ordered from the company (Abgene) who had previously made primers when the assay had previously been performed by our group. PCR product was also cleaned by treatment with SAP/exonuclease. Each of the above steps resulted in similar results with a persistent peak at 118 base pairs in all samples.

In the absence of evidence of contamination given above the possibility of fluorescence artefact related to the FAM labelled forward primer was considered. It was apparent from the multiple repeated reactions and runs that the peak around 118 base pairs had different morphology to that of the undigested product peak at 141 base pairs, being broad based and not sharply defined at the apex. This possibility was addressed by changing the fluorescence label to HEX.

Demonstrated in figure 4.4 are undigested (left) and digested (right) positive control (LoVo) samples. There is no peak at 118 bp in the undigested samples, whereas all product has been digested to generate the 118 bp peak in the digested samples (arrowed) indicating the presence of only DNA with 1 bp deletion.

Figure 4.4:



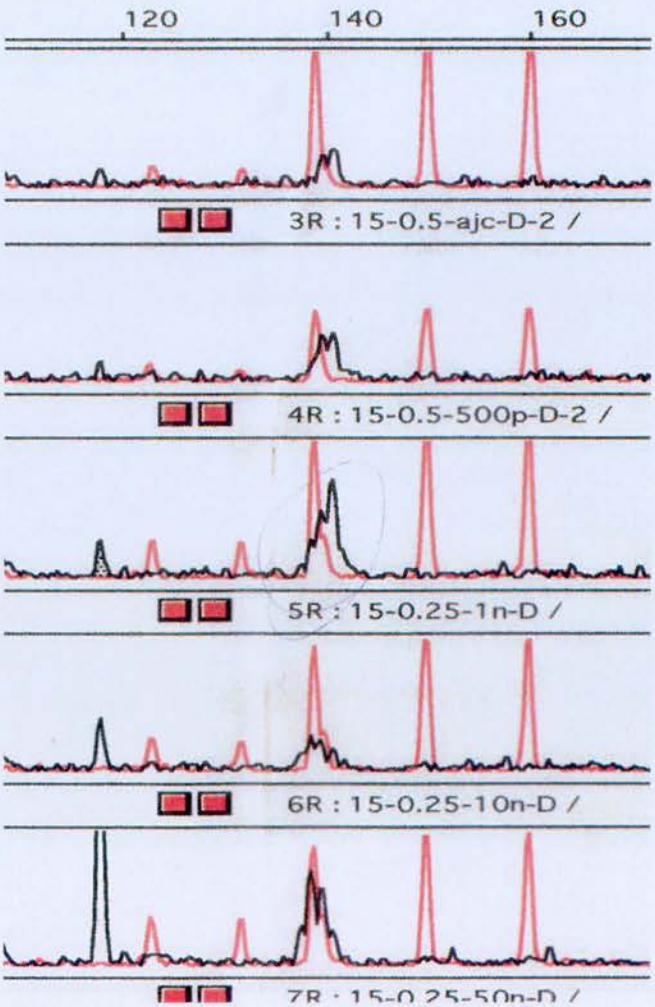
b) Determining Assay Resolution

Having ascertained that the assay gave reproducible results on positive and normal control DNA, the sensitivity of the assay for detecting mutant TGFBRII polyA tract in plasma was determined by spiking normal control plasma with serial dilutions of positive control (LoVo) DNA. LoVo DNA was added to control plasma at dilutions, described in materials and methods, consistent with levels described in the literature with respect to tumour derived DNA in plasma.

Application of the same reaction conditions as used in earlier TGFB assay experiments failed to yield PCR product demonstrable by either gel electrophoresis or fluorescence electrophoresis. Reaction conditions were therefore optimised with sequential alteration of annealing temperature (2 degree intervals) and cycle number. Discernable PCR product was reliably generated using conditions detailed in materials and methods. Annealing temperature was 58°c and cycle number 45.

The results of spiking experiments are shown in figure 4.5. A peak was visible at 141 bp in all undigested samples (left column), except the no template control (not shown), corresponding to the undigested PCR product. Digestion resulted in generation of a 118 bp peak, corresponding to digested mutant PCR product in samples spiked with greater than 1ng of LoVo DNA per ml of plasma. There was not an obviously discernable 118 bp peak in normal control sample, or with spiking below 1ng/ml (e.g.500pg/ml). These experiments suggest that this assay is able to detect down to 1ng of mutant TGFBRII DNA in 1ml that has been artificially introduced into plasma.

Fig 4.5:



c) Clinical samples

Having demonstrated that the TGFBRII assay was functioning, and was sensitive to a level relevant to tumour derived DNA in plasma, the assay was applied to patient and control plasma samples. Three healthy volunteers were used for controls. Patient population 1 constituted patients previously enrolled in genetic studies of the group, and whose tumour microsatellite status had previously been determined using standard methods. Patients with metastatic disease were selected, since stored plasma samples were taken post-operatively, and only those with residual disease could be assumed to have tumour derived DNA within

plasma post-operatively. Archived plasma samples were obtained for patient population 1, and DNA extracted from 200ul of plasma as per the Qiagen protocol.

Application of optimised reaction conditions from spiking experiments, generated a mutant peak following digestion in all plasma DNA samples except from the normal control subjects. This was the case for both the MSI case (1092) and all the MSS cases (n=9), with examples given in figure 4.6.

Figure 4.6:

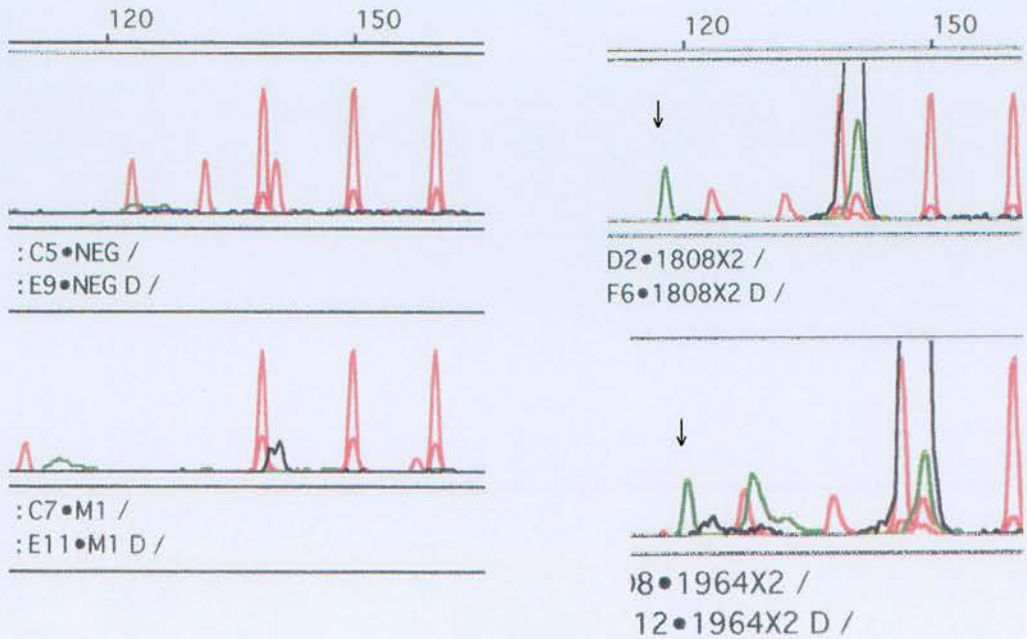


Figure 4.6 legend: Electrophoretograms from digested PCR product from negative control (top left), normal control (bottom left) and 2 stage 4 colon cancer cases plasma DNA (right). Mutant digest product (118 bp) is shown in green and arrowed.

All MSS cases had been designated MSS by microsatellite analysis with Bethsada markers, but TGFBRII polyA tract assessment was performed on these tumour samples, to exclude the

presence of a 1bp deletion in the primary tumour that might also be detectable in plasma. Assessment was performed at annealing temperature of 58 oC, and 35 cycles. Each MSS tumour sample did not demonstrate a peak consistent with digested mutant polyA tract, suggesting that the mutant peak in plasma DNA was due to artifact. Further optimisation of the plasma DNA amplification protocol was performed to try and improve the integrity of PCR products. High fidelity taq was substituted for standard Taq polymerase, but each MSS plasma DNA continued to generate a mutant peak at 118bp.

A semi-nested PCR method was therefore devised, as described in materials and methods. The number of cycles in both primary and secondary PCR were sequentially altered, as were the concentrations of the outside primers to optimise this process. However each combination resulted in a mutant peak at 118 bp being present. Reducing the cycle number resulted in neither wild-type nor mutant product being reliably detectable.

4.3.2 TGFB Real Time PCR

Taqman probes were designed according to manufacturer instructions (AppliedBiosysytems) for each of wild type (A10) and mutant (A9) TGFBRII sequences, as described in materials and methods. Submission of these sequences to Applied Biosystems led technical support to conclude that the ABI 7900 sequence detection platform was unable to discriminate a single base pair deletion with an A10 polyA tract, therefore these probes were not purchased.

4.3.3 K-ras SYBR Green Assays

a) Controls

Colo 320 cells were raised from storage and DNA extracted. The presence of PCR ready DNA for each positive and negative control template was confirmed by standard PCR cycling and conditions using primers used in microsatellite analysis for the marker D5S346:

Figure 4.7:

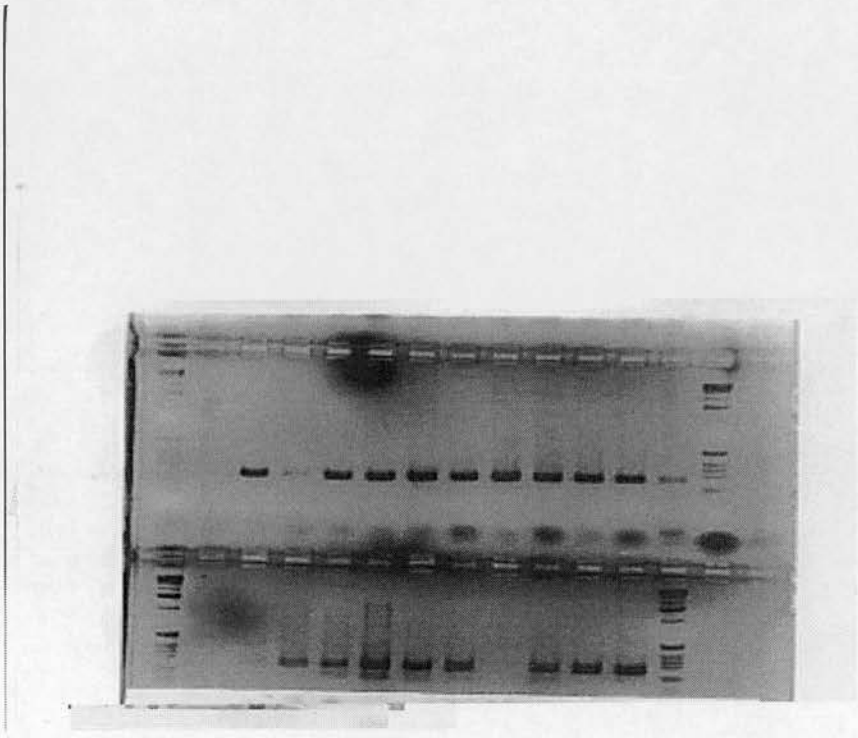


Figure 4.7 legend: Gel electrophoresis confirming PCR product for templates used in subsequent reactions. First and last columns of each row contain kilobase ladder. Column 2 of both rows contains no-template control. Thereafter triplicate results for templates : Colo 320, SW480, normal DNA from case 2530, tumour DNA from case AM 17, tumour DNA from case AM 35, tumour DNA from case 1851, tumour DNA from case 2487.

b) Primers and Probes

Sequence specific primers were designed according to manufacturer instructions for wild type and aspartate mutant K-ras sequences, yielding the following results:

Wt ras forward: ATA TAA ACT TGT GGT AGT TGG AGC TGG

Aspartate mutant: GAA TAT AAA CTT GTG GTA GTT GGA GCT GA

Common reverse TGG ATC ATA TTC GTC CAC AAA ATG

c) Assay Validation

Real-time PCR was employed using cycling described using positive and negative controls. This demonstrated no difference in Ct for the two primer sets on each of the positive and negative control samples.

The approach was adapted in an attempt to improve primer specificity by introducing a base mismatch adjacent to the mutation site for the primer pairs:

Wt ras wobble forward: ATA TAA ACT TGT GGT AGT TGG AGC AGG

Aspartate mutant wobble: GAA TAT AAA CTT GTG GTA GTT GGA GCA GA

Common reverse: TGG ATC ATA TTC GTC CAC AAA ATG

Standard PCR cycling using this primer set on postive and negative controls failed to result in successful PCR with no substrates crossing threshold upto 40 cycles, shown in figure 4.8.

Figure 4.8:

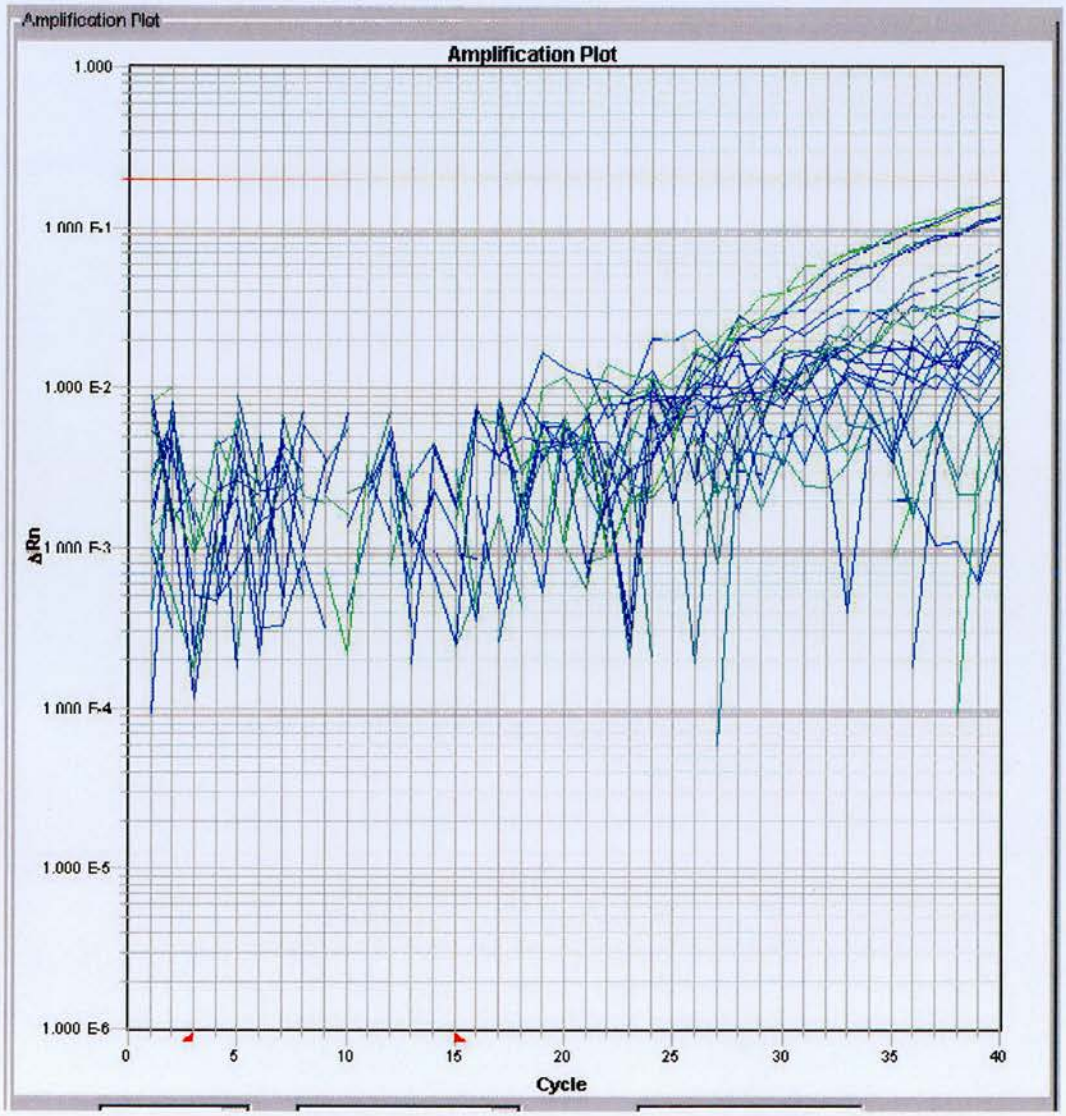


Figure 4.8 legend:

Real-time PCR amplification plot using SYBR green technology with K-ras aspartate mutant and wild type specific 'wobble' primers. By 40 cycles none of the wells have generated sufficient PCR product to cross the threshold, designated by the red line.

In view of the non-discrimination of this assay, the positive and negative controls were sequenced as described. Sequencing revealed that the colo 320 cell line DNA lacked the published (393) aspartate mutation, shown in figure 4.9. However cases AM17 and AM35 tumour DNA contained the aspartate mutation as previously established by work within the group, therefore positive and negative controls were present in the experiments described above.

Figure 4.9:

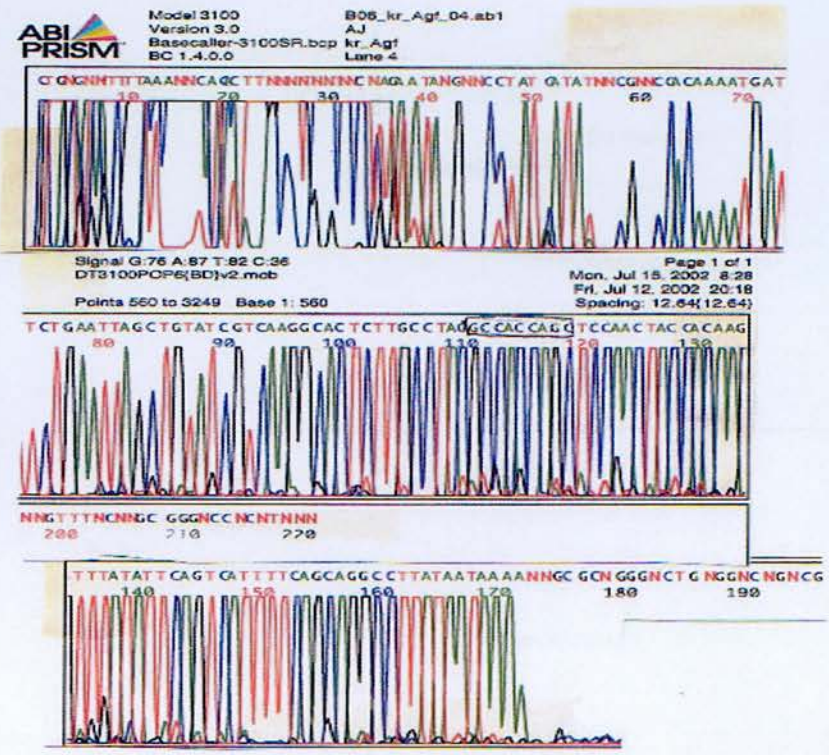


Figure 4.9 legend: Results from sequencing DNA from colo 320 cell line. Sequencing performed on the antisense strand. Demonstrated in the hand drawn box in row 2 the sequence confers to (from right to left) translation on the sense strand as GCT GGT which is wild type sequence.

4.3.4 K ras Taqman Probes

In view of the failure of the SYBR green approach to discriminate between templates it was elected to design a taqman probe assay to the mutation site. Primers and probes were designed according to manufacturers instructions and generated the following sequences:

Wild type probe (antisense strand)	CCT ACG CCA **C**CA GCT
Aspartate mutant probe (antisense strand)	CCT ACG CCA **T**CA GC
Forward primer (antisense)	TAG CTG TAT CGT CAA GGC ACT CTT
Reverse primer (sense)	AAA ATG ACT GAA TAT AAA CTT GTG GTA GTT G

Standard cycling conditions were employed to assess the same positive and negative controls as used for SYBR green experiments. These results are demonstrated in figure 4.10. There was no discrimination by fluorescence between positive and negative controls.

Figure 4.10

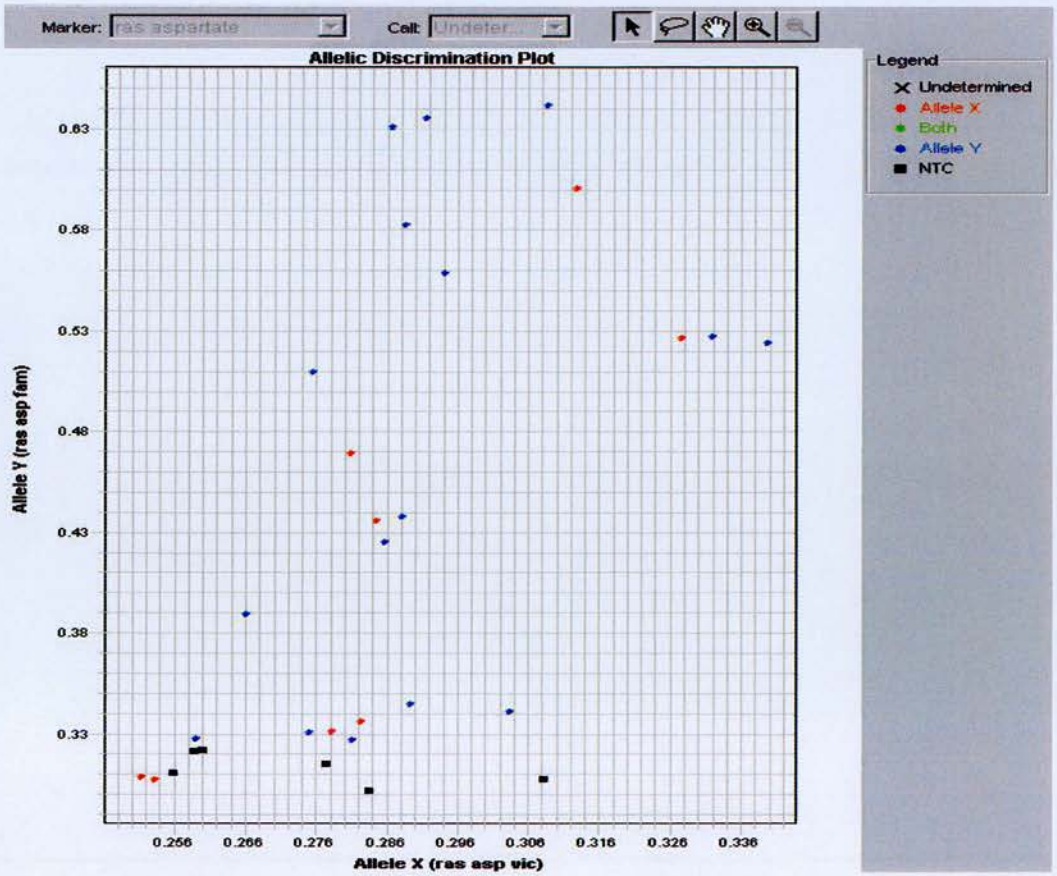


Figure 4.10 legend:

Allelic discrimination plot for K-ras MGB probes for positive and negative controls for aspartate mutant K-ras. FAM fluorescence, corresponding to the wild type probe, is presented on the y axis. VIC fluorescence, corresponding to the mutant probe, is presented on the x axis. Results from positive controls are depicted in red. Results from negative controls are depicted in blue. No template controls are depicted in black.

4.4 Discussion

4.4.1 TGFB

The experiments in this chapter assessed the detectability of mutant TGFBRII DNA in plasma with a view to use as a biomarker of microsatellite unstable colorectal cancer. To this end a previously described RFLP assay was employed, but considerable difficulties were encountered in ensuring the assay functioned reproducibly. Systematic alteration of assay reagents were required to identify an inherent problem with the fluorescent labelling of the assay primers, which when altered allowed reproducible assay results. These difficulties highlight the importance of validating published methods in the context to which they are to be applied before utilising the irreplaceable biological resource of patient samples.

Having validated the assay, its sensitivity at detecting mutant polyA tract within plasma was assessed by artificially introducing mutant DNA into control plasma. These experiments appeared to indicate that the assay was sensitive at a level of detectability of 1 ng of mutant DNA per ml of plasma. This level of sensitivity is of relevance to published data on the quantity of tumour derived mutant DNA within the plasma of cancer patients. However the method of fluorescence electrophoresis generates an uneven baseline, and close examination revealed a very small peak at 118bp, not sufficiently discernable from baseline noise to call as a positive result, that might represent a very small number of amplified mutant copies. This is important when considering the results derived from patient plasma samples, where in both microsatellite stable and unstable cases generated a positive result for mutant poly A tract was generated. Analysis of MSS tumour samples did not demonstrate a mutant digest peak indicating a deletion in the tumour and therefore suggesting that the positive plasma results might be an artefact of the amplification process. A likely explanation of these results

is that the large number of amplification cycles resulted in an induced artefactual one bp deletion in the wild type poly A tract as a result of polymerase stutter. This artefact would result in all samples being positive to some degree. In plasma samples with larger amounts of plasma DNA the quantities of PCR product might be such that both wild type and artefactual mutant products are detectable. In samples with small amounts of plasma DNA, such as normal controls and those with little mutant DNA (e.g.500pg), the quantities of PCR product are not sufficiently large for the mutant peak to be reliably detectable above baseline.

Attempts to improve the integrity of the amplification process to negate the above explanation included the use of high fidelity polymerase and the devlopment of a semi-nested PCR approach. These alterations do not negate the production of artefactual A9 polyA tract. Reduction of the number of PCR cycles resulted in insufficient PCR product for reliable detection.

These results highlight difficulties of investigating plasma DNA. Quantities of extracted DNA are small, and this in combination with the potential presence of factors affecting the PCR process that are not removed in the plasma DNA extraction process, necessitate an extended number of cycles to reliably detect PCR product. However increased number of PCR cycles compromises the fidelity of template DNA amplification, and results in generation of artefactual sequence errors. This can be expected to be a problem particularly for repetitive microsatellite tracts.

In summary these results highlight the difficulties of investigating plasma DNA, and demonstrate that the use of this RFLP assay is not sufficiently robust to detect mutant plasma TGFBRII polyA tract with a view to its use as a biomarker.

Since this work was completed a group have described an alternative method for detecting polyA tract alterations. They addressed the possibility of polymerase slippage resulting in

artefactual mutant sequences in polyA tracts by developing a so called probe-clamping primer extension PCR (PCPE-PCR), applicable to TGFBRII (418). This technique employs specific probes that preferentially produce extension products of mutant DNA, which are then extracted and hence enriched before further amplification and then analysis. Their results suggested high sensitivity with ability to detect 1 mutant copy amongst 500 normal alleles and to detect down to 3 mutant copies. Additionally the technique had some success in detecting BAT-26 mutations in stool of a small cohort of colorectal cancer cases (n=6). Assessment of this technique in relation to plasma DNA might address some of the difficulties described above.

4.4.2 K-ras

Assessment of K-ras mutation has been commonly performed in circulating DNA, and has been shown to be reproducibly demonstrable using RFLP assays. The development of a single tube assay with the potential to be performed on a large scale might facilitate the translation of circulating DNA K-ras assessment into clinically useful tests. To this end the experiments described in this chapter were aimed to develop a real-time PCR assay able to detect K-ras mutations.

Initial negative results might have resulted from lack of a true positive control. Whilst it was demonstrated by direct sequencing that DNA from the cancer cell line colo320 lacked the suggested mutation, alternative true positive controls were used, those of tumour tissue from cases archived from a previous study within the group, that had previously been demonstrated to harbour the codon 12 mutation, and confirmed again by direct sequencing.

SYBR green assay technology is a simple and relatively inexpensive form of real-time PCR assessment as described in materials and methods. Primers specific to both wild type and mutant sequences were designed, but proved to have significant cross-reactivity, with similar signal being generated on control and mutant template with either primer set. In order to try

to improve primer specificity a base mismatch was introduced one base from the mutation site, so that without the primer matching the mutation site primer annealing would not occur. This however resulted in no appreciable product occurring even in the presence of the matching template, either control or mutant.

Real-time PCR with Taqman probes potentially provides an increased assay specificity. The development of probes with a stabilising adjunct that binds in the minor groove of DNA (MGB probes) has allowed further probe specificity and reduction in probe length. Therefore Taqman MGB probes specific to the aspartate codon 12 mutation were designed and tested on mutant and control DNA. The results presented with respect to these probes indicate that it was not possible to clearly discriminate mutant from wild type DNA. Whilst there was an indication of differing fluorescence between templates, the inability to clearly discriminate large quantities of intact mutant tumour DNA from control suggest that this approach will not translate to low copy number mutant templates with admixed normal DNA. It therefore appears that the successful discrimination reported using RFLP assays is at least partly dependent on steps that result in enrichment and preferential amplification of mutant DNA. Additionally the translation of K-ras mutant assays for colorectal cancer detection is strategically flawed, since only a proportion of colorectal cancers exhibit these mutations. Given that approximately 40-50 % of colorectal cancers exhibit K-ras mutations (47), with our own studies suggesting an even lower figure in the Scottish population (~ 30%), even an assay with optimal performance would have potential to detect under half of colorectal cancers. Inclusion of K-ras assays within a panel of markers might also have inherent problems as has been found using a similar approach in stool (210), where inclusion of ras mutations resulted in decreased panel specificity. Given these limitations this approach was not explored

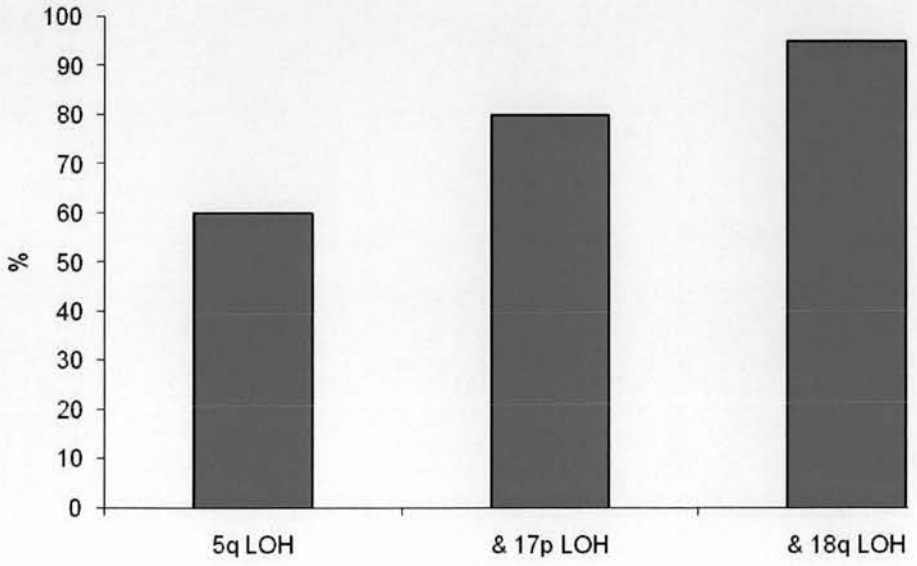
CHAPTER 5

FLUORESCENT MICROSATELLITE ANALYSIS

5.1 Introduction

Analysis of polymorphic microsatellites is a common technique in the genetic assessment of tumours, both for assessment of LOH and microsatellite instability. LOH is a defining molecular characteristic of many human cancers and hence is a common phenomenon at multiple loci(47). Information on LOH derived from microsatellite analysis has been shown to be relevant to the phenotypic characteristics of many cancers, including having prognostic relevance(419).

LOH is frequently observed at multiple sites in colorectal cancer, as discussed in section 1.2.2. Analysis of LOH at a panel of markers can strategicallybe informative for all sporadic colorectal cancers as highlighted by the graph below showing LOH frequency at 3 loci in colorectal cancer.



This graph shows the cumulative frequency of LOH at 3 commonly affected sites in colorectal cancer. Approximately 60% of colorectal cancers exhibit LOH at markers on chromosome 5q(85), demonstrated in the left hand column. A similar proportion exhibit LOH at markers on chromosome 17p(99), therefore around 80% of cancers would be expected to demonstrate evidence of LOH at either 5q or 17p, indicated in the middle column. Around 75% of colorectal cancers exhibit LOH at markers on chromosome 18q(102), therefore 95% of cancers may exhibit LOH at one of the three markers, assuming independent segregation, indicated in the right hand column. However since the completion of this thesis further refinement of the molecular classes of colorectal cancer (as discussed in section 1.2.6), has suggested a subclass of cases that would not potentially be detected by this approach, in relation to BRAF mutated cancers , that may constitute upto 20% of all sporadic colorectal cancers.

Assessment of LOH in plasma DNA using microsatellite analysis has been performed for many cancers with varying results, as discussed in section 1.5.2 d. The advantage of this approach in colorectal cancer is the potential informativity for the great majority of cases using a small panel of markers based on the same principle, which are relatively easy to analyse. With respect to microsatellite analysis of plasma DNA in colorectal cancer, 2 small studies have generated conflicting results(335;336). One of the suggested explanations of suboptimal and conflicting results in the analysis of LOH in plasma is the low copy number of DNA within samples.

There are numerous techniques for examining microsatellites, however a commonly employed method is the use of fluorescently labelled primers with capillary electrophoresis of the resultant fluorescently labelled PCR products, generating visual representation of product size and amplitude with the use of designated platforms with attendant analysis software.

This study aimed to perform fluorescent microsatellite analysis at a panel of 3 microsatellite markers on a larger cohort than previous studies of matched tumour, normal and plasma DNA, as well as control subjects. Additonally results were correlated with total plasma DNA concentration derived from experiments in chapter 3.

5.2 Materials and Methods

5.2.1 Study Population

Cases and controls were ascertained from the series of prospectively recruited patients described in section 2.2.

In total 85 cases and 24 controls were analysed.

Cases included all disease stages as indicated in table 5.1

Table 5.1

Controls	Adenomas	Stage I	Stage II	Stage III	Stage IV	Unknown (Transanal excision)
24	8	13	28	26	15	1

Controls were those normal individuals as described in chapter 2.

5.2.2 Reaction Conditions

The reaction conditions at the three chosen markers, along with the primers, constituents and methods of analysis are decribed in section 2.6.

5.3 Results

A total of 6 cases and 1 control were not fully analysed due to insufficient clinical specimens, giving full results for 77 cancer cases and 8 adenomas. Of the 23 controls, one was subsequently shown to have a haematogical malignancy and was excluded from further analysis, giving results for 22 controls.

5.3.1 Tumour DNA

Results for each marker are given in table 5.2 below with the number of cases with allelic loss.

Table 5.2:

	D5	D5	D17	D17	D18	D18
	Tumour	Adenoma	Tumour	Adenoma	Tumour	Adenoma
Non - informative	11	4	9	3	23	4
MSI	2	0	1	0	5	0
LOH	40% n=26	50% n=2	33.8% n=23	0.0%	44.0% n=22	50.0% n=2

36 cases (42.4%) of cases were either non-informative or showed no LOH at all three markers in tumour DNA. Therefore 49 cases (57.6%- 46 cancers and 3 adenomas)

were informative, with allelic alteration at one marker in 30.2%, at 2 markers in 16.3% and at all 3 markers in 10.5% of cases.

5.3.2 Plasma DNA

LOH was classified according to the allelic pattern change as follows:

1) Matching LOH in tumour and plasma DNA (Figs 5.1 and 5.2)

Fig 5.1:

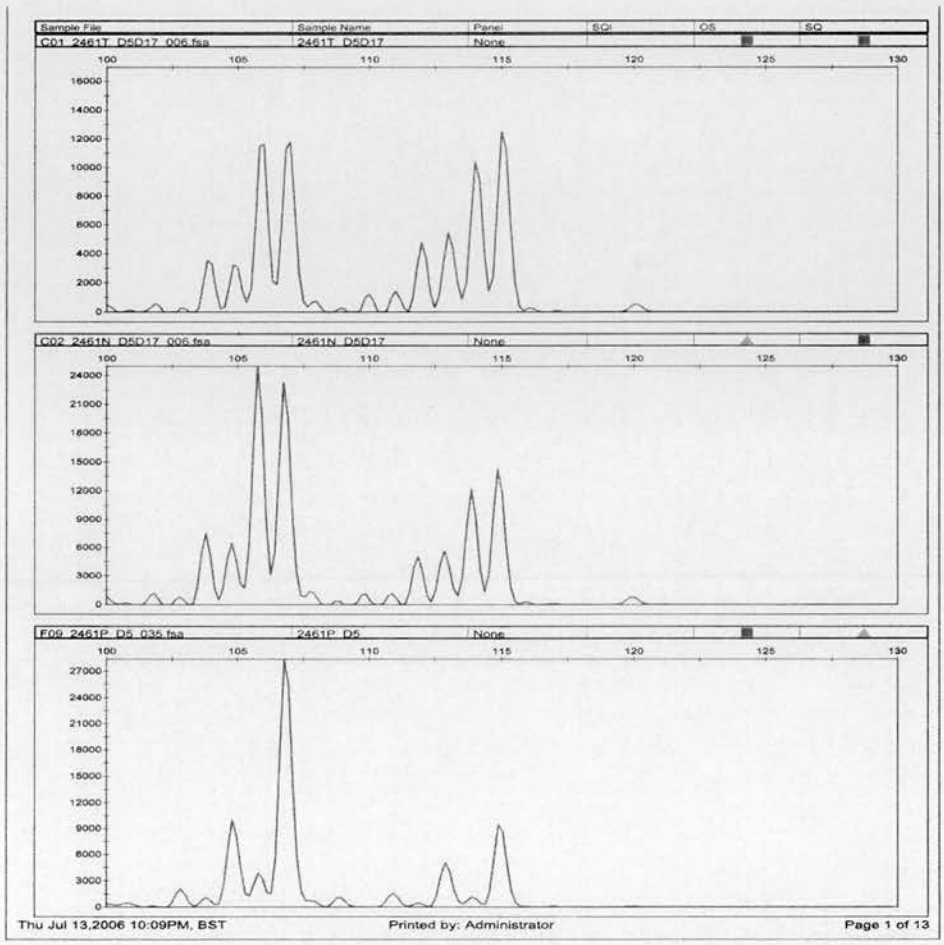


Fig 5.1 legend: Results from D5S346 for case 2461. The top row indicates normal DNA. Tumour DNA shows a relative loss of the larger allele, demonstrated in the middle row. Plasma DNA (bottom row) shows matching allelic loss to that seen in the tumour.

Fig 5.2

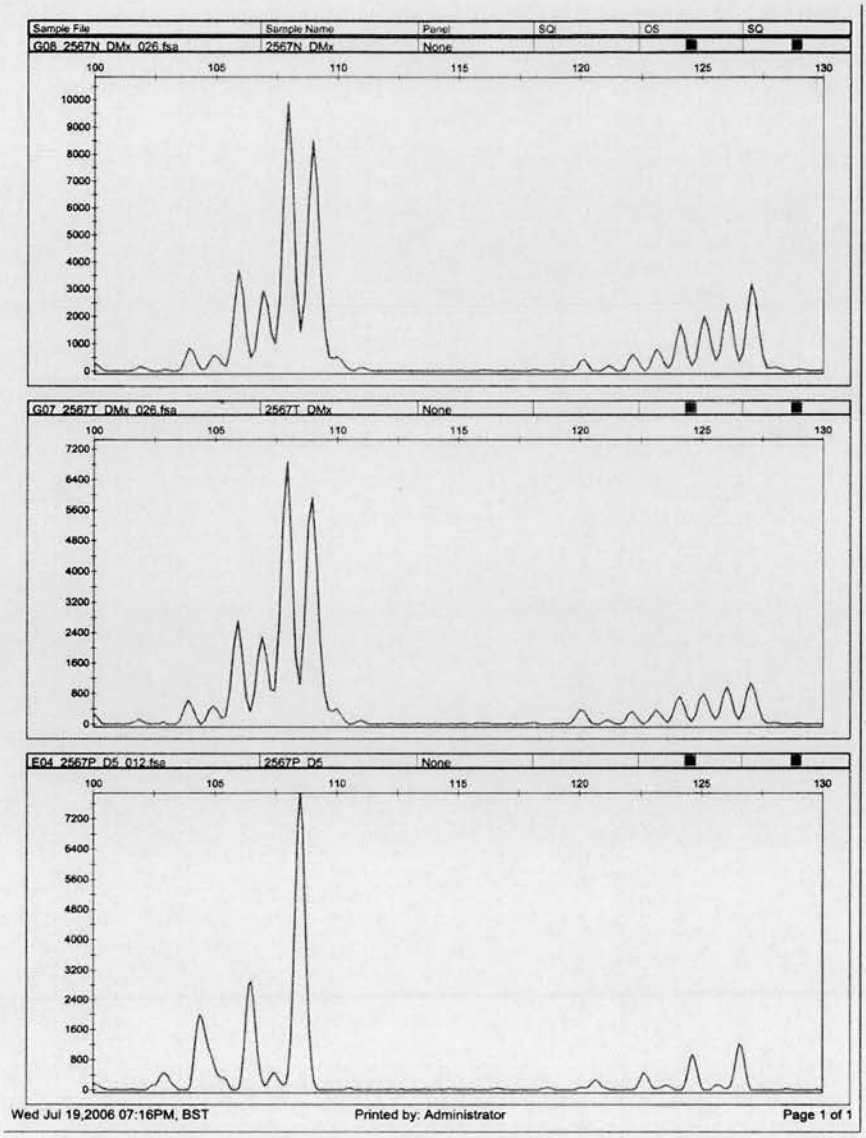


Fig 5.2 legend: Results from D5S346 for case 2567. The top row indicates normal DNA. Tumour DNA shows a relative loss of the larger allele, demonstrated in the middle row. Plasma DNA (bottom row) shows matching allelic loss to that seen in the tumour.

2) Non matching LOH: Disparate LOH with one allele having reduced signal amplitude in tumour but the other allele having reduced signal amplitude in plasma DNA (fig 5.3)

Fig 5.3:

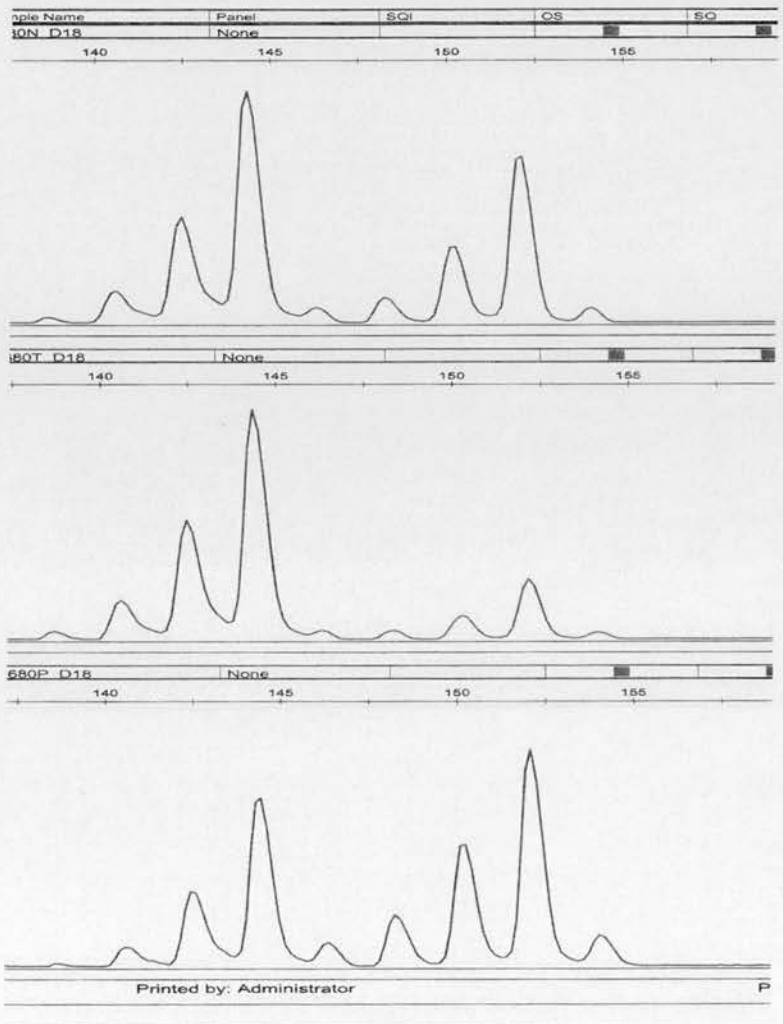


Fig 5.3 Legend: Results from D18S58 for case 2530. The top row indicates normal DNA. Tumour DNA shows a relative loss of the larger allele, demonstrated in the middle row. Plasma DNA (bottom row) shows opposing allelic loss to that seen in the tumour, with a relative loss of the smaller allele.

3) No LOH: Allelic balance maintained in plasma DNA, irrespective of allelic status in tumour (Fig 5.4).

Fig 5.4:

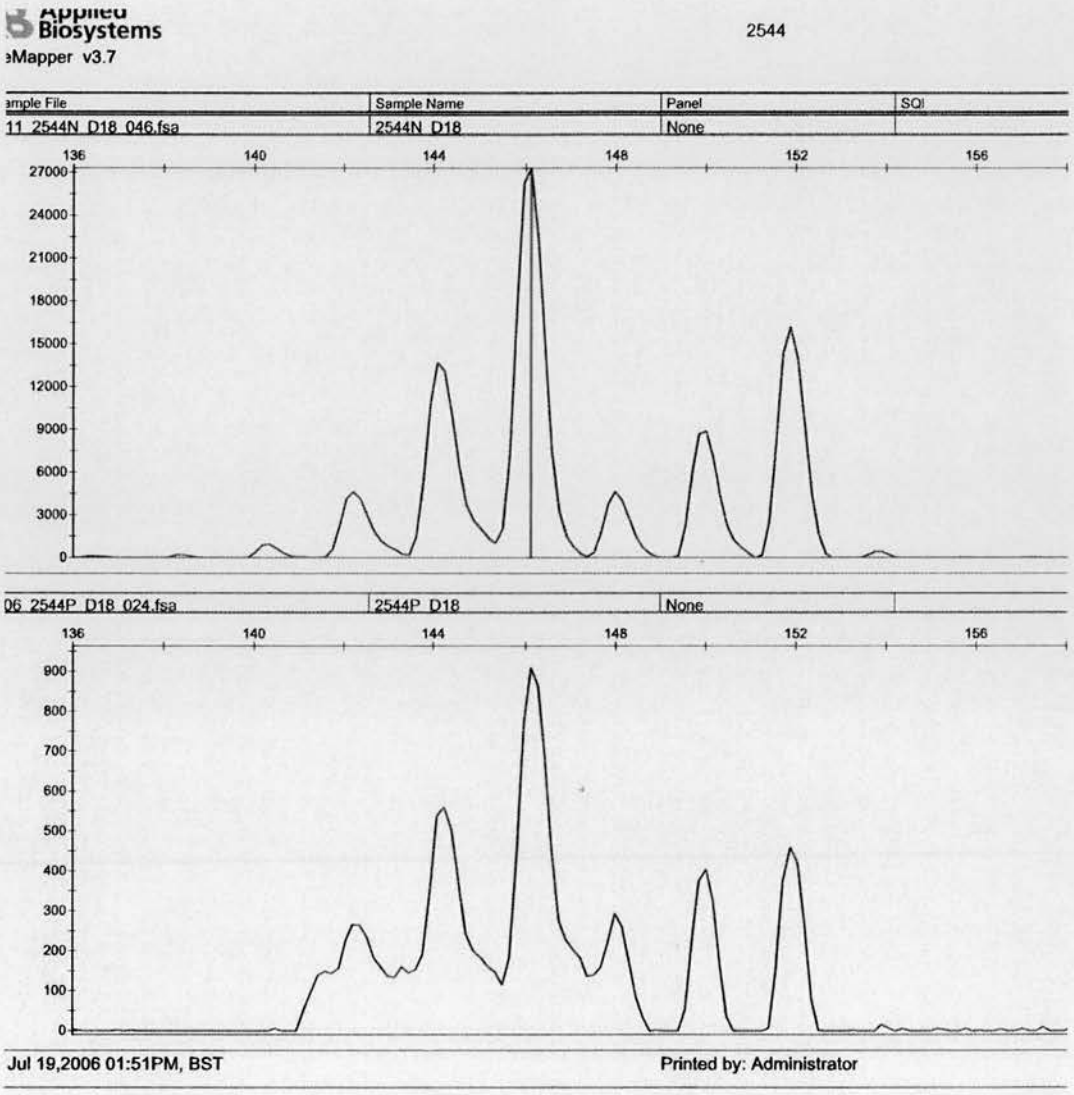


Fig 5.4 Legend: Results from D18S58 for control 2544. The top row indicates normal DNA. Plasma DNA (bottom row) shows maintained allelic balance

4) De novo LOH: AI observed in plasma for control subjects or where not present in tumour DNA for cases. (Fig 5.5)

Fig 5.5:

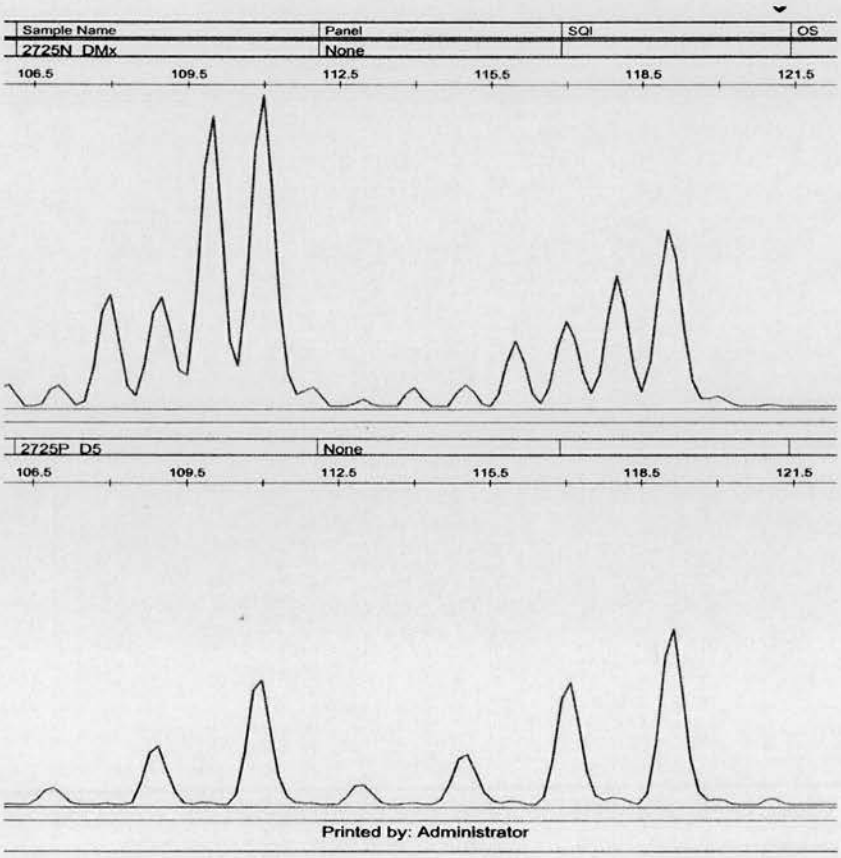


Fig 5.5 Legend: Results from D5S346 for control 2725. The top row indicates normal DNA. Plasma DNA (bottom row) shows unexpected reduction in the signal amplitude of the smaller allele.

5) Complete LOH: Absence of one of the alleles in plasma DNA that was present in both tumour and normal DNA (Figs 5.6 and 5.7)

Figure 5.6

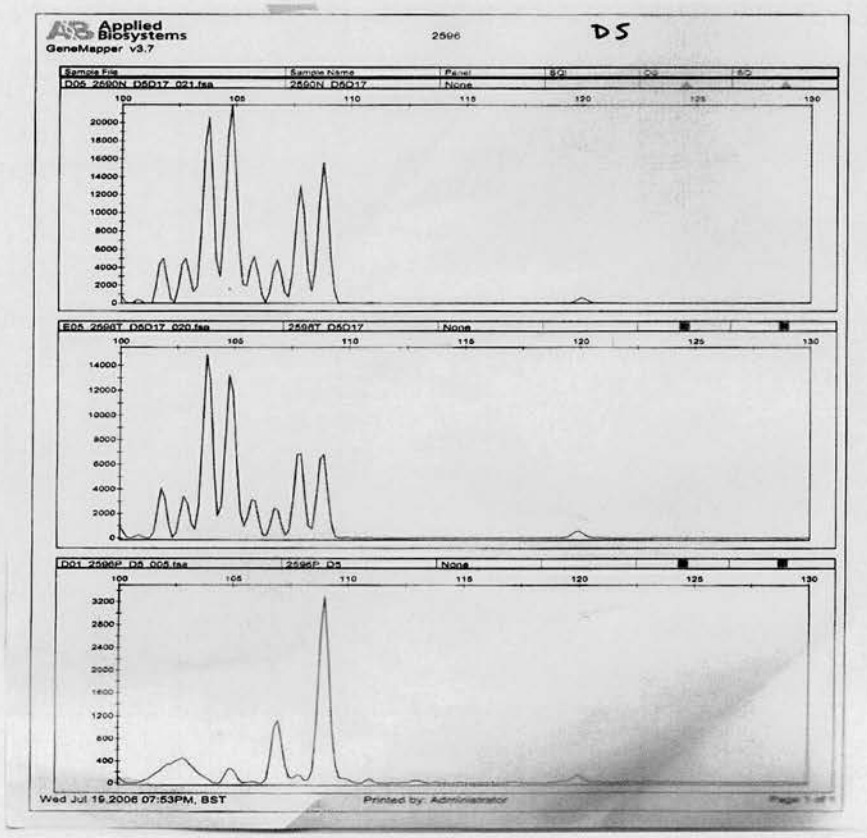


Figure 5.6 legend: Results for D5S346 for case 2596. The smaller allele at 104-105 bp is absent from plasma DNA

Figure 5.7

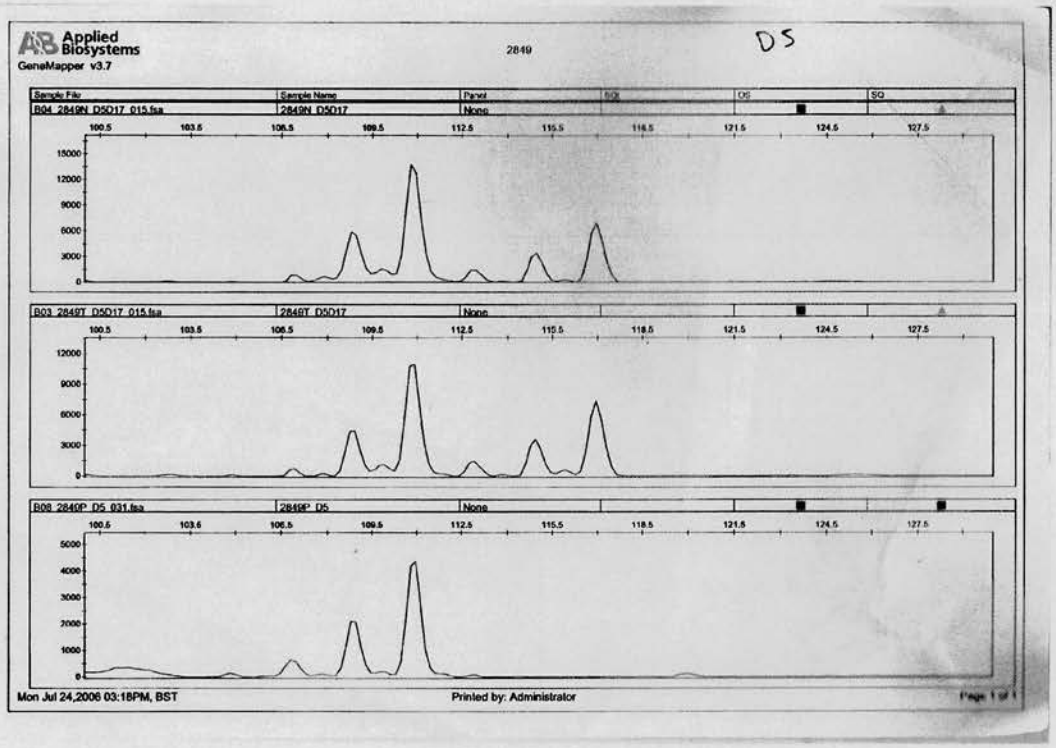
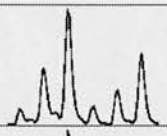
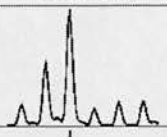
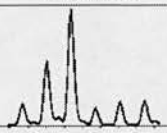
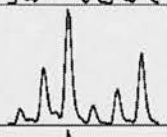
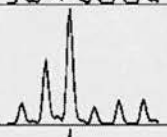
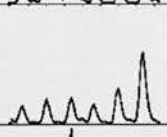
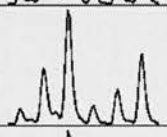
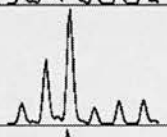
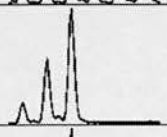
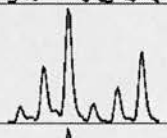
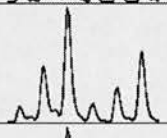
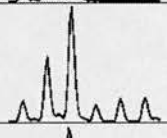
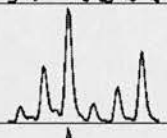
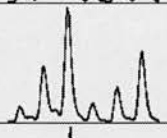
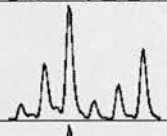
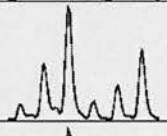
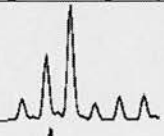
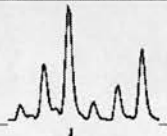
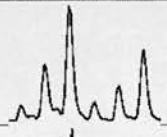
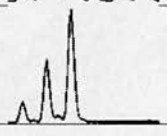
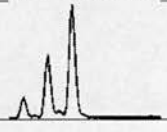


Figure 5.7 legend: Results for D5S346 for case 2849. The larger allele at 117 bp is absent from plasma DNA.

A summary of the types of LOH observed is provided in figure 5.8.

Figure 5.8:

Outcome	Normal	Tumour	Plasma	**Significance**
LOH (matching)				True positive
LOH (mismatching)				Depends on criteria
LOH (complete)				?Artefact
LOH (*de novo*)				False positive
No LOH (matching)				True negative
No LOH (mismatching)				False negative
Microsatellite unstable				Microsatellite instability
Homozygous				Non-informative

Primary data for all analysed samples is presented in appendix 4.

Summary results from analysis of matched plasma samples for cases are demonstrated in table 5.3.

Table 5.3:

	D5	***D17***	D18	Any of the 3 markers
Not informative	15	***11***	26	36
MSI (tumour)	2	***1***	5	5
Tumours with LOH	27	***23***	22	49
LOH matching	8	***7***	8	18
LOH non matching	7	***2***	7	15
No LOH	12	***12***	6	16
Tumours without LOH	36	***44***	28	34
LOH de novo	17	***13***	11	20
No LOH	18	***24***	17	14
Sensitivity	55.6%	***39.1%***	68.1%	

Table 5.3 legend: Summary data table of LOH in plasma at the 3 studied markers.

a) Sensitivity

Calculation of sensitivity from the above data is dependent on the criteria applied. Taking maximal sensitivity as the percentage of tumours with evidence of LOH that show LOH of any kind in the plasma gives sensitivity of 67.3% (33 out of 49, including adenomas). Inclusion of non-informative cases in the denominator reduces sensitivity to 38.8% (33 out of 85). However if a positive result is taken as a matching

change in tumour and plasma DNA at any of the markers (matching LOH) sensitivity is reduced to 36.7% (18 out of 49) for informative cases or 21.2% for the whole cohort.

The effect of DNA quantity on assay performance was investigated by analysis of plasma DNA concentration within the different groups of results fromplasma DNA analysis.

Average concentration of plasma DNA in samples with matching plasma DNA LOH at any one or more markers was 448.5 ng/ml. Average concentration of plasma DNA in samples with non-matching plasma DNA LOH at any one or more markers (but without concordant loss at any other marker) was 631 ng/ml (p= >0.5, t test).

Of those tumours with no LOH in tumour tissue, and correspondingly no evidence of LOH in plasma either had mean plasma [DNA] of 329.5 ng/ml, whereas those with de novo LOH in plasma had a mean plasma [DNA] of 144.5 ng/ml (t-test p=0.077).

b) Specificity

Test specificity based on the results from control subjects demonstrated that of 24 controls, 1 was not informative and one was excluded. Of the remaining 22, 7 had no de novo LOH giving specificity of 31.8%.

The mean concentration of plasma DNA for those with no LOH in plasma (true negatives) was 225 ng/ml. The mean concentration of plasma DNA for those with de novo LOH (false positives) was 83.5 ng/ml (p=0.298, t test)

Further controls from a plasma LOH perspective can be derived from cases without LOH in tumour tissue, since they would not be expected to result in LOH in plasma. Therefore cases with no LOH in tumour or plasma DNA may be considered true negatives, whilst those with no LOH in tumour but de novo LOH in plasma false positives. Including these cases as further negative controls specificity rises to 37.5%. Addition of these cases to controls gives a mean plasma DNA concentration of 289 ng/ml for true negatives, compared to 199.5 ng/ml for false positives, with a significant difference between them ($p=0.0284$ t-test)

5.4 Discussion

LOH analysis by the use of microsatellites is an attractive target for the development of a blood based biomarker in colorectal and other cancers due to its inherent informativity, with the potential for tumours demonstrating LOH also resulting in LOH in plasma DNA at multiple loci and the additional pick up of microsatellite instabilty. The experiments in this chapter investigated LOH in tumour and plasma DNA by fluorescent microsatellite analysis in the largest cohort than has been described to date. Previous similar studies in colorectal cancer have generated conflicting results, as has also been the case for studies in other cancer types.

The results presented in this chapter demonstrate that this approach is a suboptimal detection method for inferring the presence of a tumour by analysing plasma DNA LOH. Sensitivity as defined by broad criteria was acceptable at 67.3%. Sensitivity with only a matching change in tumour and plasma DNA (matching LOH) was 36.7% of informative cases. The rationale for including cases with non-matching LOH changes in plasma compared to tumour would be that the tumour cells sampled in the tumour DNA sample might not give a true reflection of the allelic status of the tumour as a whole or the predominant tumour clone. Therefore the AI detected in plasma DNA may result from tumour clones not sampled or not predominant in the portion of tumour from which tumour DNA was derived for analysis. When considering application of this technique as a clinical test, there would not be prior knowledge of the allelic status of the tumour, therefore the primary outcome would be either presence or absence of LOH in plasma DNA. A further consideration in this regard is the concept of tumour heterozygosity as discussed in section 1.2.3. Evidence from studies analysing multiple portions of microdissected tumours suggest that differing genetic aberrations occur at different sites of the same tumour. Clearly this may result

in difficulties with sampling of tumour cells and their relative ability to represent the allelic stauts of the whole tumour, as well as conflicting AI from different portions of the tumour resulting in relative allelic balance in plasma DNA. Previous studies have postulated this mechanism as an explanation of discordance between plasma DNA and tumour DNA alterations (420). A study of genetic heterogeneity and plasma DNA LOH investigated 24 lung cancer cases and 26 colorectal cancer cases (337). This study demonstrated the presence of non-matching LOH (which they termed alternate AI) in the plasma of 25% of lung carcinomas, and in 54% of liver metastasis compared to primary tumour (although plasma DNA analysis was not performed for the CRC cases).

Alternatively suboptimal results may be a reflection of artefact inherent in microsatellite analysis when performed on plasma DNA. Plasma DNA is a low copy number template that is also subject to the action of DNAse and the presence of factors that may be inhibitory to PCR. Certainly there is evidence that plasma DNA is degraded to some degree, with the majority being shorter than 181 bp (360) and evidence that microsatellite markers enrich for smaller alleles in degraded DNA (359). Therefore allelic proportions may not be truly reflected by microsatellite analysis of plasma since larger alleles may be artefactually under represented. Alternatively since the analysed aliquot of plasma DNA may contain very few alleles and as such there is evidence from mathematical modelling that the sampling of alleles within aliquots becomes subject to stochastic effects (421), a concept discussed further in the following chapters. Data presented in this chapter do not support these explanations for discordant LOH. Whilst complete allelic loss was observed in a few cases which might be explained by allele degradation this was seen for both the larger and the smaller allele. The data for plasma DNA concentration do not support

discordant LOH being a function of lower copy number since there the mean concentration for cases with discordant LOH was higher than that for cases with concordant LOH, with no significant difference between the two.

The reasons for sensitivity being suboptimal are likely multifactorial. Firstly many studies of plasma DNA have concluded that only some tumours release detectable mutant DNA into the bloodstream, hence reducing the inherent sensitivity of plasma DNA assays. Secondly any tumour derived DNA in plasma is for practical purposes 'diluted' by larger quantities of normal DNA. The exact contribution of tumour derived DNA to the totality of plasma DNA remains debated and is discussed elsewhere, and seems likely to be related in some degree to tumour load. Whilst mutation enrichment techniques for specific mutations (e.g. K-ras) may obviate the problem of tumour derived DNA being overwhelmed by normal DNA, this may be a particular problem for the detection of AI in plasma DNA, since analysis is dependent on the maintenance of relative quantities present within the original sample. However it has been suggested that fluorescent microsatellite analysis can detect DNA alterations down to a ratio of tumour to normal DNA of 0.5% (316), or even 1 in 1000 (422). Additionally it seems likely that specific characteristics of individual markers have relevance to differing sensitivity levels in detecting microsatellite alterations (301). Sensitivity levels are also affected by the defininiton used to classify LOH. There remains no consensus as to the level of allelic ratio alteration that constitutes LOH, with studies variously employing cut-offs of between 20-50% (306;318;337). In this work sensitivity was marginally improved by using a less stringent cut-off of 30% (by 4.3%) but at the expense of specificity , therefore 50% cut-off was employed in further analysis.

Sensitivity levels for the whole cohort analysed in this chapter were reduced by the proportion of cases that were not informative. Again this lack of informativity was likely multifactorial including the number of cases that were homozygous at given markers, and a few clinical samples that repeatedly yielded no detectable PCR product or artefactual peaks interfering with analysis. The rates of LOH and hence informativity in tumour samples were lower than might be predicted by the strategic rationale behind this approach. This may partly reflect that tumours were not microdissected and hence tumour samples are likely to contain a degree of 'contamination' from adjacent normal tissue that would reduce observed alterations in allelic proportions. However the rates of LOH are in keeping with other published studies.

Strategically reduced levels of sensitivity are potentially surmountable by increasing the number of analysed markers that will result in increased informativity for the whole cohort. Additionally currently employed screening methods have suboptimal sensitivity estimated at around 50%.

Of more concern to the validity and potential clinical utility of the approach are the poor levels of specificity presented in this chapter. As described previously the applicability of assays to large sections of the population is dependent on a low false positive rate, since false positives require further, invasive, investigation. Specificity based on controls alone was 31.8%. A potential source of postive results for these controls is the presence of other diseases resulting in plasma LOH. However control recruitment was stringent to prevent this confounding effect, selecting controls with a negative colonoscopy.

Further data for specificity were generated including tumours without LOH as controls. The rationale behind their inclusion was that tumours without LOH would

not be expected to result in plasma LOH, and hence lack of LOH in plasma might be considered a true negative, whilst de novo LOH might be considered a false positive. Using these criteria specificity was improved, but still suboptimal, at 37.5%. A possible explanation for poor specificity within the cohort is artefact from low copy number DNA within controls as discussed above, and would be expected to be more of an issue for controls since plasma DNA concentration is lower than in cancer cases (chapter 2). Evidence to support this from these results is the significant difference in plasma DNA concentration between true negatives and false positives. This constitutes a fundamental problem when considering applying these assays to asymptomatic populations where plasma DNA concentrations are likely to be low, and as a result analysis may be subject to inherent artefact resulting in high levels of false positives.

Investigation of techniques that have potential to negate these problems, such as whole genome amplification(423;424), seems inherently worthwhile given the optimal sensitivity and the strategic potential of these approaches.

CHAPTER 6

ASSESSMENT OF CHROMOSOME 5 LOH BY TAQMAN PCR

6.1 Introduction

Given the sub-optimal performance of fluorescence LOH detection described in the previous chapter, other approaches were explored to determine if plasma DNA LOH assessment might be a valuable biomarker. Real-time PCR has been shown to be highly sensitive, and specificity for the target sequence is improved over conventional PCR (425). Additionally post-amplification processing is not required to visualise and interpret results, hence reducing the opportunity for errors and contamination.

Taqman probes may be utilised to visualise each cycle or allow relative quantification of signal fluorescence at the end of the PCR reaction, an application designed for allelic discrimination.

Taqman PCR has been employed in investigating the associations of plasma DNA, although application has been limited to absolute quantification of plasma DNA, or the detection of mutant DNA sequences related to tumour derived DNA. The detection of differing quantities of sequences that each occur in normal subjects had yet to be described at the outset of this project. However in a normal subject that is heterozygous for a given SNP, it might be expected that each allele would be present in equal proportion in plasma DNA. If however an individual harbours a tumour that is contributing more of one allele into the circulation, on account of LOH of the region containing the other allele, there might be expected to be a relative excess of that allele in the circulation. Whilst this hypothesis is also the basis for detecting LOH in plasma by fluorescence electrophoresis, described in the previous chapter, the

potentially increased accuracy of Taqman PCR might allow detection of smaller variations in relative quantities.

Assay development was focused on a region of interest on chromosome 5. A segment of chromosome 5q21 has been shown to be frequently deleted in colorectal cancer and contains the APC gene. As described in the introduction (section 1.2.6a) the APC gene is of fundamental importance to colorectal carcinogenesis. LOH affecting the APC gene has been observed in a high proportion of colorectal cancers, and occurs at an early stage, including in pre-malignant adenomas. As such an assay able to detect small alterations in the relative quantities of chromosome 5q alleles would be an ideal potential biomarker. The ability to infer the presence of curable neoplasia by detecting tumour derived alterations in allelic ratio in plasma would allow targeted application of invasive diagnostic and therapeutic modalities such as colonoscopy.

Also included within the frequently deleted region of chromosome 5q are thousands of single nucleotide polymorphisms that can be exploited to identify one chromosome from the other copy. As such there is potential for all cases to be heterozygous at one of a few given SNPs, and hence for a small panel of taqman PCR markers to be informative. Also included within the frequently deleted region are dinucleotide repeat markers D5S346 and D5S82, which allow assessment of allelic loss within samples by conventional means, as those described in the previous chapter, and hence validation of novel assays.

Therefore a quantitative assay was designed to detect differing proportions of heterozygous alleles. The assay's sensitivity was determined by artificially inducing AI in plasma. The assay was validated using clinical samples derived from normal and tumour tissue, and then applied to the plasma of colorectal cancer patients.

6.2 Materials and methods

Assay design, primers, probes and reaction conditions are described in section 2.7, as are the methods for experiments to investigate the limit of resolution of the assay and the analysis of normal, tumour and plasma DNA

6.3 Results

6.3.1 Assay validation

a) Normal DNA samples

To determine the assay performance, analysis of normal DNA from buffy coat was performed using standard reagent volumes and concentrations and cycling conditions as indicated from manufacturer's product literature. Normal DNA from 45 colorectal cancer patients was used for this analysis, and results are depicted in Fig 6.2.

Fig 6.2:

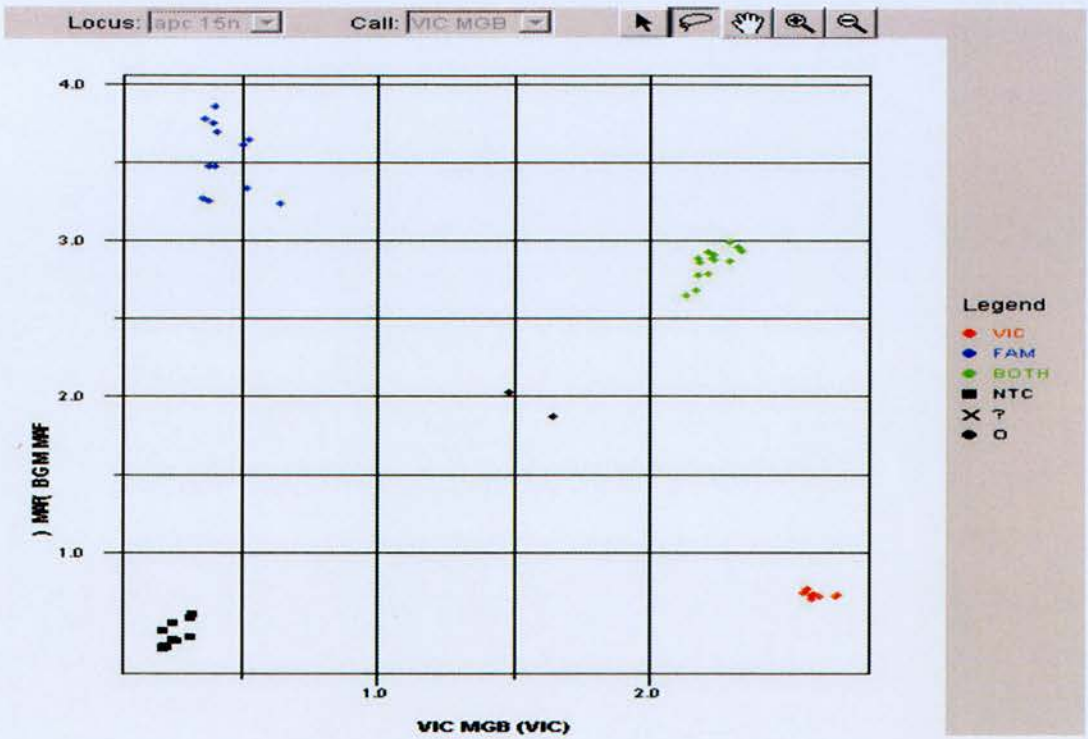


Fig 6.2 legend:

Graph depicting fluorescence results from normal DNA of colorectal cancer patients. The x axis corresponds to arbitrary units of VIC fluorescence, and similarly the y axis to FAM fluorescence. Data points in black indicate no template controls, generating small amounts of background FAM and VIC fluorescence. Blue data points indicate samples that have generated FAM fluorescence only, and correspond to samples homozygous for the allele to which the FAM probe specifically binds (CCA). Similarly red data points indicate VIC fluorescence only and correspond to samples homozygous for the CCG (VIC allele). Data points in green demonstrate FAM and VIC fluorescence and correspond to samples heterozygous at the 15n SNP.

The results shown in figure 6.2 demonstrate that the assay is able to clearly discriminate the two different alleles at the '15n' locus. The absence of the alternative fluorescent signal in homozygous samples indicates the presence of minimal cross interaction, and hence a high level of probe specificity.

These results were confirmed by sequencing of normal DNA from 10 cases, confirming presence of only CCA allele (n=3), heterozygosity (n=5) or homozygosity for CCG allele (n= 2)

6.3.2 Tumour and Normal DNA from Colorectal Cancer Cases

Having determined that the 15n assay was able to specifically discriminate between alleles, the assay's utility in determining relative proportions of the two alleles, was investigated. Assay performance was compared to the conventional LOH detection method of fluorescent electrophoresis in the analysis of tumour and normal DNA from a series of colorectal cancer cases.

i) Dinucleotide repeat analysis

Cases were deemed informative where PCR product was reproducible and two different length alleles were detectable. The number of cases informative for each marker is given in table 6.1. Only one case (2489) was not informative at either marker.

Table 6.1:

	Informative	NotInformative
D5S346	36	9
D5S82	32	13

Table 6.1 legend: number of cases informative at each of the 2 chromosome 5 short tandem repeat markers.

Details of cases and total numbers exhibiting degrees of LOH are detailed in table 6.2. LOH was classified as:

1) Clear cut LOH: reduction of relative peak intensity of one allele of greater than 50% in tumour compared to control DNA. An example of clear cut LOH is provided in figure 6.3.

Figure 6.3

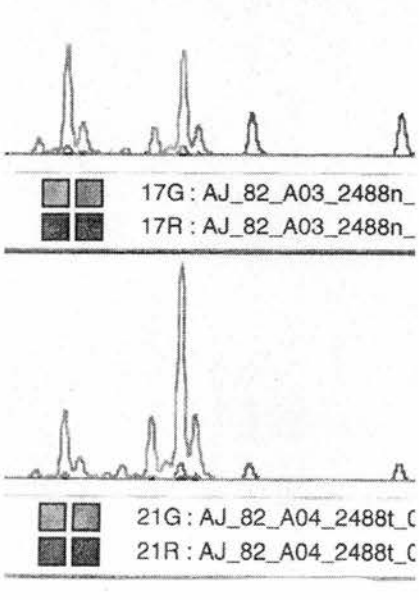


Fig 6.3legend:
Analysis of tumour and normal DNA from case number 2488. There is a reduction of the smaller allele in tumour >50% of that allele's signal amplitude from normal DNA.

2) Possible LOH: reduction of relative peak intensity of one allele of between 20-50% in tumour compared to control DNA. Example provided in figure 6.4.

Fig 6.4:

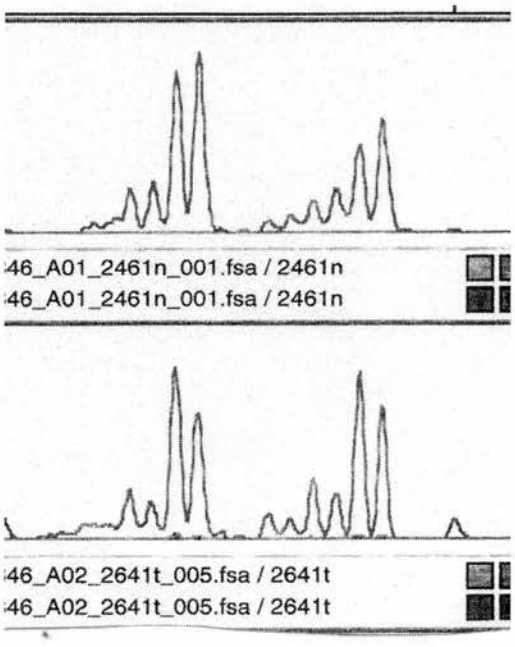


Fig 6.4 legend:

Analysis of tumour and normal DNA from case number 2641. There is a reduction of the smaller allele in tumour by 25% of that allele's signal amplitude from normal DNA.

3) No LOH: reduction of relative peak intensity of one allele of less than 20% in tumour compared to control DNA. An example is provided in figure 6.5.

Fig 6.5:

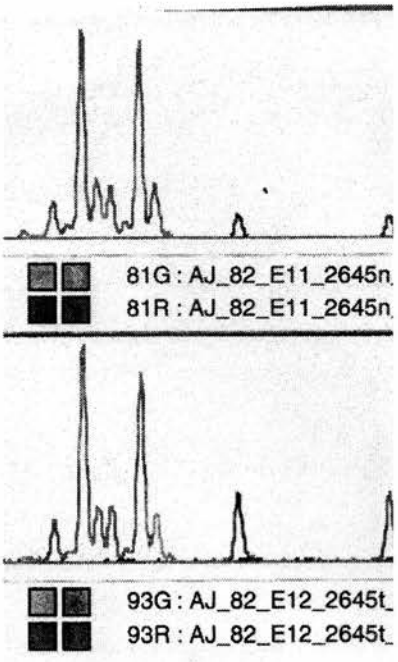


Fig 6.5 legend: Analysis of tumour and normal DNA from case number 2645. The allelic proportions are maintained in tumour and normal samples.

4) Allelic Shift: Was identified on the basis of a different size allele in the tumour that was not present in normal DNA. This was detected in only one case (2565) as demonstrated in Figure 6.6.

Fig 6.6

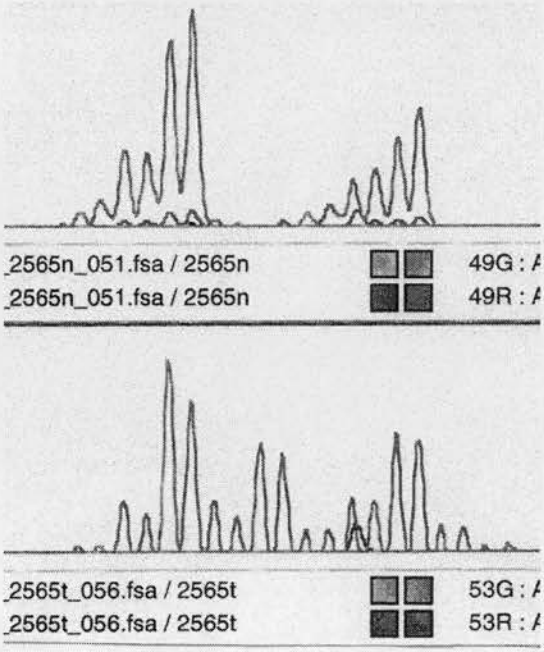


Figure 6.6 legend: Analysis of case 2565, showing the presence of an extra allele (bottom trace), sized between the 2 normal allele sizes whichseen in the normal DNA (top trace)

Summary data of the number of cases exhibiting evidence of differing degrees of LOH in tumour are detailed in Table 6.2

Primary data for this chapter are presented in appendix 5.

Table 6.2

	ClearcutLOH	PossibleLOH	NoLOH	Shift	NotInformative
D5S346	5	15	15	1	9
D5S82	3	10	19	0	13

ii) 15n SNP analysis

Analysis of the same 45 colorectal cancer cases was performed using the taqman '15n' assay. Standard reaction conditions were as described in the materials and methods. Normal DNA was analysed to determine allele status and results are provided in table 6.3.

Table 6.3:

	CCA homozygotes	CCGhomozygotes	Heterozygotes
Number of cases	16	5	24

A total of 24 of 45cases (53.3%) were informative (heterozygous) at the 15n SNP. The tumour and normal DNA from these cases was therefore analysed with the Taqman '15n' assay, using standard conditions, in duplicate.
Fluorescent electrophoresis analysis of dinucleotide repeat markers described in the previous section had identified evidence of LOH at either marker of:

Clearcut LOH: 5

Possible LOH: 7

No LOH: 12

Results of 15n realtime analysis of tumour and normal DNA are provided in figure 6.7.

Figure 6.7:

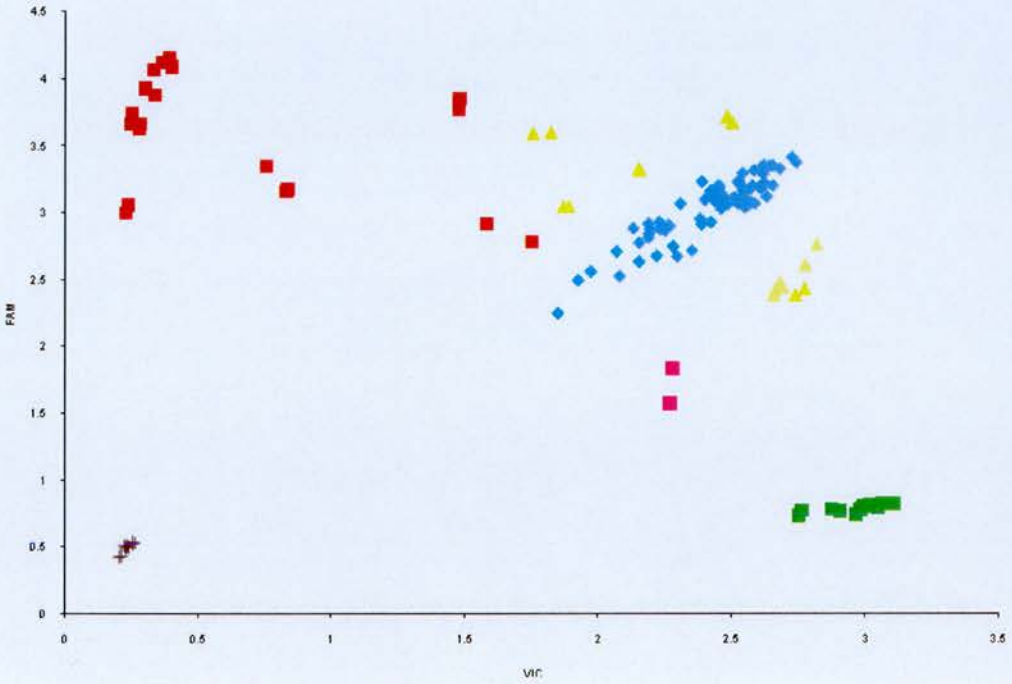


Fig 6.7 legend:

Graph demonstrating the results of 15n assay analysis of tumour and normal DNA from heterozygous cases (n=24). The x-axis exhibits units of VIC fluorescence and the Y axis units of FAM fluorescence. Red squares (■) indicate a positive control for FAM fluorescence. Green squares (■) indicate a positive control for VIC fluorescence. Green crosses (+) indicate no template controls. Dark blue diamonds (♦) indicate normal DNA from cases. Light blue diamonds (♦) indicate tumour DNA from cases exhibiting no evidence of LOH by fluorescence electrophoresis. Purple squares (■) indicate tumour DNA from cases with clearcut LOH by fluorescence electrophoresis. Yellow triangles (▲) indicate tumour DNA from cases with possible LOH by fluorescence electrophoresis.

Clearly those cases exhibiting some evidence of LOH by fluorescence electrophoresis fall into 2 separate groups on analysis with the 15n assay, according to which allele (CCA or CCG) has been lost. This classification is demonstrated graphically in fig 6.8.

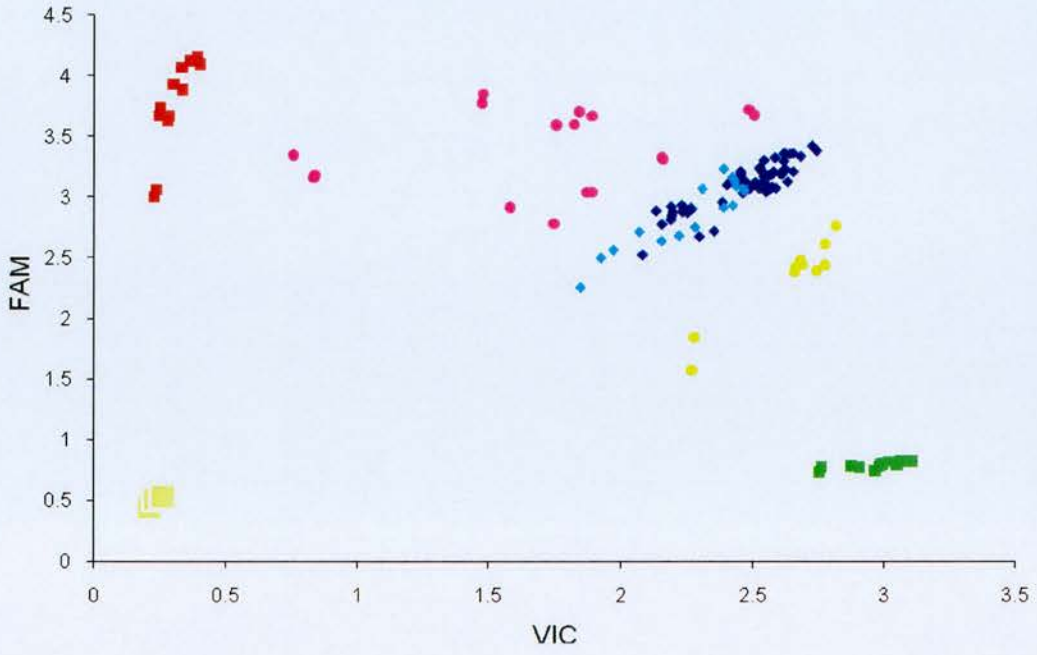


Fig 6.8:

Figure 6.8 legend:

Graph demonstrating the results of 15n assay analysis of tumour and normal DNA from heterozygous cases (n=24). The x-axis exhibits units of VIC fluorescence and the Y axis units of FAM fluorescence. Red squares (■) indicate a positive control for FAM fluorescence. Green squares (■) indicate a positive control for VIC fluorescence. Green crosses (+) indicate no template controls. Dark blue diamonds (♦) indicate normal DNA from cases. Light blue diamonds (♦) indicate tumour DNA from cases exhibiting no evidence of LOH by fluorescence electrophoresis. Purple circles

(●) indicate cases with loss of the CCG allele.Yellow circles (●) indicate cases with loss of the CCA allele

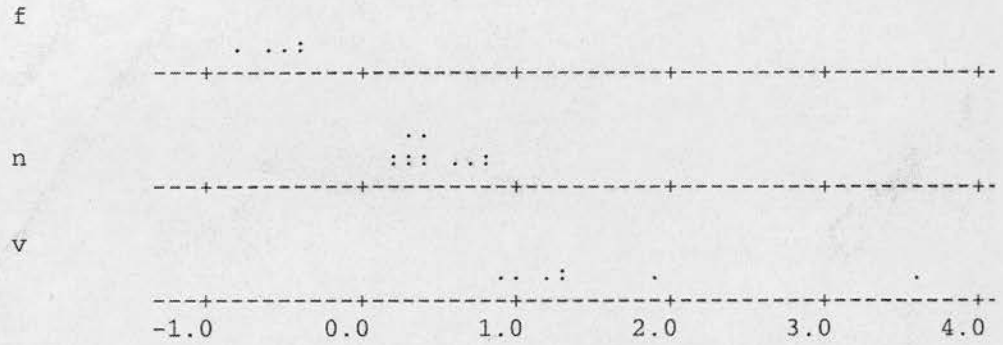
Generating an allelic ratio by dividing the FAM signal amplitude by the VIC signal amplitude allows analysis of these results numerically. There is no overlap in allelic ratio between those cases exhibiting some evidence of LOH (clearcut and possible), and those with no evidence of LOH by fluorescence electrophoresis. Numerical results are demonstrated in table 6.4, and in alternative format in fig 6.9.

Table 6.4:

	ARMin	ARMax
NoLOH(n=12)	1.27	2.28
CCA Loss(n=5)	0.47	0.67
CCG loss(n=7)	2.50	37.8

Table 6.4 legend: Table presenting allelic ratios from tumour DNA analysis. Rows represent cases with each of allelic balance (No LOH), loss of the CCA (FAM labelled) allele and loss of the CCG (VIC labelled) allele. Columns demonstrate the minimum (ARMin) and maximum (ARMax) alleic ratios for each group.

Fig 6.9:



	N	Mean	StDev
f	5	-0.5482	0.1713
n	12	0.4600	0.2163
v	7	1.6009	0.9488
All	24	0.5827	0.9294

Fig 6.9 Legend:

Data presented on a log scale, with data points corresponding to the allelic ratio from each case given as a dot. The three rows indicate tumours with CCA allele loss (f), no LOH (n), and CCG allele loss (v). Numbers of cases, mean allelic ratio and standard deviation for each group are given below.

These data demonstrate that the novel Taqman '15n' assay is able to quantify relative allelic proportions within clinical samples of tumour DNA from colorectal cancer cases. The assay is able to clearly categorise cases exhibiting LOH in the tumour, including in those samples where conventional analysis provides ambiguous results.

6.3.3 Application of 15n Assay to Plasma DNA

Having determined that the assay was able to determine relative proportions of alleles within clinical samples of tumour DNA, its ability to detect AI in plasma DNA was investigated.

a) Determining Sensitivity of Detecting Allelic Imbalance Induced in Plasma

To delineate the sensitivity of the 15n assay at detecting AI within plasma DNA, quantities of DNA homozygous for the FAM allele was introduced into plasma from heterozygous control subjects.

Normal DNA from the buffy coat of a control subject that was homozygous for the FAM allele was extracted and quantified using spectrophotometry. Serial dilutions of this DNA were added to 1ml of plasma from a healthy control heterozygous at the 15n SNP, and then the plasma DNA extracted as described. Analysis of these spiked plasma DNA samples with the 15n assay were performed using standard reagent concentrations and reaction conditions. Spiking, extraction and analysis were performed in triplicate. Collated results are presented in figure 6.10:

Fig 6.10:

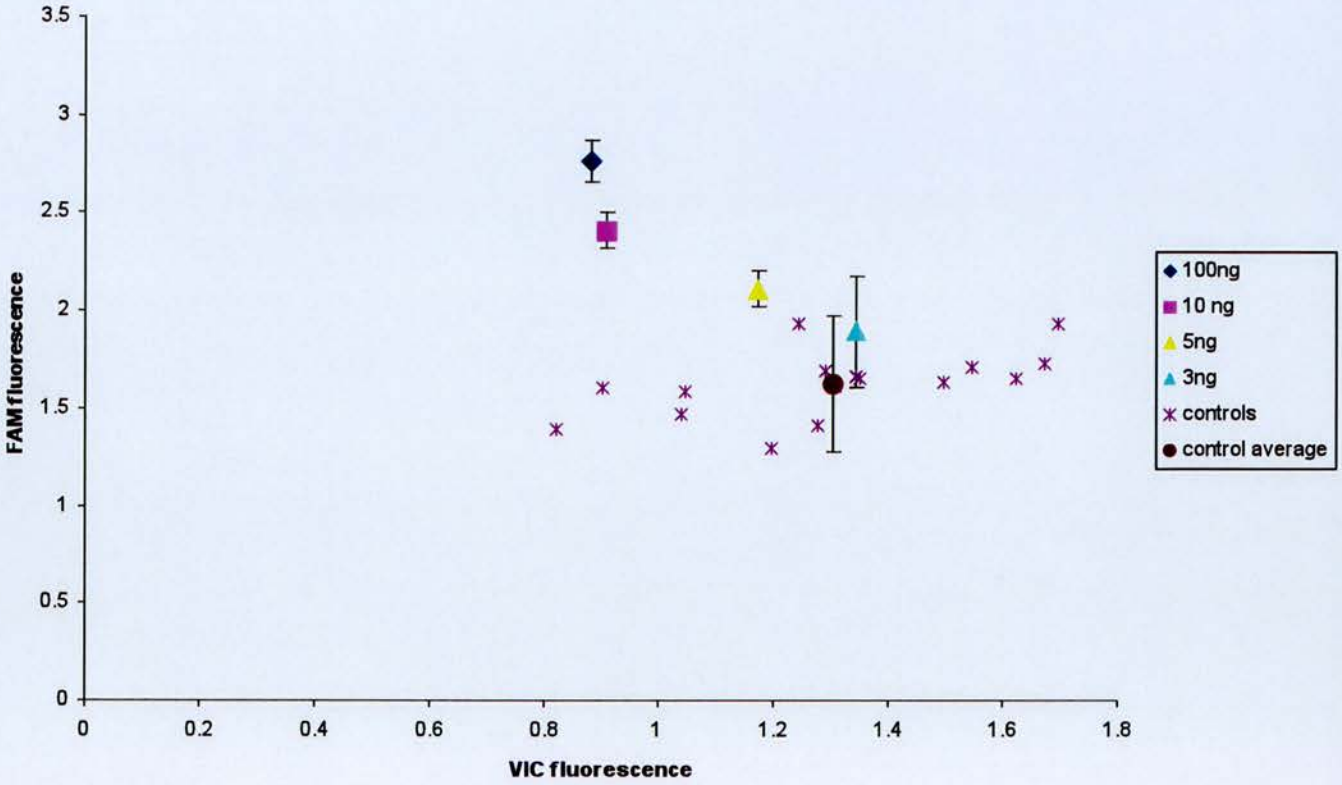


Fig 6.10 legend

Graph demonstrating results of real-time analysis of plasma DNA where AI has been introduced by spiking. VIC fluorescence is demonstrated on the x-axis and FAM fluorescence on the y-axis. Data points shown are average results from replicates, with vertical errorbars indicating 95% confidence intervals.

♦ Plasma DNA spiked with 100ng of FAM homozygous DNA

■ Plasma DNA spiked with 10ng of FAM homozygous DNA

▲ Plasma DNA spiked with 5ng of FAM homozygous DNA

▲ Plasma DNA spiked with 3ng of FAM homozygous DNA

* Indicate all individual results from control (unspiked) samples

● Indicates mean from all controls

These results indicate that the 15n assay is able to discriminate, with statistical significance, plasma DNA with an artificially induced AI down to a level of 5ng/ml of plasma. These levels are consistent with those described in the literature for tumour derived DNA in plasma, and indicate this assay is sufficiently sensitive to potentially detect tumour derived AI in plasma.

b) Application of 15n assay to clinical plasma samples from Colorectal Cancer Cases and Controls

Having established the level of sensitivity of AI detection using the Taqman assay, the abilityof theassay to detect AI in plasma DNA of colorectal cancer cases was tested, using the same reaction conditions. Results are presented in Fig 6.11.

Fig 6.11

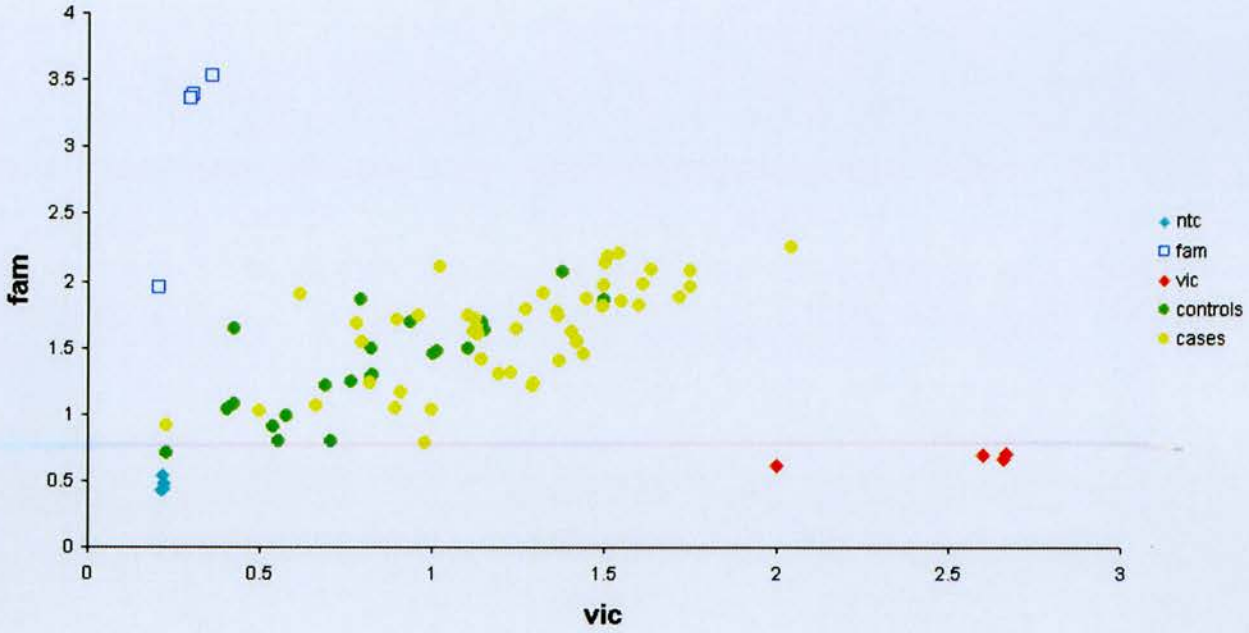


Fig 6.11 legend:

Graph demonstrating results of 15n assay analysis of plasma DNA from colorectal cancer cases and controls.

♦ no template controls

♦ positive controls (VIC)

□ positive controls (FAM)

● controls

● cases

These results are presented in an alternative way in figure 6.12

Fig 6.12:

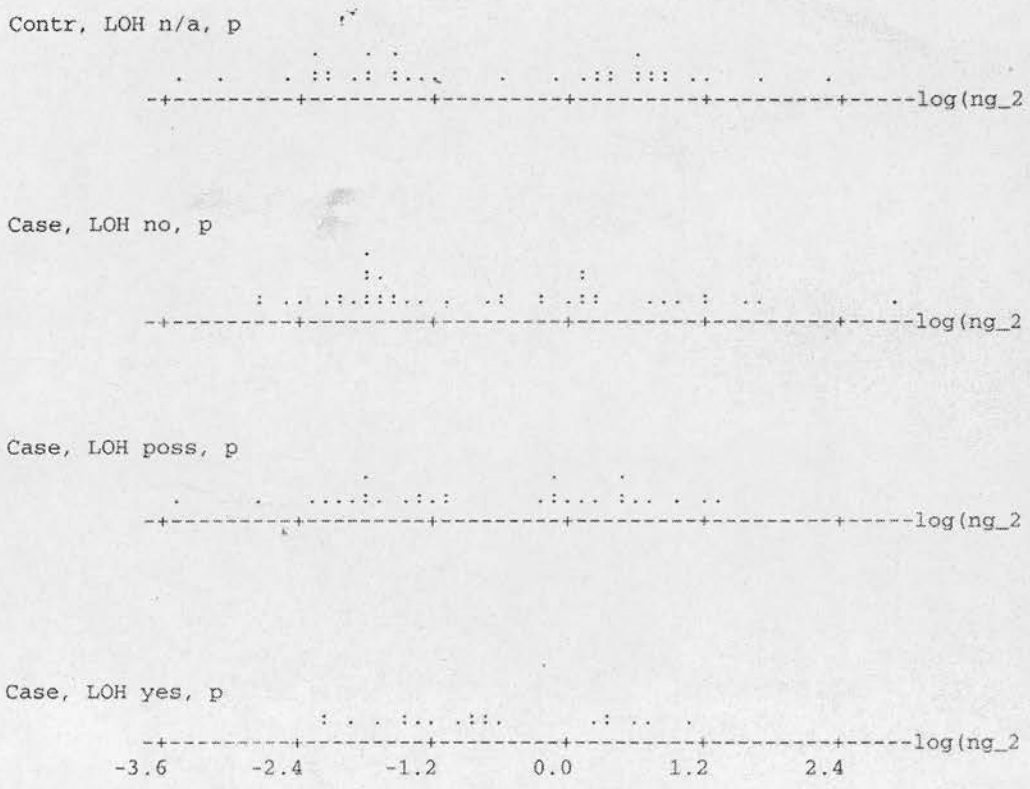


Fig 6.12 legend: Data presented on a log scale, with data points corresponding to the allelic ratio from each case given as a dot. The four rows indicate plasma results from controls, tumours without LOH, those with ambiguous LOH and those with clear cut LOH by microsatellite analysis.

As can be seen from figures 6.11 and 6.12 there is overlap in the allelic ratios between all groups. Therefore using these methods, realtime PCR using the 15n assay is not able to discriminate plasma DNA from cases with tumour LOH from cases without tumour LOH or from controls.

6.4 Discussion

The experiments in this chapter were designed to further develop the strategy to exploit the near ubiquitous phenomenon of LOH in colorectal cancer. Given the limitations of standard fluorescence electrophoresis described in the previous chapter, an approach was developed to utilise the high performance characteristics of Taqman PCR.

To this end a novel assay was designed and validated, with the ability to discriminate different alleles of a commonly heterozygous SNP within exon 15 of the APC gene. As part of assay validation the assay's performance was compared to that of conventional LOH detection in detecting LOH resulting in AI in tumour tissue from colorectal cancer cases. Previous studies of LOH in tumour tissue have used varying definitions of what degree of loss is counted as demonstrating LOH. Classically LOH has been scored as being present when the signal indicating one allele is reduced by 70% or more than that detectable in corresponding normal DNA. This stringent classification is likely to result in few false positives, but may result in some tumours being classified as not exhibiting LOH that do to lesser degrees. In order to meet these stringent criteria studies have by necessity employed microdissection of tumour samples to prevent inclusion of portions of normal DNA within the tumour sample. This process is both time consuming and expensive, and was not performed in the work presented in this thesis. Studies have also used smaller decreases in allele signal to indicate LOH, including 50% or more, which was taken in this work to indicate clear cut LOH. Less than 20% change in allele signal cannot be termed relevant to LOH detection due to inherent variability and artefact within analysis by fluorescent electrophoresis. However cases demonstrating between 20-50% alteration in allele

signal may indicate a level of LOH within the tumour, and in this work were classified as possible LOH. These cases of possible LOH are of particular relevance to the detection of AI in plasma DNA, since within plasma DNA only a proportion can be expected to be derived from the tumour, with the rest constituting 'contaminating' normal DNA. As such stringent LOH criteria cannot be expected to detect subtle AI in plasma caused by a tumour, and more accurate methods of quantifying allelic proportions would be beneficial. The novel assay described in this chapter appear to fulfil this requirement with the clear and statistically significant classification of tumour DNA samples to exhibiting LOH, where conventional analysis is ambiguous. This approach may also have application to studies investigating LOH in tumour samples only, as it may obviate the need for tumour microdissection, and allows single step, high throughput analysis of samples.The principal is applicable to innumerate genetic loci, present on any given chromosomal region of interest.

In view of the assay's ability to discriminate lower levels of AI in tumour tissue the assay was then applied to plasma DNA. The results presented in this chapter indicate that this approach is able to sensitively detect AI that has been artificially introduced into plasma DNA. A level of sensitivity of 5ng of 'foreign' DNA per millilitre of plasma is in keeping with levels of tumour DNA that have been described in plasma. However the results in this chapter demonstrate that the inter-sample variability increases when there is less plasma DNA available to act as template within a reaction. This is indicated by the widening error bars with lower levels of spiked DNA, with the largest confidence interval occurring for control plasma samples. This is likely to be a reflection of the low quantities of plasma DNA expected in normal controls, and was not negated by the high number of repeat samples from controls. As such this approach will favour the detection of AI in samples with higher

concentrations of plasma DNA, and hence favour the detection of advanced cases, where levels of plasma DNA have been shown to be highest.

A further difficulty with artificially inducing AI in plasma by spiking is that the introduced DNA has a high degree of integrity. Conversely plasma DNA has been exposed to the milieu within plasma, including the action of endonucleases, and has been shown to be largely composed of small, partially digested fragments. As such the ability to detect low levels of intact DNA may have limited applicability to the detection of native tumour derived DNA. However determining the level of detectability of the assay is a necessary process in assessing assay performance and indicated a high level of sensitivity. Additionally fragmentary DNA may be less of an issue using a Taqman PCR assay such as the 15n assay, as the primers and probes are targeted at a relatively short DNA sequence when compared to the longer sequences involved in fluorescent microsatellite analysis.

Application of the assay using the same reaction conditions to plasma DNA from cases and controls did not result in discrimination of cases with tumour LOH from those with no tumour LOH or from controls. Therefore the assay using these methods does not appear to have potential clinical utility. There are a number of possible explanations for these negative results, some of which are discussed above and in the discussion of the previous chapter. However there are a number of technical considerations that might explain the failure to detect any AI that might be present. There was a wide variation in the allelic ratio from control plasma DNA, precluding the generation of a narrow range of allelic ratio outside of which a sample might be categorised as having AI. This coupled with the variability within cancer cases results and within replicates of the same samples indicate that template plasma DNA

introduced into the reactions was variable. A possible explanation of these results is sampling error, whereby only a small number of plasma DNA alleles are included in each well, on account of the low plasma DNA concentration. Therefore the numbers of each allele in each well may vary sufficiently by chance, to result in a large percentage change in generated signal and hence allelic ratio.

Approaches considered to address this possibility included the use of an initial round of nested PCR to increase the template copies in the analysis reaction. However if the initial round of PCR equally amplified both alleles, as would be desired, then the template for the secondary PCR would simply contain an extrapolation of the sampling error introduced in the primary PCR. An alternative approach is count a large number of alleles individually, and this was investigated and described in the following chapter.

CHAPTER 7

ALLELE COUNTING

7.1 Introduction

An alternative approach to detecting AI is a modification of the assay described in the previous chapter that allows counting of individual alleles within a sample. This approach is able to address the possibility of sampling error as the cause for poor discrimination of plasma DNA from cases and controls using the 15n assay, since many alleles may be assessed by repeating reactions on a large scale.

In order to count alleles individually DNA template is diluted such that there is only one DNA copy present in every other reaction well. This approach has previously been employed to detect low levels of genetic changes that might otherwise be undetectable within the signal from a large number of normal alleles (392).

With regard to allelic ratios, with the template diluted to this level each reaction well is either empty or contains one or the other allele. Therefore positive wells can simply be counted and reflect the relative quantities of either allele within the undiluted template. This process can be repeated on a large scale, and hence allow sampling of numerous alleles from a template. The description of this approach has been termed digital SNP analysis (426), and has been applied to tumours (427), ascitic fluid (320) and plasma DNA (52) to determine allelic ratios within samples. In the study of DNA extracted from malignant ascitic fluid associated with several intra-abdominal malignancies, including colorectal cancer, digital SNP analysis was used to compare allelic ratios from 20 cases to 20 controls. This study employed 7 SNP markers and

counted around 100 alleles per sample, and was able to demonstrate AI in 95% of the cytology proven malignant samples, compared to 5% of the cytology negative samples.

The potential application of this technique to plasma DNA has been investigated with respect to ovarian cancer, as a subset of a large study of controls and cases with a variety of conditions. 330 plasma samples from cases with benign and malignant diseases, as well as healthy controls, were assessed for plasma DNA concentration. Of these 54 ovarian cancer cases and 31 controls, had a plasma DNA concentration greater than 50ng/ml, and were assessed for AI by digital SNP analysis using 8 SNP markers. An average of 200 individual alleles were counted per plasma DNA sample and varying levels of AI were used, from 55% to 95%, to generate receiver-operating characteristic curves. Optimal performance was generated using an allelic ratio of 0.6, giving an overall sensitivity of 93% (87% for stages I or II cancer) and a specificity of 100%. In 15 out of 17 matched tumour samples available the AI was concordant between tumour and plasma (52). The authors of this study concluded that this approach was a promising method for detecting cancer and should be tested more extensively on ovarian and other cancers.

Therefore an allele counting approach was assessed in its ability to detect allelic imbalance in clinical samples from a subset of the prospectively collected series of cases and controls.

7.2 Materials and methods

Reaction conditions, assay validation, study subjects and means of analysis are presented in section 2.8.

7.3 Results

7.3.1 Validation

Allele counting of normal DNA from control heterozygote was performed to assess whether this approach resulted in allelic balance in clinical samples. Triplicate data is presented in table 7.1. There was no significant difference in expected compared to observed numbers of each allele ($p>0.05$, Chi squared).

Table 7.1

	Observed CCA	Observed CCG	Expected CCA	Expected CCG
Replicate 1	521	512	516.5	516.5
Replicate 2	499	503	501	501
Replicate 3	507	502	504.5	504.5

7.3.2 Test case

To test the approach, one case was selected from the cohort analysed using the 15n assay, as described in the previous chapter, which had demonstrated clear cut LOH in the tumour by microsatellite marker analysis and confirmed by analysis using the 15n assay (case number MD 2530). This was a case of advanced cancer, with metastatic disease, and a corresponding high plasma DNA concentration allowing sufficient template for multiple reactions.

Allele counting in tumour DNA was performed at dilution of 1:50 stock tumour DNA: dH_20.

FAM alleles:	81	
VICalleles	35	χ^2=9.494, p<0.01

Serial dilution of plasma DNA from case 2530, to a level whereby 1 allele was present in every other 1ul aliquot was performed (1 in 200), figures 7.1 and 7.2. Reactions were repeated until over 1000 individual alleles were counted.

Figure 7.1:

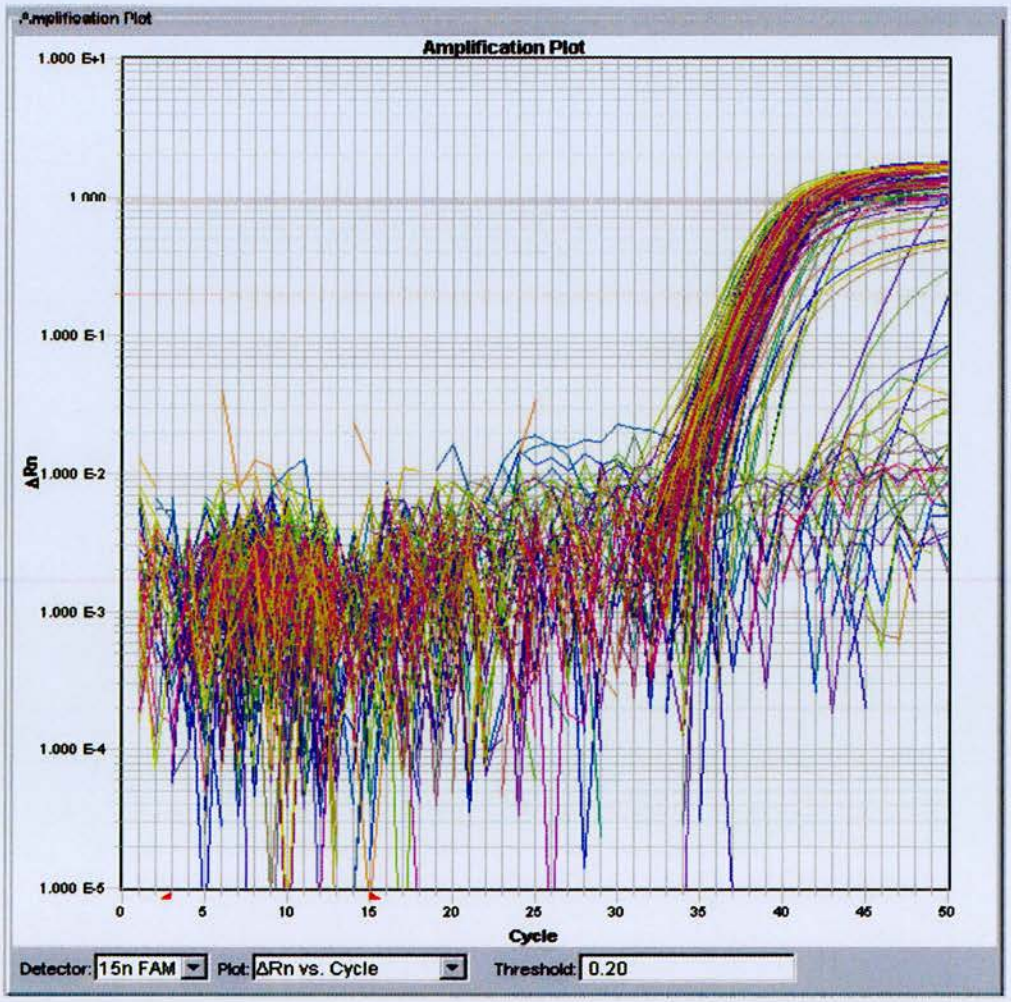


Figure 7.1 legend: Raw amplification plot for test case (2530)

Figure 7.2:

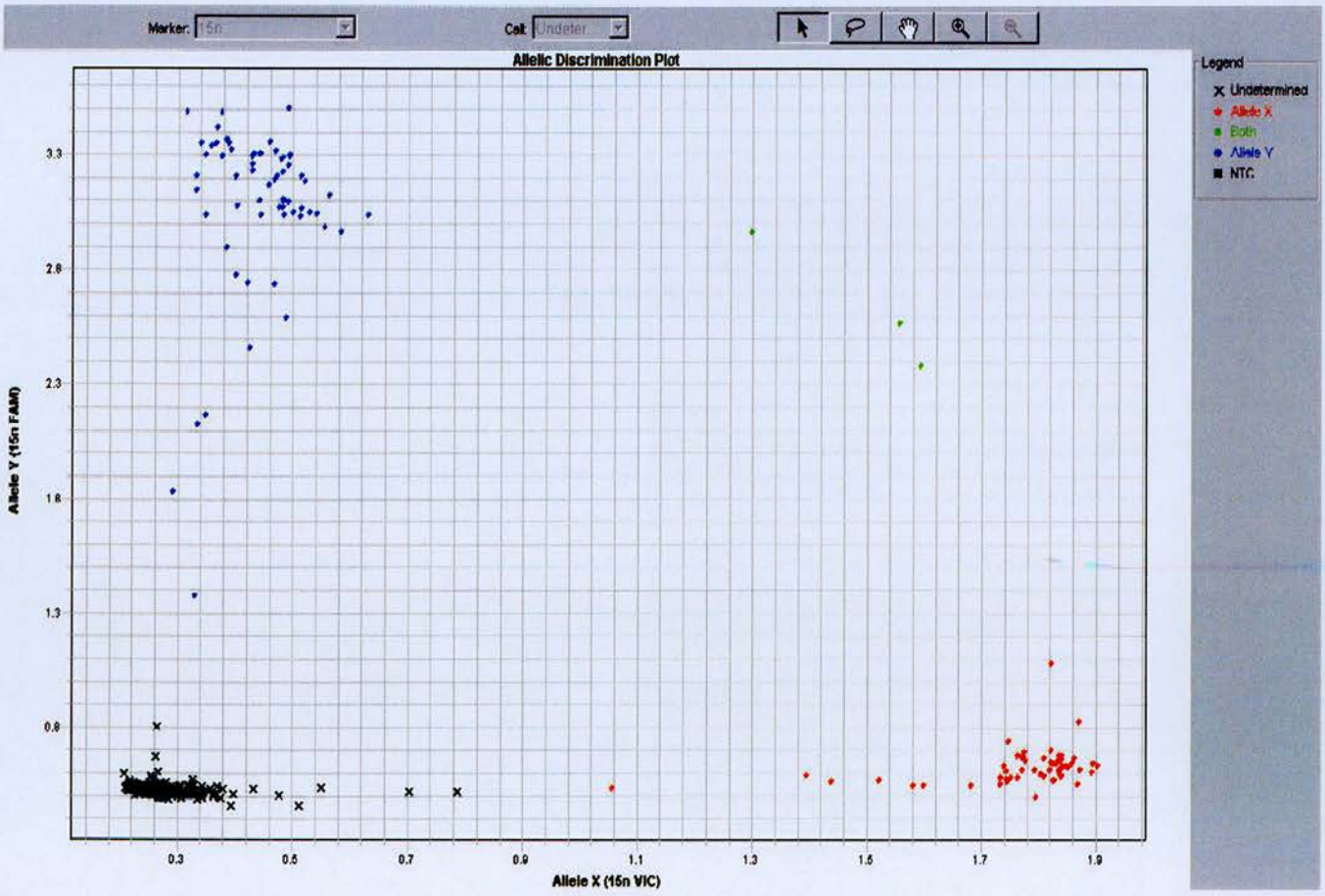


Figure 7.2 legend: Allelic discrimination plot for plasma from test case 2530. y axis = FAM fluorescence, x axis = VIC fluorescence. Dilution of template plasma DNA has been performed to detect individual alleles (FAM in blue, VIC in red), with only 3 wells containing a CCA and a CCG allele and hence registering as heterozygotes.

The number of each allele present within each 384 well plate in analysis of plasma DNA from case 2530 by allele counting is presented in table 7.2.

Table 7.2

	CCA allele	CCG allele	Negative	Both
Plate1	67	65	238	14
Plate2	60	52	269	3
Plate3	108	49	216	11
Plate4	66	37	272	9
Plate5	61	70	234	19
Plate6	64	45	262	13
Plate7	85	56	223	20
Plate8	70	74	224	16
TOTAL	**581**	**448**		

Comparing these allele totals to those expected if the sample were in allelic balance gives the following table (7.3).

Table 7.3:

	FAM	VIC
Expected	514.5	514.5
Observed	581	448

Performing the Chi squared (χ^2) test (with Yates correction) on these values gives χ^2 8.38 (p=0.004) indicating that the plasma DNA from case 2530 has significant AI, with a relative excess of the same allele observed in the tumour (FAM).

Therefore using this allele counting approach it was possible to demonstrate matching AI in plasma and tumour DNA.

7.3.2 Prospective cohort

Having demonstrated that allele counting is able to detect tumour derived AI in plasma DNA from a colorectal cancer case, the principle was further investigated by analysing a prospective cohort in a blinded fashion.

The ascertainment and enrolment of cases remained unchanged from earlier cases in this study, but a larger volume of plasma was collected from cases and controls, to allow counting of a large number of alleles. 13 cases and 7 controls with normal colonoscopy were recruited. Blood was collected and processed, with plasma DNA extracted, as described in materials and methods.

In all 8 patients (38%) were informative at this SNP, with 4 cancer cases (31%) and 4 controls (50%). These cases and controls underwent counting of over 1000 alleles without prior knowledge of the allelic status in the 4 corresponding tumours.

Serial dilution of plasma DNA was performed to allow an individual allele in every other well or less. Dilutions required are provided in chapter 2.

Total number of individual alleles counted in each plasma DNA sample, along with the χ^2 value and significance of difference from allelic balance (total number of FAM and VIC alleles counted / 2) are provided in table 7.4:

Table 7.4:

	FAM	VIC	χ^2	p value
5011	571	565	0.01	0.920
5508	527	485	0.79	0.374
5509	480	484	0.00	1.0
6341	537	519	0.12	0.73
5504	539	547	0.01	0.92
5505	498	527	0.36	0.55
6335	511	512	0.00	1.0
6336	534	518	0.09	0.76

As can been seen from the above results neither the 4 controls or the 4 cases had a plasma DNA allelic ratio that differed significantly from allelic balance. This would suggest that this approach is unable to identify cases from controls within this small cohort. It was however possible that these results simply reflected an un-informative population sample. Therefore the paired tumours for the 4 informative cancer cases were analysed for AI by counting >100 alleles at the 15n SNP, and the results presented in the table below (7.5):

Table 7.5:

	FAM	VIC	χ^2	pvalue
5011	105	75	2.19	0.14
5508	62	58	0.02	0.89
5509	82	70	0.33	0.57
6341	81	94	0.35	0.55

As such none of the 4 informative cancer cases demonstrated AI in tumour tissue by allele counting (>100 alleles), and hence none of the cohort was truly informative. Additionally microsatellite analysis of these cases as described in chapter 5 did not show evidence of LOH by assessment with the marker D5 S346.

7.4 Discussion

There are a number of potential advantages to the allele counting approach described in this chapter. As with other applications of the 15n assay, PCR products for each allele are small and the same size, unlike in assessment with microsatellite markers where there may be bias against detection of the larger allele due to DNA degradation in plasma. Additionally any given number of alleles may be counted giving the ability to increase sensitivity and specificity, and the potential to detect more subtle examples of AI. As described in the introduction in this chapter, promising results have been described in ovarian cancer, but this approach had not been previously described in relation to colorectal cancer.

The results described in this chapter investigate the potential of allele counting to detect tumour derived AI within plasma. Analysis of case 2530 demonstrated that matching AI in tumour and normal tissue is detectable using this approach. This case was of advanced colorectal malignancy with a high plasma DNA concentration, where tumour related AI might be expected to be more readily demonstrated than in cases with earlier disease stage. Additionally plasma DNA analysis was performed with prior knowledge of the allelic status of the tumour, which might constitute a source of potential bias. However the allele counting approach is less susceptible to interpretation bias on account of the simple nature of the generated results, whereby samples are either negative (empty) or contain one or the other allele. As such results are not generated within a continuous spectrum that could be open to interpretation. The ability to detect only advanced colorectal cancer cases via a blood test would have limited clinical utility and have no potential for disease screening, therefore analysis was performed on a prospective cohort that included earlier stage disease.

This series of 4 heterozygous cancer cases, from a range of cancer stages, did not show significant plasma DNA AI by allele counting. However allele counting of matched tumour DNA for these cases did not show AI, hence these tumours could not be expected to result in plasma AI. Whilst the number of alleles counted from tumour specimens was a degree smaller than for plasma DNA, analysis of a similar number of alleles from case 2530 tumour DNA did demonstrate significant AI. Additionally it seems unlikely that tumours that do not show appreciable AI, as would be detected by analysing smaller numbers of alleles, would result in detectable levels of plasma DNA AI in a background of normal DNA. It seems therefore that this small series of cases and controls was simply not informative. Therefore although proof of principle is demonstrated by case 2530, analysis of the further prospective cohort neither disproves nor enhances the potential for detecting tumour derived AI within plasma DNA of colorectal cancer cases.

Importantly however these cases and controls in plasma allelic balance demonstrate a specificity for the approach whereby those individuals expected to have no AI were observed to maintain allelic balance. This contrasts with the poor specificity of microsatellite analysis described in chapter 5. As such this approach may negate a technical difficulty relating to assessment of LOH in very low copy number samples, where observed AI may be the result of artefact.

The previous reports of the allele counting approach discussed in the introduction to this chapter used stastical modelling to determine how many alleles required to be counted before it was possible to determine if the sample was in allelic balance or imbalance, with an average number of 200 alleles counted per plasma DNA sample. Whilst this method has potential to reduce the number of replicates required it

assumes the use of a representative sample of alleles. However sampling error is likely to remain an issue, as demonstrated by the variation in allelic ratios between plates of the same sample as shown for case 2530 in table 7.1. The proof of principle work in this chapter looked to circumvent this potential sampling error by performing large numbers of replicates and hence giving a high probability of analysing samples representative of the allelic content within the plasma DNA.

There are also a number of disadvantages to the allele counting approach. Having to repeat very many replicates is inherently expensive. This is particularly the case since by the nature of the dilution at least every other well is negative, resulting in non-informative use of reagents. Previous studies have estimated the cost at over $200 per test (52). Added to this are labour costs, with the technique being time consuming and labour intensive. There is potential for these costs to be reduced with streamlining of processing and high throughput technologies, that were employed in this work, including the use of robots to prepare 384 well plates and automated analysis with realtime PCR platforms such as ABI 7900 HT. Coupled to this is the need to take relatively large amounts of plasma from patients to ensure a pool of sufficient alleles to sample. Previous studies have drawn 3-5mls of plasma per patients (~7.5–12.5 mls of blood), and in this work 40 mls of blood was taken for the allele counting cohort. The principle of allele counting is applicable to innumerate SNP loci, which would enable informativity for very nearly 100% of any cohort with analysis of a panel of sufficient numbers of markers. This work describes analysis of a cohort of 13 cases at a commonly heterozygous SNP with a published high rate of LOH of the region in which it is contained. However none of the 13 cases were informative, therefore it is likely that a relatively large panel of markers would be required to provide a

sufficiently sensitive blood test. Given the financial and workload constraints this would entail it seems unlikely that this approach will yield a viable and practical test to identify tumour related AI in plasma DNA with clinical utility.

However since the completion of the laboratory work for this project a recent paper examined low copy number APC mutations in plasma DNA from colorectal cancer and adenoma patients (323). Whilst the intent was not to detect LOH per se, the results looked to determine the relative contribution of tumour DNA in the circulation. They utilised an elegant technique termed BEAMing, that utilises microemulsions to count many alleles individually within a single reaction. This approach clearly has advantages over manual dilution and allele counting in terms of time, labour intensity and cost. BEAMing might therefore constitute the necessary technological advance that facilitates the assessment of the allele counting strategy into wider application. However the results from Diehl's paper suggest that the relative contribution of tumour DNA to the totality of plasma DNA is low, particularly in respect to early stage disease. These findings warrant further investigation, since should they prove reproducibly true, then only approaches that contain a considerable degree of mutation enrichment have the potential to be sensitive enough to detect tumour derived DNA in plasma of colorectal cancer patients.

CHAPTER 8

FINAL DISCUSSION

In Chapter 1 I presented the rationale for this project. Colorectal cancer is undeniably an important disease both in terms of the clinical implications of the disease and the public health challenges it represents. As such further approaches to influence the incidence and disease survival are a major focus of research interest. One approach to influencing the effects of disease is to identify means by which to detect disease at an early stage, when surgical treatment alone effects cure in the majority of patients. This approach has been extensively tested with regard to screening of asymptomatic populations for colorectal neoplasia using FOBT, and is now adopted as a national screening programme for bowel cancer. This national programme and review of the literature for FOBT establishes the rationale for screening for bowel cancer, with real benefits within the screened arm of trials, including an improved stage distribution of disease, improved cancer specific survival and a reduced cancer incidence in the long term. FOBT screening therefore constitutes a significant step forward in the management of colorectal cancer. However there are a number of limitations to FOBT, as there are with other potential methods of screening, including suboptimal test accuracy as well as limited acceptability to populations offered screening, manifest as poor compliance rates. There is therefore a need for the development of other markers of disease to supplement or replace FOBT by enhancing the diagnostic accuracy of tests, and by being more acceptable to screened populations and hence widening the net to the potential benefits of being screened.

It remains the 'Holy Grail' of molecular biology in general, and genetics in particular, to translate the vast expansion of knowledge ascertained in recent decades

into increasing numbers of clinically useful tests. The development of biomarkers for disease detection and monitoring would appear to be an ideal avenue in this regard. As such there have been considerable efforts directed into this field over recent years. The discovery and characterisation of free circulating nucleic acids has naturally become a focus for this type of translational research. The development of clinically relevant tests on the basis of tumour derived DNA are inherently attractive, not least since such tests would be essentially non-invasive and hence likely to be highly acceptable to at risk populations, but also potentially applicable to tumours at multiple anatomical sites. This said there have published studies in the field of circulating DNA in cancer patients for more than 15 years, but no tests that are in clinical practice as yet, although there are encouraging data from head and neck cancer and EBV circulating DNA that this approach may soon be of clinical use in the monitoring of disease response and recurrence.

Certainly there is much that remains to be elucidated regarding the biology of circulating tumour derived DNA. It is clear that not all tumours result in detectable tumour derived DNA in circulation using currently available methods. With respect to colorectal cancer it has been suggested that first pass metabolism from the portal circulation has a role in clearing tumour derived DNA, and that this might explain the negative results from some assays. It is counterintuitive however that the liver would clear some mutant sequences whilst others are preserved, and tumour derived DNA has been reproducibly detected using k-ras assays.

Additionally the source, characteristics, kinetics and clearance of plasma DNA remain to be determined. It is evident that circulating DNA is a less than ideal template with which to perform molecular genetic tests. There is evidence that DNA is low copy number, fragmented, susceptible to inhibitors of assay performance, and that tumour

derived template may be sparse amongst a relatively much larger quantity of normal circulating DNA, all of which may have implications to the performance of assays looking to detect mutant sequences as a surrogate of disease states.

In chapter 2 the generic materials and methods utilised in this project are documented, with reference to experiment specific methods within individual chapters.

Chapter 3 examined extraction and quantification of plasma DNA, with correlation to disease stage and survival. The technical difficulties of working with small volume plasma DNA were manifest in the experiments of extraction of plasma DNA, and there remains no consensus on optimal extraction methods in the literature. This certainly constitutes a major hurdle for the field of circulating nucleic acids research, limiting comparisons between studies on account of methodological differences. However it appears to be too early to settle on any given method since technological advances in extraction techniques and commercially available kits are likely to continue to generate improving yields and quality templates with which to perform analysis. That said a reproducible and widely employed commercial extraction kit was employed and allowed generation of results presented throughout this work.

I subsequently quantified plasma DNA using a number of techniques, and demonstrated the picogreen assay to be the most accurate of these for assessment of quantity in relation to plasma DNA. Analysis of a prospective series of patients employing this method demonstrated a significant difference in total plasma DNA concentration between cases and controls. Subsequent to the completion of the laboratory work for this project, a number of papers, examining smaller series of cases, have demonstrated similar results, with a degree of variation in the sensitivity and specificity of plasma DNA quantity as a marker of colorectal cancer. The ability

of plasma DNA concentration alone to detect colorectal cancer was suboptimal, and likely multifactorial. There remains to be sufficient work detailing the effects of benign and inflammatory diseases on plasma DNA concentration which might inform the selection of super controls and hence improve specificity of testing.

In this work there was not an association between disease stage or suvival and plasma DNA quantity, although in subset analysis numbers were small. There was a trend to poorer survival with the highest levels of plasma DNA, which may be a reflection of a tendency for advanced disease to generate the highest concentrations. However this trend was also observed in cases with early stage disease where survival would be expected to be good. This association warrants further investigation with a view to application clinically, as may be of relevance to disease states where clinical staging is suboptimal such as polyp cancers. The potential ability of plasma DNA quantification to augment current screening modalities such as FOB would require assessment on large asymptomatic populations which might be accessed through current screening programmes. Each of the potential applications of plasma DNA assessment would again require further knowledge of plasma DNA levels in asymptomatic populations, which may alter with other disease states or vary within different demographic subsets of the population.

Chapter 4 details experiments designed to detect tumour specific mutations within plasma DNA. Assays were developed to the poly A tract of the TGFBR II gene and to a mutational hotspot of the K ras gene. Initial experiments to validate the TGFB assay were hampered by technical difficulties that necessitated multiple reagent changes, and ultimately a change of marker fluoresence. Subsequently I demonstrated a limit of detectability of this assay to levels applicable to mutant plasma DNA in spiking experiments using exogenous positive control DNA.

However these experiments utilised DNA derived directly from cell culture, which is likely to be non-fragmentary in nature. Application of the assay to clinical plasma samples resulted in positive results for all samples likely reflecting an artefact from the multiple numbers of replication cycles. This would suggest that this approach has limited applicability to plasma DNA when assessing repetitive polyA tracts. Negative results were also obtained from the assay designed to detect K-ras mutations.

Chapter 5 presents results of fluorescent microsatellite analysis, at 3 polymorphic markers on 3 chromosomes, in matched normal, tumour and plasma DNA. Using this small panel of markers gave good levels of informativity for a relatively large series of patients, with approaching two thirds of cases having LOH in tumour tissue at one or more, highlighting the strategic rationale for this approach. Analysing for changes in allelic ratio in plasma as a marker of the underlying tumour had suboptimal sensitivity and low specificity. These findings have been previously described in relation to LOH in plasma, although the maximal sensitivity of 67.3% is encouraging. Of more concern for the applicability of this approach is the poor specificity with a large number of controls exhibiting unexplained changes in plasma allelic ratios. However further data from experiments quantifying plasma DNA suggest that poor specificity may be partly a function of low copy number DNA, since there was a significantly lower plasma DNA concentration from false positive controls compared to true negative controls. This might suggest that the very low copy number DNA in some controls results in artifact in this form of plasma DNA analysis hampering the identification of normal subjects.

Experiments in chapter 6 looked to exploit the potentially increased accuracy afforded by real time PCR, with the development of a SNP based real time PCR chromosome 5 assay. A functioning assay was designed and validated to clearly

discriminate homozygotes and heterozygotes. The assay was then assessed in its ability to detect allelic imbalance within tumour samples from a series of colorectal cancer patients. The assay was shown to clearly categorise tumours according to allelic proportion, including in a subset of patients in whom fluorescent microsatellite analysis was ambiguous. The assay was then assessed in its limit of detectability by artificially introducing allelic imbalance in control plasma by the addition of homozygous normal DNA, showing a limit of detectability of between 3-5ng /ml of added exogenous DNA. The assay was then applied to a series of cancer cases and controls, but was unable to discriminate between these plasmas using this approach. Whilst the results of this assay in tumour samples and in artificially induced allelic imbalance were encouraging, the assay was not sufficiently accurate to be discriminatory in regard to plasma DNA. It appears that this approach, in itself, is not sufficient to negate the difficulties of using plasma DNA substrates. A potential explanation of these findings is a function of the low copy number DNA in plasma, resulting in a non-proportional number of alleles being introduced into each reaction well.

Therefore in Chapter 7 experiments were performed to try to obviate this potential sampling error, by counting large numbers of individual alleles. This approach was initially validated on normal control DNA, and then performed on a test case, with high levels of plasma DNA and LOH in the tumour. This analysis demonstrated matching allelic imbalance in plasma and tumour tissue, showing proof of principle for this approach. On the basis of this allele counting was assessed in a small series of heterozygous cases and controls. Plasma analysis was performed in a blinded fashion in order to limit potential bias. All of these cases and controls demonstrated allelic balance within plasma, but subsequent analysis showed allelic

balance in the cases tumours also. In retrospect therefore it would have been better to perform unblinded analysis to ensure that the series included cases demonstrating allelic imbalance at the relevant locus, which might then be detected in the plasma. That said this non-informativity of the series would be potentially circumvented by assessment at further loci. These results also constitute an important negative, since this approach demonstrated high specificity, in that assessment of each plasma demonstrated allelic balance and hence the presence of no false positives. This would suggest that assessing sufficient numbers of alleles for each plasma sample improves test accuracy. This improved specificity was however hard fought, requiring labour intensive and expensive methods that would not be practical to apply to clinical practice. That said recent developments that allow examination of multiple individual alleles by in a single reaction tube, or by chip based arrays, might circumvent some of these limitations in the future. Pursuing these approaches seems worthwhile given the strategic informativity of LOH at a panel of markers both in colorectal and other cancers.

At present it appears positive results from plasma DNA assays are to a large degree dependent on steps that produce enrichment of mutant sequences. Certainly the wealth of literature regarding k-ras mutations in colorectal and other cancers, with reasonable sensitivity and specificity, employ methods that select and amplify mutant sequence. As such low copy number mutant template within an excess of normal sequences may become detectable, but these assays have ability to be qualitative only and provide little information regarding relative proportions of tumour derived DNA within plasma. These difficulties may be obviated by technological advances that allow the assessment of multiple alleles or markers in single experiments. Alternatively techniques that enable improved extraction or quality of template such

as whole genome amplification may result in more robust template with which to assess quantitative assays.

There will remain however some important strategic questions, particularly since assessments of plasma based assays to date have been performed on subjects with proven cancer detected by other means rather than on asymptomatic populations. Many cancers have been shown to demonstrate genetic alterations the same genes, such as p53. If an asymptomatic person was demonstrated to harbour such a mutation in the plasma, much research remains to be done to inform how that patient might be further investigated clinically to confirm or exclude any of these disease states. It is likely that any test that comes to clinical utility will involve a panel of markers, and some information as to likely disease process may be determined from the pattern of changes across the panel, such as patterns of LOH at given chromosomal locations, but data is lacking in this regard at present.

CHAPTER 10:

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Information Sheet:

Full project title

Development of techniques for detecting gene mutations in plasma of patients undergoing investigation and treatment for benign or malignant colorectal tumours

Short lay title

Gene mutations in plasma of patients undergoing investigation and treatment for suspected bowel conditions

Principal Investigator

Professor MG Dunlop MD FRCS
Department of Surgery
Western General Hospital, Edinburgh EH4 2XU

Contact number 0131 537 1546 (or 467 8439 in event of any urgent problem relating to the study)

Independent advisor (who is not involved in the research but can provide further information)

Mr IMC Macintyre FRCS
Department of Surgery
Western General Hospital, Edinburgh EH4 2XU
Tel 0131 537 1549

Clinical protocol
Detecting changes in DNA in the blood of patients undergoing investigation and treatment for benign or malignant colorectal tumours

Invitation:
You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You'. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, London N16 0BW.

Thank you for reading this.

What is the purpose of this study?
Certain diseases of the large bowel result from changes in the genetic code (DNA) in the cells lining the bowel. This study is designed to determine how often, and for which diseases, the same genetic changes seen in the bowel can be detected in the blood. The ultimate aim is to be able to detect bowel diseases, including growths in the bowel, by way of performing a blood test.

Why have I been chosen?
We need to test people who are undergoing investigation and/or treatment for a suspected large bowel condition. We also need to test people who do not have any known bowel condition in order to be sure that our test results don't simply occur in everyone whether they have bowel disease or not.

Do I have to take part?
It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen if I take part?
The study will make no difference to how your condition is treated, or how often you are followed up after treatment. If you agree to take part in the study you will be asked to sign a form confirming your consent to be involved. We will then require a blood sample, which can be taken at the same time as routine blood tests that occur as part of your tests or treatment anyway. Similarly we would take a further blood test following your investigation or treatment. Should you be found to have a disease of the large bowel during your tests, or you are undergoing an operation for a known large bowel disease, then we would take a portion of the diseased tissue. We will perform genetic tests on both the diseased tissue and the blood samples. We also require to examine specific parts of your medical notes, namely your operation and pathology reports, so that we can relate findings in these to the results of your genetic tests.

What do I have to do?
Allow us to take two blood samples from you, and a piece of tissue at your operation or test. There are no other requirements of this study, and you do not need to take any further action.

What is the procedure being tested?
This is a study looking at a series of new tests, in order to determine if they might be able to provide additional information about diseases of the large bowel. For example, it might be possible to tell whether people have a benign or malignant tumour simply using a blood test.

Are there any side effects or risks of taking part?
There are no side effects or risks to taking part. You will have blood taken routinely as part of your care, and the study only requires some additional samples.

What are the potential benefits of taking part?
There is no direct benefit to yourself for taking part in this study. The information we get from this study may help us diagnose and treat future patients with large bowel diseases better.

What if something goes wrong?
It is not foreseen that you can come to any harm by taking part in this study, however if you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or

treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you.

Will my taking part in this study be kept confidential?
All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it. Because your participation has no implications for your clinical care, we will not inform your General Practitioner of your participation in this study.

What will happen to the results of the research study?
You will **not** be informed of the results of the genetic tests. As this is a study of tests at an early stage of their development, the results of them will not have a proven meaning that would relate to *your* health or your treatment. Similarly doctors involved in your care will not be informed of the results.
The results of the study may be published in medical journals, but it will not be possible to identify you from these. Should you be interested in the collective results you may get details of where they have been published (probably in 2003) from the contact person given below.

Who is Organising and Funding the Research?
The study is being undertaken by researchers employed by the University of Edinburgh.
The study is funded by grants from Cancer Research UK (formerly the Cancer Research Campaign) and the Chief Scientists Office (CSO). The doctors involved in your care are not paid for your inclusion into this study.

Who has reviewed the study?

This work forms part of a major project aimed at combating cancer of the large bowel and funded to Professor Dunlop by the largest cancer charity in the UK, Cancer Research UK. This work has been the subject of intensive peer review by Cancer Research UK and has gone forward for funding for a further 5 years. The Lothian Research Ethics Committee has also reviewed the ethical aspects of the study.

Contact for further information:

Dr Andrew Clark
Clinical Research Fellow
MRC Human Genetics Unit
Western General Hospital
Edinburgh

Tel: 0131 332 2471 Ext. 2106

You will be given a copy of this information sheet and a copy of the consent form to keep.

Centre Number: :
Study Number:
Patient Identification Number for this trial:

Colon Cancer Genetics Group
Division of Molecular and

Department of Surgery
Western General Hospital
Edinburgh EH4 2XU
United Kingdom

CONSENT FORM

Full project title

Development of techniques for detecting gene mutations in plasma of patients undergoing investigation and treatment for benign or malignant colorectal tumours

Short lay title

Gene mutations in plasma of patients with colorectal tumours

Clinical protocol

Detecting changes in DNA in the blood of patients undergoing investigation and treatment for benign or malignant colorectal tumours

Principal Investigator Surgery 2XU	*Prof.* MG Dunlop MD FRCS, Department of Western General Hospital, Edinburgh EH4 Tel. number 0131 537 1546
Independent advisor Surgery *(a doctor not involved in the research* 2XU *but able to provide further information)*	Mr IMC Macintyre FRCS, Department of Western General Hospital, Edinburgh EH4 Tel 0131 537 1549

- **I have read and understood this consent form and Information Sheet and had opportunity to ask questions about them**
- **I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.**
- **I agree to participate in this study which requires taking a blood sample before and after a bowel test or operation and for a tissue sample/biopsy to be collected and analysed.**
- **I agree to review of my medical and pathology records, as well as records held on computer files and databases.**

Signature ..... **Name of Patient/Subject**.....

Date: .....

Investigator .....

Date: .....
Date: .....

Appendix 2

Applied Biosystems

CERTIFICATE

THIS IS TO CERTIFY THAT **Dr Andrew J Clark**

HAS SATISFACTORILY COMPLETED A COURSE ON **ABI PRISM® 7900 HT Sequence Detection System Course**

DATE **8 – 9 January 2002**

SIGNED

TRAINING MANAGER

MD/AJC number	Age	Sex	Stage	CANCER OR POLYP	SITE	alive	date censored	survival days	Picogreen 1	2	3	mean	RT PCR plasma DNA quantuty ng/ml
1051	41	F	ADENOMA	POLYP	RECTUM	y	16/08/2005	1404					
2461	45	M	II	CA	RECTUM	yes	30/04/2007	2026	1.047	1.107	0.928	1.027333333	467
2488	47	M	III	CA	RECTUM	yes	30/04/2007	2015	0.379	0.318	0.303	0.333333333	16.9
2487	43	M	II	CA	RECTUM	no	05/12/2004	1112	0.142	0.18	0.153	0.158333333	
2489	50	M	ADENOMA	POLYP	RIGHT	yes	08/08/2008	2450	0.581	0.472	0.559	0.537333333	
2490	73	F	IV	CA	RIGHT	no	05/09/2002	286	0.666	0.595	0.613	0.624666667	331
2491	66	M	III	CA	SIGMOID	yes	11/07/2007	2056	1.028	0.854	0.9	0.927333333	
2494	54	F	IV	CA	RECTUM	no	23/01/2002	12	3.851	2.109	2.123	2.694333333	182.4
2530	73	F	IV	CA	RIGHT	no	07/12/2002	317	5.141	4.847	4.74	4.909333333	1808.6
2532	62	M	II	CA	SIGMOID	yes	30/04/2007	1900	0.489	0.402	0.399	0.43	303.6
2533	76	M	IV	CA	RECTUM	no	15/06/2002	120	0.325	0.327	0.273	0.308333333	
2534	73	M	IV	CA	RECTUM AND LIVER	yes	11/02/2008	2187	0.253	0.233	0.236	0.240666667	
2535	47	M	N/A	CONTROL	N/A				0.259	0.245	0.427	0.310333333	26.4
2541	85	F	II	CA	RECTUM				0.515	0.411	0.441	0.455666667	157.6
2536	68	M	IV	CA	SIGMOID	no	09/04/2003	415	1.95	1.623	1.62	1.731	
2537	73	F	II	CA	RIGHT	yes	03/04/2008	2236	0.505	0.443	0.415	0.454333333	176.8
2538	63	M	II	CA	RIGHT	yes	30/04/2007	1897	0.33	0.348	0.384	0.354	
2539 (2540)	66	M	III	CA	RIGHT	yes	30/04/2007	1895	1.094	1.049	1.039	1.060666667	309
(531)	32	F	N/A	CONTROL	N/A	yes	14/03/2006	1478	1.016	0.588	0.309	0.637666667	497.4
2543	44	F	N/A	CONTROL	N/A	yes	08/10/2007	2051	0.3	0.23	0.21	0.246666667	217.8
2544	23	F	N/A	CONTROL	N/A	yes	23/07/2008	2340	0.27	0.289	0.305	0.288	
2565	79	F	II	CA	CAECUM	no	04/11/2003	588	3.94	2.703	2.832	3.158333333	

2566	80	F	IV	CA	ASC	no	20/06/2002	73	0.411	0.351	0.409	0.390333333	
2567	75	M	III	CA	ASC				0.431	0.384	0.39	0.401666667	291
2568	78	F	IV	CA	SIGMOID	no	20/02/2003	311	0.674	0.699	0.613	0.662	279.6
2569	60	F	III	CA	ASC	yes	30/04/2007	1838	0.794	0.821	0.68	0.765	
2596	37	F	II	CA	RECTUM	yes	30/04/2007	1820	0.174	0.171	0.13	0.158333333	
2597	62	M	II	CA	SIGMOID	yes	30/04/2007	1820	13.957	12.569	14.214	13.58	5097
2599	69	F	III	CA	SIGMOID	no	26/06/2004	780	0.387	0.525	0.321	0.411	
2601	80	M	I	CA	RIGHT	yes	21/08/2007	1919	0.227	0.216	0.234	0.225666667	
2602	59	F	III	CA	RIGHT	yes	26/09/2008	2321	0.208	0.236	0.207	0.217	
2603	81	M	IV	CA	SIGMOID	no	28/04/2003	343	0.097	0.135	0.119	0.117	
2604	71	M	II	CA	RIGHT	no	09/04/2004	683	0.37	0.359	0.32	0.349666667	
2605	76	M	II	CA	SIGMOID	yes	30/04/2007	1799	0.506	0.606	0.576	0.562666667	186.4
2606	80	M	III	CA	RECTUM	yes	10/10/2008	2328	0.217	0.233	0.249	0.233	
2607	74	F	I	CA	SIGMOID	yes	30/04/2007	1799	24.688	0.2	0.196	8.361333333	
2608	64	F	N/A	POLYP/leio	SIGMOID	yes	13/10/2008	2330	0.025	-0.005	0.021	0.013666667	
2609	58	M	II	CA	SPLEN FLEX	yes	19/08/2008	2268	0.176	0.31	0.175	0.220333333	39
2610	73	F	I	CA	RECTUM	yes	30/04/2007	1790	0.323	0.273	0.258	0.284666667	
2644	68	M	II	CA	RECTUM	yes	14/10/2008	2310	0.178	0.255	0.231	0.221333333	
2645	71	F	IV	CA	RIGHT	no	26/03/2003	280	1.986	1.784	1.671	1.813666667	737
2657	56	F	II	CA	RECTUM	yes	19/08/2008	2247	0.243	0.192	0.212	0.215666667	
2661	70	F	I	CA	SIGMOID	yes	30/04/2007	1757	1.144	1.593	2.045	1.594	146
2662	78	F	III	CA	RECTUM	no	02/02/2005	940	0.648	0.627	0.682	0.652333333	221.2
2678	70	M	III	CA	TRANS	yes	16/07/2008	2193	0.732	0.809	0.694	0.745	
2679	86	F	II	CA	RIGHT	yes	25/01/2008	2019	0.272	0.546	0.218	0.345333333	78.2
2680	78	M	II	CA	SIGMOID	no	04/05/2005	1017	0.271	0.265	0.243	0.259666667	

2681	79	M	IV	CA	RECTUM	no	12/05/2003	294	0.433	0.451	0.444	0.442666667	
2682	57	M	III	CA	RIGHT	yes	30/04/2007	1736	0.429	0.317	0.358	0.3375	
2683	73	F	I	CA	RECTUM	yes	10/07/2008	2173	0.416	0.373	0.407	0.39	111.2
2684	63	M	I	CA	RECTUM	yes	30/04/2007	1735	0.409	0.338	0.364	0.351	117.8
2699	58	F	III	CA	SPLEN FLEX	no	19/09/2003	409	0.215	0.231	0.198	0.2145	22.4
2700	72	F	IV	CA	RECTUM	no	14/12/2003	495	0.311	0.248	0.318	0.283	82.8
579	50	M	TWO	SB CA	SMALL BOWEL & ureter	yes	29/08/2008	2209					
2710	74	F	III	CA	SIGMOID	yes	20/10/2005	1158	0.144	0.19	0.052	0.121	138.8
2711	58	F	III	CA	RECTOSIG	yes	05/09/2008	2209	0.158	0.149	0.137	0.143	
2713	66	F	II	CA	RIGHT	yes	18/04/2005	966	6.76	5.608	5.847	5.7275	
2714	71	F	III	CA	RIGHT	yes	17/10/2008	2242	0.171	0.089	0.076	0.0825	43
2715	80	M	?	CA	RECTUM	no	23/01/2003	148	1.073	0.966	1.008	0.987	
2719	88	F	ADENOMA	?	HEPATIC	yes	10/07/2006	1412	0.18	0.183	0.173	0.178	
2720	55	F	ADENOMA	POLYP	SIGMOID	yes	07/07/2008	2126	0.226	0.238	0.178	0.208	
2721	74	F	N/A	CONTROL	N/A	yes	01/09/2008	2182	0.142	0.145	0.119	0.132	47.4
2722	74	M	IV	CA	SIGMOID	no	20/12/2002	95	0.094	0.159	0.187	0.173	
2723	69	M	ADENOMA	POLYP	RECTUM	yes	07/07/2008	2119	0.167	0.167	0.129	0.148	
2724	63	F	I	POLYP/IN SITU	SIGMOID	no	31/08/2007	1808	2.441	2.217	2.177	2.197	
2725	62	M	N/A	CONTROL	N/A	yes	21/07/2007	1767	0.308	0.331	0.295	0.313	99.8
2726	59	F	N/A	CONTROL	N/A	yes	27/02/2006	1258	0.127	0.164	0.133	0.1485	27.2
68	29	M	N/A	CONTROL	N/A	yes	30/04/2007	2021	-0.004	0.06	0.021	0.0405	
69	29	F	N/A	CONTROL	N/A	yes	30/04/2007	2021	0.13	0.23	0.127	0.1785	17.8
70	29	F	N/A	CONTROL	N/A	yes	30/04/2007	2021	0.104	0.153	0.167	0.16	

71	27	M	N/A	CONTROL	N/A	yes	30/04/2007	2021	0.197	0.184	-0.025	0.0795	48.2
72	37	F	N/A	CONTROL	N/A	yes	30/04/2007	2021	0.135	0.159	0.118	0.1385	77
73	22	M	N/A	CONTROL	N/A	yes	30/04/2007	2021	0.27	0.19	0.245	0.2175	
74	47	M	N/A	CONTROL	N/A	yes	30/04/2007	2021	0.128	0.173	0.165	0.169	
75	35	F	N/A	CONTROL	N/A	yes	30/04/2007	2021	0.148	0.14	0.134	0.137	29.8
2781	56	M	II	CA	SPLEN FLEX	yes	30/04/2007	1624	0.074	0.118	0.081	0.0995	
2782	68	F	ADENOMA	CA/ADENOMA	RECTUM	yes	30/04/2007	1624	0.127	0.161	0.173	0.167	
2793	70	F	I	CA	RECTUM	yes	07/02/2007	1535	0.38	0.133	0.106	0.1195	
2803	70	F	I	CA	RIGHT	yes	07/07/2008	2008	0.114	0.127	0.094	0.1105	
2804	59	M	II	CA	RIGHT	yes	07/07/2005	912	0.989	0.826	0.901	0.8635	
2805	56	M	I	CA	RECTOSIG	yes	19/09/2008	2082	0.088	0.066	0.069	0.0675	
2820	49	M	N/A	CONTROL	N/A	yes	09/07/2007	1636	0.131	0.147	0.146	0.1465	
2821	76	F	N/A	CONTROL	N/A	no	24/02/2007	1501	0.121	0.124	0.181	0.1525	
2822	61	M	IV	CA	RIGHT	no	26/07/2003	192	0.123	0.136	0.212	0.174	
2833	50	F	N/A	CONTROL	N/A	yes	01/10/2005	969	0.063	0.121	0.093	0.107	
2834	73	M	N/A	CONTROL	N/A	yes	20/06/2008	1962	2.451	2.268	2.135	2.2015	
2835	53	M	N/A	CONTROL	N/A	yes	05/02/2007	1461	0.428	0.392	0.378	0.385	
2836	70	F	N/A	CONTROL	N/A	yes	06/06/2008	1948	0.106	0.094	0.07	0.082	
2848	60	M	III	CA	RECTUM	yes	30/04/2007	1526	0.095	0.121	0.054	0.0875	
2849	67	M	III	CA	RIGHT	no	02/02/2004	343	0.007	0.031	0.01	0.0205	
2850	54	M	I	CA	RECTUM	yes	30/04/2007	1525	8.453	8.312	8.091	8.2015	
2851	84	F	II	CA	RIGHT	no	20/05/2004	444	0.019	0.078	0.052	0.065	
2852	82	M	II	CA	SIGMOID	no	14/08/2006	1260	1.42	1.401	0.787	1.094	
2886	33	M	ADENOMA	POLYP	RIGHT				-0.011	0.073	0.041	0.057	
2914	62	F	II	CA	RECTUM	yes	30/04/2007	1475	0.017	0.073	0.014	0.0435	

2915	62	M	II	CA	RECTUM	yes	30/04/2007	1475	0.048	0.048	0.049	0.0485
2917	69	M	II	CA	RECTUM	yes	30/04/2007	1469	0.096	0.103	0.112	0.1075
2918	74	F	I	CA	RECTUM	yes	30/04/2007	1469	0.119	0.137	0.065	0.101
2954	52	M	II	CA	RECTOSIG	yes	30/04/2007	1421	0.021	0.084	0.071	0.0775
2955	69	M	I	CA	RECTOSIG	no	31/08/2006	1179	0.01	0.026	-0.017	0.0045
2979	37	F	III	CA	RIGHT	yes	30/04/2007	1398	0.031	0.07	0.034	0.052
2980	81	F	II	CA	RECTUM	no	25/12/2004	529	0.095	0.02	0.008	0.014
2981	79	F	III	CA	RECTUM	no	23/07/2004	374	0.028	0.016	0.026	0.021
5004	61	F	II	CA	RECTUM	no	24/11/2003	41	2.062	2.046	4.329	3.1875
5005	67	M	III	CA	RIGHT	no	19/01/2006	828	0.58	0.616	0.578	0.597
5006	68	M	III	CA	RECTOSIG	no	14/02/2005	476	0.14	0.164	0.117	0.1405
5007	57	F	IV	CA	RECTUM	no	06/07/2004	252	0.801	0.885	0.741	0.813
5008	79	F	III	CA	RIGHT	no	11/12/2006	1139	0.183	0.182	0.156	0.169
5009		F				yes	30/04/2007	1272	0.332	0.357	0.307	0.332
5010	70	F	III	CA	DESC	no	18/05/2006	925	0.227	0.27	0.197	0.2335
5011	55	F	ADENOMA	CA/POLYP	RECTUM	y	07/03/2005	488	0.15	0.135	0.053	0.094
5012	81	M	III	CA	TRANS				0.345	0.393	0.325	0.359
5504 (5699)	44	M	N/A	CONTROL	N/A				0.213	0.166	0.11	0.138
5505 (5700)	58	F	N/A	CONTROL	N/A				0.246	0.285	0.231	0.258
5506 (5701)	40	F	N/A	CONTROL	N/A	yes	18/07/2006	958	0.053	0.112	0.039	0.0755
5507 (5702)	70	M	III	CA	RECTUM	no	18/10/2007	1414	0.108	0.141	0.086	0.1135
5508 (5703)	74	F	III	CA	RIGHT	no	15/08/2004	250	0.378	0.396	0.33	0.363
5509	78	M	II	CA	DESC	yes	03/03/2008	1546	0.922	1.112	0.86	0.986

(5704)												
6335	72	M	N/A	CONTROL	N/A	yes	23/04/2008	1534	0.168	0.173	0.124	0.1485
6336	68	F	N/A	CONTROL	N/A	y	09/09/2005	577	-0.178	-0.13	0.192	0.031
6337	59	M	I	CA	RECTUM	no	05/03/2004	17	0.38	0.422	0.315	0.3685
6338	55	M	N/A	CONTROL	N/A	yes	10/01/2008	1422	0.547	0.644	0.42	0.532
6339	73	F	N/A	CONTROL	N/A	yes	16/10/2008	1702	0.313	0.311	1.001	0.656
6340	55	F	N/A	CONTROL	N/A	y	27/09/2005	587	-0.017	0.012	0.013	0.0125
6341	80	F	I	CA?	RECTUM	no	08/09/2005	562	0.597	0.618	0.493	0.5555
6342	74	M	II	CA	RECTUM	yes	15/07/2008	1603	0.303	0.31	0.195	0.2525
6343	63	M	II	CA	RECTUM	yes	30/04/2007	1154	0.272	0.243	0.183	0.213

MD number	Age	Sex	Stage	SITE	D5S346 allelic height ratios N	T	% change	Call	P	Call (50%)
1051	41	F	ADENOMA	RECTUM	1.63712	no amp	n/a	NI	2.06	NI
2461	45	M	II	RECTUM	1.63716	0.941223	73.9	LOH	1.7394	LOH (dis)
2488	47	M	III	RECTUM	1.52084412	5.931238	390	LOH	1.18427	no LOH (dis)
2487	43	M	II	RECTUM	1.58357601	1.50176	5.5	no LOH	1.3534	no LOH (con)
2489	50	M	ADENOMA	RIGHT	1.67415523	no amp	n/a	NI	2.02357	NI
2490	73	F	IV	RIGHT						
2491	66	M	III	SIGMOID						
2494	54	F	IV	RECTUM	0.49685817	2.908509	585	LOH	2.48214	LOH (con)
2530	73	F	IV	RIGHT	1.64447404	9.542373	480.3	LOH	2.52875	LOH (con)
2532	62	M	II	SIGMOID	2.04890713	1.977046	3.6	no LOH	3.4116	LOH (de novo)
2533	76	M	IV	RECTUM	1.85587071	7.394351	398	LOH	1.49593	no LOH (dis)
2534	73	M	IV	RECTUM AND LI\	1.41857346	0.942321	50.5	LOH	1.17981	no LOH (dis)
2535	47	M	N/A	N/A	2.1230744	n/a	n/a	n/a	3.10699	LOH (de novo)
2541	85	F	II	RECTUM	1.74065266	0.639338	272	LOH	3.78567	LOH (dis)
2536	68	M	IV	SIGMOID	1.4723637	0.417819	352	LOH	1.29865	no LOH (dis)
2537	73	F	II	RIGHT	0.84229717	0.842297	0	no LOH	1.51398	LOH (de novo)
2538	63	M	II	RIGHT	1.5104	1.477168	2.3	no LOH	1.37825	no LOH (con)
2539 (2540)	66	M	III	RIGHT	1.76851198	2.12381	20.1	no LOH	2.4203	LOH (de novo)
(531)	32	F	N/A	N/A	1.83331218	n/a	n/a	n/a	3.26168	LOH (de novo)
2543	44	F	N/A	N/A	2.15472666	n/a	n/a	n/a	3.26311	LOH (de novo)
2544	23	F	N/A	N/A	homozygous					
2565	79	F	II	CAECUM	1.8804763			msi		no msi (dis)
2566	80	F	IV	ASC	1.89723212	1.709246	11	no LOH	2.83303	LOH (de novo)
2567	75	M	III	ASC	2.67830189	5.499537	105	LOH	6.47049	LOH (con)
2568	78	F	IV	SIGMOID	2.61849495	5.652295	115.9	LOH	1.91917	no LOH (dis)
2569	60	F	III	ASC	1.63922102	3.039406	85.4	LOH	1.06014	LOH (dis)
2596	37	F	II	RECTUM	1.40910838	1.947686	38.2	no LOH		
2597	62	M	II	SIGMOID	2.43063584	0.652787	272.4	LOH	3.81921	LOH (dis)

2599	69	F	III	SIGMOID	1.52645788	1.615919	5.9	no LOH	3.39108 LOH (de novo)
2601	80	M	I	RIGHT	homozygous				
2602	59	F	III	RIGHT	1.97721158	0.700894	182.1	LOH	2.4194 no LOH (dis)
2603	81	M	IV	SIGMOID	1.5570011	1.071526	45.3	no LOH	1.94592 no LOH (con)
2604	71	M	II	RIGHT	homozygous				
2605	76	M	II	SIGMOID	1.92666343	1.829056	5.3	no LOH	2.63828 LOH (de novo)
2606	80	M	III	RECTUM	1.94413018	0.575245	238	LOH	2.48455 no LOH (dis)
2607	74	F	I	SIGMOID	1.6366098	0.916583	78.6	LOH	1.61988 no LOH (dis)
2608	64	F	N/A	SIGMOID	homozygous				
2609	58	M	II	SPLEN FLEX	1.4116101	1.429806	1.3	no LOH	1.26267 no LOH (con)
2610	73	F	I	RECTUM	homozygous				
2644	68	M	II	RECTUM	1.54239431	1.081601	42.6	no LOH	0.9903 LOH (dis)
2645	71	F	IV	RIGHT	1.66544154	1.577522	5.6	no LOH	1.28448 no LOH (con)
2657	56	F	II	RECTUM	homozygous				
2661	70	F	I	SIGMOID	no normal				
2662	78	F	III	RECTUM	1.56263397	1.06606	46.6	no LOH	1.84031 no LOH (con)
2678	70	M	III	TRANS	1.59054054	1.329442	19.6	no LOH	1.45425 no LOH (con)
2679	86	F	II	RIGHT	1.67271941	1.775841	6.2	no LOH	1.10593 LOH (de novo)
2680	78	M	II	SIGMOID	homozygous				
2681	79	M	IV	RECTUM	1.72024867	1.839551	6.9	no LOH	1.67082 no LOH (con)
2682	57	M	III	RIGHT	1.48900142	1.496396	0.5	no LOH	1.88061 no LOH (con)
2683	73	F	I	RECTUM	1.8869863	1.754391	7.6	no LOH	3.29716 LOH (de novo)
2684	63	M	I	RECTUM	1.82840394	4.404348	140.9	LOH	1.03027 LOH (dis)
2699	58	F	III	SPLEN FLEX	1.78150499	2.882512	61.8	LOH	2.03551 no LOH (dis)
2700	72	F	IV	RECTUM	1.6710783	0.62805	166.1	LOH	1.07921 LOH (con)
579	50	M	TWO	SMALL BOWEL &	homozygous				
2710	74	F	III	SIGMOID	1.54731979	3.096303	100.1	LOH	1.59934 no LOH (dis)
2711	58	F	III	RECTOSIG	1.53068493	1.283814	19.2	no LOH	NI NI
2713	66	F	II	RIGHT	2.4012129	6.260831	160.7	LOH	2.66614 no LOH (dis)

2714	71 F	III	RIGHT	1.87720403	1.760502	6.6	no LOH	complete	LOH (de novo)
2715	80 M	?	RECTUM	2.28677667	2.145889	6.6	no LOH	2.56775	no LOH (con)
2719	88 F	ADENOMA	HEPATIC	homozygous					
2720	55 F	ADENOMA	SIGMOID	1.55109489	1.50969	2.7	no LOH	2.02634	LOH (de novo)
2721	74 F	N/A	N/A	1.87572143	n/a	n/a	n/a	1.77434	no LOH (con)
2722	74 M	IV	SIGMOID	1.96094188	1.796966	9.1	no LOH	0,851612	LOH (de novo)
2723	69 M	ADENOMA	RECTUM	1.6857184	2.219428	31.7	no LOH	2.10075	no LOH (con)
2724	63 F	I	SIGMOID	1.51110356	1.409812	7.2	no LOH	1.44981	no LOH (con)
2725	62 M	N/A	N/A	1.75975863	n/a	n/a	n/a	0.71135	LOH (de novo)
2726	59 F	N/A	N/A	1.77684662	n/a	n/a	n/a	homozygou	NI
68	29 M	N/A	N/A	2.02072055	n/a	n/a	n/a	NI	NI
69	29 F	N/A	N/A	2.03272324	n/a	n/a	n/a	NI	NI
70	29 F	N/A	N/A	1.47212149	n/a	n/a	n/a	homozygou	NI
71	27 M	N/A	N/A	1.70540098	n/a	n/a	n/a	1.11358	LOH (de novo)
72	37 F	N/A	N/A	2.38188976	n/a	n/a	n/a	homozygou	NI
73	22 M	N/A	N/A	homozygous	n/a	n/a	n/a	homozygou	NI
74	47 M	N/A	N/A	1.68924303	n/a	n/a	n/a	1.21042	LOH (de novo)
75	35 F	N/A	N/A	1.96990146	n/a	n/a	n/a	NI	NI
2781	56 M	II	SPLEN FLEX	homozygous			NI	homozygou	NI
2782	68 F	ADENOMA	RECTUM	1.46724446	3.364337	129.3	LOH	1.16292	no LOH (dis)
2793	70 F	I	RECTUM	1.53525984	0.434744	253.1	LOH	homozygou	NI
2803	70 F	I	RIGHT	1.39727729	1.72664	23.6	no LOH	0.73371	LOH (de novo)
2804	59 M	II	RIGHT	2.24395342	2.900738	29.3	no LOH	2.86906	no LOH (con)
2805	56 M	I	RECTOSIG	1.72142473	2.418214	40.5	no LOH	NI	NI
2820	49 M	N/A	N/A				NI		NI
2821	76 F	N/A	N/A	1.36768552	n/a	n/a	n/a	homozygou	NI
2822	61 M	IV	RIGHT	homozygous			NI	homozygou	NI
2833	50 F	N/A	N/A	1.57408944	n/a	n/a	n/a	0.98963	LOH (de novo)
2834	73 M	N/A	N/A	2.06292178	n/a	n/a	n/a	1.99261	no LOH (con)

2835	53	M	N/A	N/A	1.75498678	n/a	n/a	n/a	0.97143 LOH (dis)
2836	70	F	N/A	N/A	1.6696466	n/a	n/a	n/a	complete LOH (de novo)
2848	60	M	III	RECTUM	1.80225492	1.422132	26.7	no LOH	0.99948 LOH (de novo)
2849	67	M	III	RIGHT	1.98663409	1.489813	33.4	no LOH	homozygou NI
2850	54	M	I	RECTUM	1.61747951	2.03557	25.9	no LOH	1.54945 no LOH (con)
2851	84	F	II	RIGHT	2.17418627	homozygous	n/a	NI	homozygou NI
2852	82	M	II	SIGMOID	3.20727792	42.52431	1225.9	LOH	1.34803 LOH (dis)
2886	33	M	ADENOMA	RIGHT	2.75163734	3.157317	14.7	no LOH	homozygou LOH (de novo) com
2914	62	F	II	RECTUM	homozygous			NI	homozygou NI
2915	62	M	II	RECTUM	1.67223764	1.439001	16.2	no LOH	homozygou NI
2917	69	M	II	RECTUM	homozygous			NI	homozygou NI
2918	74	F	I	RECTUM	2.95832764	3.84236	29.9	no LOH	2.05676 LOH (de novo)
2954	52	M	II	RECTOSIG	homozygous			NI	homozygou NI
2955	69	M	I	RECTOSIG	2.063106	2.524602	22.4	no LOH	4.34966 LOH (de novo)
2979	37	F	III	RIGHT	2.10913999	2.085268	1.1	no LOH	0.84447 LOH (de novo)
2980	81	F	II	RECTUM	homozygous			NI	homozygou NI
2981	79	F	III	RECTUM	homozygous			NI	homozygou NI
5004	61	F	II	RECTUM	2.00409571	0.709121	181	LOH	5.53484 LOH (con)
5005	67	M	III	RIGHT	1.99239905	4.719462	135.5	LOH	1.2893 LOH (con)
5006	68	M	III	RECTOSIG	3.08900052	1.874238	64.8	LOH	1.35803 LOH (con)
5007	57	F	IV	RECTUM				NI	NI
5008	79	F	III	RIGHT	1.98797312	1.981452	0.3	no LOH	1.6334 no LOH (con)
5009		F			1.66520553	1.722693	3.5	no LOH	1.54518 no LOH (con)
5010	70	F	III	DESC	1.72269259	1.025501	68	LOH	1.65386 no LOH (dis)
5011	55	F	ADENOMA	RECTUM	1.84362851	1.792346	2.9	no LOH	0.29984 LOH (de novo)
5012	81	M	III	TRANS	homozygous			NI	homozygou NI
5504 (5699)	44	M	N/A	N/A	2.45363518	n/a	n/a	n/a	1.80411 no LOH (con)
5505 (5700)	58	F	N/A	N/A	2.82631014	n/a	n/a	n/a	2.15517 no LOH (con)

5506 (5701)	40	F	N/A	N/A	1.87647522	n/a	n/a	n/a	3.72554 LOH (de novo)
5507 (5702)	70	M	III	RECTUM	homozygous			NI	homozygou NI
5508 (5703)	74	F	III	RIGHT	2.51389854	6.864665	173.1	LOH	0.17729 LOH (dis)
5509 (5704)	78	M	II	DESC	2.78380062	2.570085	8.3	no LOH	1.18963 LOH (de novo)

MD number	D17S250 allelic height ratios					
	N	T	% change	Call	P	Call (50%)
1051	1.519857		NI	NI	NI	NI
2461	1.602997	4.01362862	150.4	LOH	1.711462451	no LOH (dis)
2488	1.7364351	0.73714195	133.5	LOH	2.014314928	no LOH (dis)
2487	1.7009161	3.3222222	95.3	LOH	1.120056497	LOH (dis)
2489	1.8564381	no tumour trace	n/a	NI	2.490566038	NI
2490						
2491						
2494	1.7157107	1.362208988	26	NO LOH	1.860107495	NO LOH (CON)
2530	1.6458982	no tumour trace	n/a	NI	1.957240728	NI
2532	homozygous	n/a	n/a	NI	homozygous	NI
2533	homozygous	n/a	n/a	NI	homozygous	NI
2534	1.8686235	2.88888889	54.6	LOH	1.828496042	no LOH (dis)
2535	1.709941	n/a	n/a	n/a	0.820037106	LOH (de novo)
2541	1.4622054	1.885822306	29	NO LOH	1.781609195	NO LOH (CON)
2536	1.5671563	2.794019934	78.3	LOH	3.45631068	LOH (con)
2537	0.374376	1.738572323	364.4	LOH	1.614493771	LOH (con)
2538	1.8039807	1.826213819	1.2	NO LOH	4.731448763	LOH (de novo)
2539 (2540)	2.1612102	2.515397083	16.4	NO LOH	3.689312977	LOH (de novo)
(531)	no normal	n/a	n/a	n/a		NI
2543	1.3816672	n/a	n/a	n/a	1.972375691	NO LOH (CON)
2544	homozygous	n/a	n/a	NI	homozygous	NI
2565	1.6239769	1.534223232	5.9	NO LOH	4.102372035	LOH (de novo)
2566	1.5653438	1.776537335	13.4	NO LOH	1.660468876	NO LOH (CON)
2567	1.6441894	1.904882155	15.9	NO LOH	3.933832709	LOH (de novo)
2568	1.4087275	8.398876404	496.2	LOH	5.201197605	LOH (con)
2569	1.4269635	5.115404169	258.5	LOH	1.601656886	no LOH (dis)
2596	homozygous	n/a	n/a	NI	homozygous	NI
2597	1.8971689	2.356519507	24.2	NO LOH	3.149957155	LOH (de novo)

2599	2.1253961		2.068313253		2.8	NO LOH	2.268137848 NO LOH (CON)
2601	1.4583333		4.002214839		174.4	LOH	2.690738474 LOH (con)
2602	1.4728482		2.24126485		52.2	LOH	1.7030185 no LOH (dis)
2603	1.6520503		1.93966898		17.4	NO LOH	1.603993776 NO LOH (CON)
2604	1.4914152		1.841407151		23.5	NO LOH	1.88526373 NO LOH (CON)
2605	1.8755556		1.83286119		2.3	NO LOH	0.633674255 LOH (de novo)
2606	1.5596554		1.199150118		30.1	NO LOH	homozygous NI
2607	1.5799025		1.381733021		14.3	NO LOH	1.751148816 NO LOH (CON)
2608	1.839345	n/a		n/a	n/a		NI
2609	2.3051541		2.619934283		13.7	NO LOH	no amp NI
2610	1.7404422		1.243696254		39.9	NO LOH	1.608733624 NO LOH (CON)
2644	1.7725768		1.562972292		13.4	NO LOH	0.7219593 LOH (de novo)
2645	1.8418848		1.77694357		3.7	NO LOH	1.622454254 NO LOH (CON)
2657	1.8319892		1.660933053		10.3	NO LOH	2.94084507 LOH (de novo)
2661					NI		NI
2662	1.6652502		3.662849873		120	LOH	1.692772277 no LOH (dis)
2678	1.5088706		1.224588939		23.2	NO LOH	1.449419569 NO LOH (CON)
2679	2.3497942		2.207916667		6.4	NO LOH	1.391712275 LOH (de novo)
2680	1.9886307		5.510703364		177.1	LOH	3.838178295 LOH (con)
2681	1.6796537		1.459912136		15.1	NO LOH	1.429275362 NO LOH (CON)
2682	1.5749913		5.174515235		228.5	LOH	1.863082863 no LOH (dis)
2683	2.2209583		2.080213904		6.8	NO LOH	3.605813953 LOH (de novo)
2684	1.552586		0.790518569		96.4	LOH	2.099423631 no LOH (dis)
2699	1.5642702		6.123966942		291.5	LOH	complete LOH (con)
2700	1.4131305		1.181563126		19.6	NO LOH	1.241118229 NO LOH (CON)
579	1.8330286		1.163580247		65.3	LOH	no amp NI
2710	1.8002272		2.386792453		32.6	NO LOH	1.948295455 NO LOH (CON)
2711	1.4136823		1.689179596		19.5	NO LOH	0.990180033 LOH (de novo)
2713	1.6669866		1.229398664		35.5	NO LOH	1.87947736 NO LOH (CON)

2714	no amp	no amp	n/a	NI	no amp	NI
2715	2.6126915	3.575055188		36.8 NO LOH	2.116948378	NO LOH (CON)
2719	1.5063682	1.330313015		13.2 NO LOH	0.163953763	LOH (de novo)
2720	1.7116513	1.637610186		4.5 NI	no amp	NI
2721	1.2340895	?complete	complete	NI	?complete	NI
2722	homozygous	n/a	n/a	NI	homozygous	NI
2723	1.7835821	1.739308462		2.5	no amp	NI
2724	1.4901528	1.608142214		7.9 NO LOH	1.349431568	NO LOH (CON)
2725	1.8458574	no amp	n/a	NI	no amp	NI
2726	1.4622578	n/a	n/a	n/a	no amp	NI
68	no amp	n/a	n/a	n/a	no amp	NI
69	1.6739905	n/a	n/a	n/a	no amp	NI
70	1.6901108	n/a	n/a	n/a	no amp	NI
71	1.6295681	n/a	n/a	n/a	1.904458599	NO LOH (CON)
72	1.7766423	n/a	n/a	n/a	no amp	NI
73	1.686853	n/a	n/a	n/a	no amp	NI
74	1.4954853	n/a	n/a	n/a	0.586330935	LOH (de novo)
75	1.5233083	n/a	n/a	n/a	no amp	NI
2781	1.8370338	1.91206544		4.1 NO LOH	1.558788362	NO LOH (CON)
2782	1.6384142	1.55634896		5.3 NO LOH	no amp	NI
2793	1.4133074	1.270102177		11.3 NO LOH	no amp	NI
2803	1.8158865	2.055929919		13.2 NO LOH	3.176340275	LOH (de novo)
2804	1.6448751	1.52744186		7.7 NO LOH	2.14412678	NO LOH (CON)
2805	homozygous	n/a	n/a	NI	homozygous	NI
2820	2.6313686	n/a	n/a	n/a	no amp	NI
2821	1.7328214	n/a	n/a	n/a	0.984050367	LOH (de novo)
2822	1.811098	2.246777164		24.1 NO LOH	no amp	NI
2833	3.6867679	n/a	n/a	n/a	no amp	NI
2834	1.9032258	n/a	n/a	n/a	2.063860493	NO LOH (CON)

2835	1.539231	n/a	n/a	n/a		2.707889708	LOH (de novo)
2836	homozygous	n/a	n/a	NI		homozygous	NI
2848	2.0640987	1.315041646	57	LOH		3.411550152	LOH (dis)
2849	1.6458091	1.624845194	1.3	NO LOH		2.364791988	NO LOH (CON)
2850	1.7585327	0.562385014	212.7	LOH		1.530462185	no LOH (dis)
2851	homozygous	n/a	n/a	NI		homozygous	NI
2852	1.627923	1.794010635	10.2	NO LOH		1.969194313	NO LOH (CON)
2886	1.9394144	2.298227676	18.5	NO LOH		5.700507614	LOH (de novo)
2914	1.5505828	0.930572473	66.6	LOH		0.277474196	LOH (con)
2915	0.9455113	1.034538586	9.4	NO LOH		1.035472973	NO LOH (CON)
2917	1.6583954	1.677768526	1.2	NO LOH		no amp	NI
2918	1.709077	1.587078652	7.7	NO LOH		no amp	NI
2954	1.5693391	1.790640394	14.1	NO LOH		no amp	NI
2955	1.657784	1.80634501	9	NO LOH		0.1864	LOH (de novo)
2979	1.6997516	1.247050659	36.3	NO LOH		no amp	NI
2980	homozygous	n/a	n/a	NI		homozygous	NI
2981	homozygous	n/a	n/a	NI		homozygous	NI
5004	1.7632445	8.021558872	354.9	LOH		2.617221999	no LOH (dis)
5005	1.2782225	3.917849141	122.2	LOH		no amp	NI
5006	3.5889175	1.654690071	116.9	LOH		no amp	NI
5007	homozygous	n/a	n/a	NI		homozygous	NI
5008	homozygous	n/a	n/a	NI		homozygous	NI
5009	homozygous	n/a	n/a	NI		homozygous	NI
5010	1.4504263	7.875	442.9	LOH		1.38803681	no LOH (dis)
5011	homozygous	n/a	n/a	NI		homozygous	NI
5012	1.8200524	1.486763911	22.4	NO LOH		1.403631787	NO LOH (CON)
5504 (5699)	homozygous	n/a	n/a	NI		homozygous	NI
5505 (5700)	homozygous	n/a	n/a	NI		homozygous	NI

5506 (5701)	homozygous	n/a	n/a		NI	homozygous	NI
5507 (5702)	1.87523	0.6137043	205.6	LOH	2.094288114	no LOH (dis)
5508 (5703)	homozygous	n/a	n/a		NI	homozygous	NI
5509 (5704)	1.7134856	1.732821422	1.1	NO LOH	2.016067146	NO LOH (CON)

MD number	D18 height ratios						survival day	Picogreen			Picogreen
	N	T	% change	call	P	call		1	2	3	mean
1051	NI	NI	n/a	NI	3.205323194	NI	1404				
2461	2.125995399	0.315553412	573.7	LOH	2.792607803	LOH (dis)	2026	1.047	1.107	0.928	1.02733333
2488	1.624660466	1.707712205	5.1	no LOH	1.643769968	no LOH (con)	2015	0.379	0.318	0.303	0.3333333
2487	1.304794521	0.909168736	43.5	no LOH	1.881147541	no LOH (con)	1112	0.142	0.18	0.153	0.1583333
2489	homozygous	NI	n/a	NI	homozygous	NI	2450	0.581	0.472	0.559	0.5373333
2490	NI	NI	NI	NI	NI	NI	286	0.666	0.595	0.613	0.6246667
2491	NI	NI	NI	NI	2.65630713	NI	2056	1.028	0.854	0.9	0.9273333
2494	homozygous	1.684363728	n/a	MSI	10.2584493	MSI	12	3.851	2.109	2.123	2.6943333
2530	1.627582286	0.1051965	1447.2	LOH	3.173955296	LOH (dis)	317	5.141	4.847	4.74	4.9093333
2532	1.675624093	1.682137238	0.4	no LOH	3.493349456	LOH (de nov	1900	0.489	0.402	0.399	0.43
2533	homozygous	NI	n/a	NI	homozygous	NI	120	0.325	0.327	0.273	0.3083333
2534	1.702579306	2.028658214	19.2	no LOH	1.03279939	LOH (de nov	2187	0.253	0.233	0.236	0.2406667
2535	2.036829699	n/a	n/a	n/a	0.190766551	LOH (de novo)		0.259	0.245	0.427	0.3103333
2541	1.692744553	1.60990502	5.1	no LOH	1.37060861	no LOH (con)		0.515	0.411	0.441	0.4556667
2536	2.486517831	0.186	1236.8	LOH	12.33437014	LOH (dis)	415	1.95	1.623	1.62	1.731
2537	12.67479675	0.800954592	1482.5	LOH	0.257789061	LOH (con)	2236	0.505	0.443	0.415	0.4543333
2538	1.130878518	1.135187897	0.4	no LOH	0.696557971	LOH (de nov	1897	0.33	0.348	0.384	0.354
2539 (2540)	1.493886358	1.8108039	21.2	no LOH	2.07029703	no LOH (con)	1895	1.094	1.049	1.039	1.0606667
-531	1.87795323	n/a	n/a	n/a	1.649519503	no LOH (con)	1478	1.016	0.588	0.309	0.6376667
2543	homozygous	n/a	n/a	NI	homozygous	NI	2051	0.3	0.23	0.21	0.2466667
2544	1.686870559	n/a	n/a	n/a	1.97826087	no LOH (con)	2340	0.27	0.289	0.305	0.288
2565				MSI		MSI	588	3.94	2.703	2.832	3.1583333
2566	1.180313958	1.394242184	18.1	no LOH	5.745980707	LOH (de nov	73	0.411	0.351	0.409	0.3903333
2567	homozygous	n/a	n/a	NI	homozygous	NI		0.431	0.384	0.39	0.4016667
2568	1.762151777	10.55235602	498.8	LOH	9.288461538	LOH (con)	311	0.674	0.699	0.613	0.662
2569				MSI		MSI	1838	0.794	0.821	0.68	0.765
2596	homozygous	n/a	n/a	NI	homozygous	NI	1820	0.174	0.171	0.13	0.1583333
2597	2.097547116	3.033094099	44.6	no LOH	1.105128205	LOH (de nov	1820	13.957	12.569	14.214	13.58

2599	1.566978193	1.514802829	3.4	no LOH	1.141019956	no LOH (con	780	0.387	0.525	0.321	0.411
2601	homozygous	n/a	n/a	NI	homozygous	NI	1919	0.227	0.216	0.234	0.2256667
2602	homozygous	n/a	n/a	NI	homozygous	NI	2321	0.208	0.236	0.207	0.217
2603	1.623150432	1.064773831	52.4	LOH	2.920552677	LOH (dis)	343	0.097	0.135	0.119	0.117
2604	1.926985514	1.518382353	26.9	no LOH	2.381093058	no LOH (con	683	0.37	0.359	0.32	0.3496667
2605	1.729278428	0.377878874	357.6	LOH	1.667342799	no LOH (dis)	1799	0.506	0.606	0.576	0.5626667
2606	1.825892269	0.9618701	89.8	LOH	3.01986755	LOH (dis)	2328	0.217	0.233	0.249	0.233
2607	1.257359402	1.285398571	2.2	no LOH	1.504751985	no LOH (con	1799	24.688	0.2	0.196	8.3613333
2608	1.605849241	n/a	n/a	n/a		NI	2330	0.025	-0.005	0.021	0.0136667
2609	2.506285714	2.103648617	19.1	no LOH	1.93429777	no LOH (con	2268	0.176	0.31	0.175	0.2203333
2610	homozygous	n/a	n/a	NI	homozygous	NI	1790	0.323	0.273	0.258	0.2846667
2644	1.264219323	0.845539883	49.5	no LOH	2.068965517	LOH (de nov	2310	0.178	0.255	0.231	0.2213333
2645	homozygous	n/a	n/a	NI	homozygous	NI	280	1.986	1.784	1.671	1.8136667
2657	1.613076923	1.737925775	7.7	no LOH	2.215606509	no LOH (con	2247	0.243	0.192	0.212	0.2156667
2661	homozygous	n/a	n/a	NI	homozygous	NI	1757	1.144	1.593	2.045	1.594
2662	homozygous	n/a	n/a	NI	homozygous	NI	940	0.648	0.627	0.682	0.6523333
2678	1.555981502	1.822887592	17.2	no LOH	1.083599057	no LOH (con	2193	0.732	0.809	0.694	0.745
2679	homozygous	n/a	n/a	NI	homozygous	NI	2019	0.272	0.546	0.218	0.3453333
2680	1.396563973	3.781271596	170.8	LOH	0.778283979	LOH (dis)	1017	0.271	0.265	0.243	0.2596667
2681	1.497038281	2.296333003	53.4	LOH	1.114810073	no LOH (dis)	294	0.433	0.451	0.444	0.4426667
2682	2.664355062	1.929371599	38.1	no LOH	1.665283541	LOH (de nov	1736	0.429	0.317	0.358	0.3375
2683	1.865769582	1.655725922	19.2	no LOH	1.049443758	LOH (de nov	2173	0.416	0.373	0.407	0.39
2684	1.087442003	1.05231352	3.3	no LOH	0.852340145	no LOH (con	1735	0.409	0.338	0.364	0.351
2699	1.288985695	6.687059577	418.8	LOH	8.44858156	LOH (con)	409	0.215	0.231	0.198	0.2145
2700	1.547470052	1.00489335	54	LOH	0.436382218	LOH (con)	495	0.311	0.248	0.318	0.283
579	1.667306569			NI	#DIV/0!	NI	2209				
2710	homozygous	n/a	n/a	NI	homozygous	NI	1158	0.144	0.19	0.052	0.121
2711	1.266857072	0.809662268	56.5	LOH	1.402717391	no LOH (dis)	2209	0.158	0.149	0.137	0.143
2713	1.570701265			MSI	1.600019826	no MSI	966	6.76	5.608	5.847	5.7275

2714	homozygous	n/a	n/a	NI	homozygous	NI	2242	0.171	0.089	0.076	0.0825
2715	1.212275636	1.128377595	7.4	no LOH	1.77594442	no LOH (con	148	1.073	0.966	1.008	0.987
2719	1.354819977	1.52688172	12.7	no LOH	1.70573108	no LOH (con	1412	0.18	0.183	0.173	0.178
2720	0.98415722	3.858943906	292.1	LOH	0.635603345	LOH (dis)	2126	0.226	0.238	0.178	0.208
2721	1.114692536	n/a	n/a	n/a	2.759281437	LOH (de nov	2182	0.142	0.145	0.119	0.132
2722	homozygous	n/a	n/a	NI	homozygous	NI	95	0.094	0.159	0.187	0.173
2723	homozygous	n/a	n/a	NI	homozygous	NI	2119	0.167	0.167	0.129	0.148
2724	1.364746756	1.366978326	0.2	no LOH	1.253570242	no LOH (con	1808	2.441	2.217	2.177	2.197
2725	homozygous	n/a	n/a	NI	homozygous	NI	1767	0.308	0.331	0.295	0.313
2726	1.585460292	n/a	n/a	n/a	1.086440678	no LOH (con	1258	0.127	0.164	0.133	0.1485
68	2.201560232	n/a	n/a	n/a		NI	2021	-0.004	0.06	0.021	0.0405
69	1.062300678	n/a	n/a	n/a	7.7	LOH (de nov	2021	0.13	0.23	0.127	0.1785
70	1.352882261	n/a	n/a	n/a	5.596837945	LOH (de nov	2021	0.104	0.153	0.167	0.16
71	1.713189212	n/a	n/a	n/a	1.470873786	no LOH (con	2021	0.197	0.184	-0.025	0.0795
72	1.637222005	n/a	n/a	n/a	0.127731092	LOH (de nov	2021	0.135	0.159	0.118	0.1385
73	1.54216386	n/a	n/a	n/a	1.353467562	no LOH (con	2021	0.27	0.19	0.245	0.2175
74	1.604863404	n/a	n/a	n/a	1.293693694	no LOH (con	2021	0.128	0.173	0.165	0.169
75	1.513622421	n/a	n/a	n/a	0.834029624	LOH (de nov	2021	0.148	0.14	0.134	0.137
2781	1.507707399	1.36137163	10.7	no LOH		NI	1624	0.074	0.118	0.081	0.0995
2782	1.742975146	1.690701769	3.1	no LOH	2.6	no LOH (con	1624	0.127	0.161	0.173	0.167
2793	homozygous	n/a	n/a	NI	homozygous	NI	1535	0.38	0.133	0.106	0.1195
2803	1.432533792	1.084585337	32.1	no LOH	3.928864569	LOH (de nov	2008	0.114	0.127	0.094	0.1105
2804	1.511133775	1.518059002	0.5	no LOH	1.423355025	no LOH (con	912	0.989	0.826	0.901	0.8635
2805	1.097787959	0.685486131	60.1	LOH		NI	2082	0.088	0.066	0.069	0.0675
2820	1.989374156	n/a	n/a	n/a	1.871932515	no LOH (con	1636	0.131	0.147	0.146	0.1465
2821	2.133560187	n/a	n/a	n/a	homozygous	NI	1501	0.121	0.124	0.181	0.1525
2822	1.583903092	6.661111111	320.6	LOH	2.254649499	no LOH (dis)	192	0.123	0.136	0.212	0.174
2833	1.964304885	n/a	n/a	n/a	1.974609375	no LOH (con	969	0.063	0.121	0.093	0.107
2834	1.621750765	n/a	n/a	n/a	1.991068361	no LOH (con	1962	2.451	2.268	2.135	2.2015

2835	1.40614961	n/a	n/a	n/a	1.605967078	no LOH (con)	1461	0.428	0.392	0.378	0.385
2836	homozygous	n/a	n/a	NI	homozygous	NI	1948	0.106	0.094	0.07	0.082
2848	1.298254664	1.361671764	4.9	no LOH	5.645914397	LOH (de nov	1526	0.095	0.121	0.054	0.0875
2849	homozygous	n/a	n/a	NI	homozygous	NI	343	0.007	0.031	0.01	0.0205
2850	homozygous	n/a	n/a	NI	homozygous	NI	1525	8.453	8.312	8.091	8.2015
2851	1.34523428	3.90702	190.4	LOH	0.255681818	LOH (dis)	444	0.019	0.078	0.052	0.065
2852	1.398295916	3.214897825	129.9	LOH	1.691225296	no LOH (dis)	1260	1.42	1.401	0.787	1.094
2886	homozygous	n/a	n/a	NI	homozygous	NI		-0.011	0.073	0.041	0.057
2914	1.703441104	1.876850606	10.2	no LOH	homozygous	NI	1475	0.017	0.073	0.014	0.0435
2915	1.738737917	1.728219368	0.6	no LOH	1.828888889	no LOH (con)	1475	0.048	0.048	0.049	0.0485
2917	1.340680402	0.273564166	390.1	LOH	#DIV/0!	LOH (con)	1469	0.096	0.103	0.112	0.1075
2918	homozygous	n/a	n/a	NI	homozygous	NI	1469	0.119	0.137	0.065	0.101
2954	homozygous	n/a	n/a	NI	homozygous	NI	1421	0.021	0.084	0.071	0.0775
2955	1.339285714	0.725543478	84.6	LOH	#DIV/0!	LOH (con)	1179	0.01	0.026	-0.017	0.0045
2979	homozygous	n/a	n/a	NI	homozygous	NI	1398	0.031	0.07	0.034	0.052
2980	homozygous	n/a	n/a	NI	homozygous	NI	529	0.095	0.02	0.008	0.014
2981	homozygous	n/a	n/a	NI	homozygous	NI	374	0.028	0.016	0.026	0.021
5004	4.675389408	0	complete	LOH	0	LOH (con)	41	2.062	2.046	4.329	3.1875
5005	1.527759822	1.699262899	11.1	no LOH	2.221252974	no LOH (con	828	0.58	0.616	0.578	0.597
5006	3.101979828	1.626779847	90.7	LOH	homozygous	NI	476	0.14	0.164	0.117	0.1405
5007	homozygous	n/a	n/a	NI	homozygous	NI	252	0.801	0.885	0.741	0.813
5008	homozygous	n/a	n/a	NI	homozygous	NI	1139	0.183	0.182	0.156	0.169
5009				NI		NI	1272	0.332	0.357	0.307	0.332
5010	1.630440887	5.909166667	210.8	LOH	1.075145805	LOH (dis)	925	0.227	0.27	0.197	0.2335
5011	1.592945503	0.23408414	580.5	LOH	0.891570739	LOH (con)	488	0.15	0.135	0.053	0.094
5012	homozygous	n/a	n/a	NI	homozygous	NI		0.345	0.393	0.325	0.359
5504 (5699)	1.71755035	n/a	n/a	n/a	2.351918159	no LOH (con)		0.213	0.166	0.11	0.138
5505 (5700)	1.170239051	n/a	n/a	n/a	1.086884644	no LOH (con)		0.246	0.285	0.231	0.258

5506 (5701)	homozygous	n/a	n/a	NI	homozygous	NI	958	0.053	0.112	0.039	0.0755
5507 (5702)	1.682391724	0.721505481	133.2	LOH	0.578343949	LOH (con)	1414	0.108	0.141	0.086	0.1135
5508 (5703)	1.33812299	0.843038	58.7	LOH	2.409820585	LOH (dis)	250	0.378	0.396	0.33	0.363
5509 (5704)	homozygous	n/a	n/a	NI	homozygous	NI	1546	0.922	1.112	0.86	0.986

MD number	Age	Sex	Stage	CANCER OR PC	SITE	APC 15n	LOH 310 346		LOH 310 82	
							%	call	%	call
2461		45 M	II	CA	RECTUM	HETERO	48.3	poss	NI	NI
2488		47 M	III	CA	RECTUM	HETERO	264.9	clear	198.2	clear
2487		43 M	II	CA	RECTUM	FAM	23.1	poss	18.6	N
2489		50 M	ADENOMA	POLYP	RIGHT	VIC	NI	NI	NI	NI
2490		73 F	IV	CA	RIGHT	HETERO	11.8	N	5.9	N
2491		66 M	III	CA	SIGMOID	FAM	37.7	poss	31.4	poss
2494		54 F	IV	CA	RECTUM	HETERO	NI	NI	2.1	N
2530		73 F	IV	CA	RIGHT	HETERO	364.6	clear	NI	NI
2532		62 M	II	CA	SIGMOID	HETERO	NI	NI	8.2	N
2535		47 M	N/A	CONTROL	N/A	HETERO	N/A	N/A	N/A	N/A
2541		85 F	II	CA	RECTUM	HETERO	46.1	poss	28.5	poss
2536		68 M	IV	CA	SIGMOID	FAM	48.4	poss	46.2	poss
2537		73 F	II	CA	RIGHT	HETERO	27.9	poss	41	poss
2538		63 M	II	CA	RIGHT	FAM	16.6	N	10.8	N
2539		66 M	III	CA	RIGHT	HETERO	15.2	N	18.5	N
(531)		32 F	N/A	CONTROL	N/A	HETERO	N/A	N/A	N/A	N/A
2543		44 F	N/A	CONTROL	N/A	HETERO	N/A	N/A	N/A	N/A
2544		23 F	N/A	CONTROL	N/A	FAM	N/A	N/A	N/A	N/A
2565		79 F	II	CA	CAECUM	VIC	MSI	MSI	34.8	poss
2566		80 F	IV	CA	ASC	VIC	NI	NI	17.2	N
2567		75 M	III	CA	ASC	HETERO	97.3	clear	89.5	clear
2568		78 F	IV	CA	SIGMOID	HETERO	44.8	poss	49.7	poss
2569		60 F	III	CA	ASC	FAM	39	poss	34.6	poss
2596		37 F	II	CA	RECTUM	FAM	19.2	N	NI	NI
2597		62 M	II	CA	SIGMOID	HETERO	199.4	clear	92.8	clear
2599		69 F	III	CA	SIGMOID	FAM	NI	NI	11.1	N
2601		80 M	I	CA	RIGHT	FAM	33.5	poss	NI	NI
2602		59 F	III	CA	RIGHT	FAM	17.3	N	12.8	N
2603		81 M	IV	CA	SIGMOID	FAM	42.7	poss	39.6	poss
2604		71 M	II	CA	RIGHT	FAM	NI	NI	2.4	N
2605		76 M	II	CA	SIGMOID	HETERO	1.8	N	NI	NI
2606		80 M	III	CA	RECTUM	FAM	46.2	poss	NI	NI

2609		58	M	II	CA	SPLEN FLEX	HETERO		4.3	N	NI	NI
2610		73	F	I	CA	RECTUM	FAM		14.7	N	10.1	N
2644		68	M	II	CA	RECTUM	FAM		19.3	N	NI	NI
2645		71	F	IV	CA	RIGHT	HETERO	NI		NI	9.8	N
2657		56	F	II	CA	RECTUM	FAM		23.5	poss	17.2	N
2661		70	F	I	CA	SIGMOID	HETERO		63.9	clear	NI	NI
2662		78	F	III	CA	RECTUM	HETERO	NI		NI	45.2	poss
2678		70	M	III	CA	TRANS	FAM		6	N	12.1	NI
2679		86	F	II	CA	RIGHT	HETERO		3.7	N	9.2	N
2680		78	M	II	CA	SIGMOID	FAM	NI		NI	NI	NI
2681		79	M	IV	CA	RECTUM	VIC		25.7	poss	18.1	N
2682		57	M	III	CA	RIGHT	FAM		7.8	N	4.3	N
2683		73	F	I	CA	RECTUM	HETERO	NI		NI	11.9	N
2684		63	M	I	CA	RECTUM	HETERO		42.5	poss	NI	NI
2699		58	F	III	CA	SPLEN FLEX	HETERO		13.3	N	NI	NI
2700		72	F	IV	CA	RECTUM	HETERO		39.5	poss	33.1	poss
2710		74	F	III	CA	SIGMOID	HETERO		8.8	N	11.7	N
2714		71	F	III	CA	RIGHT	HETERO		1.1	N	7.4	N
2721		74	F	N/A	CONTROL	N/A	HET	N/A		N/A	N/A	N/A
2725		62	M	N/A	CONTROL	N/A	HET					
2726		59	F	N/A	CONTROL	N/A	HET					
	68	29	M	N/A	CONTROL	N/A	HET					
	69	29	F	N/A	CONTROL	N/A	HET					
	70	27	M	N/A	CONTROL	N/A	HET					
	71	37	F	N/A	CONTROL	N/A	HET					

MD number	7900 N FAM/VIC				7900 T FAM/VIC			
	1	2	3	mean	1	2	3	mean
2461	1.2341887	1.1909365	1.18875104	1.204625413	0.65461313	0.589864	0.579833	0.5798325
2488	1.308634331	1.269368619	1.3261553	1.301386083	2.292416	2.715149	2.808793	2.8087925
2487								
2489								
2490	0.67929816	0.663266377	0.66134292	0.667969152	1.99501	1.807036	1.956214	1.956214
2491								
2494	1.236334549	1.280620185	1.2266844	1.247879711	2.234418	2.32929	2.312827	2.3128266
2530	1.128126284	1.09763848	1.1113255	1.112363421	37.75452	25.45927	18.45189	21.955579
2532	1.165192872	1.047018302	1.09873719	1.103649455	1.32347106	1.20722755	1.208128	1.2081282
2535	1.2354631	1.309284655	1.2509097	1.265219152	n/a	n/a	n/a	n/a
2541	0.960617655	1.000500623	1.03059345	0.997237243	0.49327719	0.5826579	0.45929	0.45929
2536								#DIV/0!
2537	0.74023969	0.741874896	0.748188823	0.74343447	3.658341	3.704023	3.683318	3.683318
2538								
2539	1.215113608	1.368642045	1.20994878	1.264568144	1.991592	1.920399	2.001792	2.0017924
-531								
2543	1.008673928	0.982847287	0.974773728	0.988764981	n/a	n/a	n/a	n/a
2544								
2565								
2566								
2567	1.168593716	1.330848599	1.35627773	1.285240015	2.983133	3.746587	2.231124	2.2311238
2568	1.224684162	1.192807589	1.189287664	1.202259805	2.66283863	2.63334682	2.667927	2.6679273
2569								
2596								
2597	1.170619632	1.185986105	1.15090874	1.169171492	0.344903	0.570683	0.487853	0.487853
2599								
2601								
2602								
2603								
2604								
2605	1.148352063	1.212746769	1.2252845	1.195461111	2.15217	2.239076	2.118564	2.1185635
2606								

2609	1.167596609	1.160603047	1.166846333	1.16501533	1.724278	1.125745	1.10095	1.1009497
2610								
2644								
2645	0.983766397	1.035334982	1.05287936	1.02399358	1.28625445	1.2857658	1.276826	1.2768263
2657								
2661	1.007981525	1.013016249	0.9992733	1.006757025	6.69133026	6.4100281	6.358267	6.3582675
2662	0.91786442	1.082645406	1.10939635	1.036635392	0.66982322	0.6769657	0.683699	0.6836988
2678								
2679	0.991845751	1.010556512	1.00089392	1.001098728	1.27939954	1.1847442	1.121178	1.1211778
2680								
2681								
2682								
2683	1.060433466	1.118036789	1.05837465	1.078948302	1.5240231	1.48417257	1.364857	1.364857
2684	1.076597667	1.079533627	1.07352718	1.076552825	3.47719356	3.8511834	3.397556	3.397556
2699	1.005555494	1.068682096	1.039298348	1.037845313	1.463836	1.647892	1.56383	1.5638296
2700	0.989257834	0.994567475	1.005839276	0.996554862	0.63664638	0.6599963	0.659888	0.6598878
2710	1.331734335	1.399516565	1.300583729	1.343944876	1.49911762	1.4551122	1.398698	1.3986983
2714	1.210966357	1.155310143	1.2009989	1.1890918	1.36351768	1.4302285	1.330372	1.3303719
2721	0.99836727	1.0287368	0.927281894	0.984795321	n/a	n/a	n/a	n/a
2725	1.14638736	1.123783654	1.16990277	1.146691261	n/a	n/a	n/a	
2726	1.2381272	1.21828466	1.19291873	1.21644353	n/a	n/a	n/a	
68	1.22192655	1.23665983	1.20183312	1.220139833	n/a	n/a	n/a	
69	0.9878992	1.02097897	1.031323289	1.013400486	n/a	n/a	n/a	
70	1.098674757	1.109175047	1.14224635	1.116698718	n/a	n/a	n/a	
71	1.200988894	1.18736233	1.14286559	1.177072271	n/a	n/a	n/a	

MD number	LOH 7900 CALL	7900 P FAM/VIC 1	2	3	MEAN	survival days	RT PCR plasma DNA quantuty ng/ml
2461	yes to vic	0.167301	0.07824	0.59354	0.2796937	2026	467
2488	yes to fam	0.142335	0.164825	0.758812	0.355324	2015	16.9
2487						1112	
2489						2450	
2490	no	0.117019	0.101425	0.862075	0.360173	286	331
2491						2056	
2494	yes to fam	0.08457	0.074732	1.84087	0.666724	12	182.4
2530	yes to fam	0.067529	0.079239	1.939478	0.6954153	317	1808.6
2532	n	0.118711	0.163378	0.304834	0.195641	1900	303.6
2535	n/a	0.430781	0.191343	0.7309256	0.4510166		26.4
2541	yes to vic	0.779633	0.644009	0.1355998	0.5197473		157.6
2536						415	
2537	yes to fam	0.115692	0.531793	1.1913981	0.6129609	2236	176.8
2538						1897	
2539	no	0.094948	0.109813	0.9669238	0.3905615	1895	309
-531		0.099295	0.093073	0.7850884	0.3258187	1478	497.4
2543	n/a	0.084847	0.166313	0.7637562	0.3383051	2051	217.8
2544						2340	
2565						588	
2566						73	
2567	yes to fam	0.105809	0.087844	0.683914	0.2925222		291
2568	yes to fam	0.101382	0.11667	0.3960222	0.2046913	311	279.6
2569						1838	
2596						1820	
2597	yes to vic	0.045595	0.055169	2.2467215	0.7824951	1820	5097
2599						780	
2601						1919	
2602						2321	
2603						343	
2604						683	
2605	no	0.120003	0.110112	0.5054065	0.2451738	1799	186.4
2606						2328	

2609	no	0.298506	0.174289	0.2197095	0.2308347	2268	39
2610						1790	
2644						2310	
2645	no	0.097717	0.058175	0.8527505	0.336214	280	737
2657						2247	
2661	yes to fam	0.079665	0.181463	0.3457907	0.2023061	1757	146
2662	yes to vic	0.073029	0.084278	0.4990991	0.2188021	940	221.2
2678						2193	
2679	no	0.208113	0.116559	0.2907455	0.205139	2019	78.2
2680						1017	
2681						294	
2682						1736	
2683	no	0.155972	0.246953	1.9726458	0.7918568	2173	111.2
2684	yes to fam	0.159668	0.055577	0.609082	0.2747754	1735	117.8
2699	yes to fam	0.6693	0.189697	0.9030984	0.5873653	409	22.4
2700	yes to vic	0.067777	0.304895	1.0174643	0.4633788	495	82.8
2710	no	0.319422	0.118815	0.0568015	0.1650127	1158	138.8
2714	no	0.131904	0.401813	0.2341864	0.2559678	2242	43
2721	n/a	0.07687	0.654581	0.415881	0.3824441	2182	47.4
2725	n/a	0.063432	0.249233	0.8690917	0.3939189	1767	99.8
2726	n/a	0.197965	0.309992	0.1863246	0.2314273	1258	27.2
-	n/a	0.120554	0.186155	0.4898377	0.2655155	2021	
-	n/a	0.096508	0.143477	0.3605057	0.2001637	2021	17.8
-	n/a	0.176279	0.12322	0.45816	0.2525533	2021	48.2
-	n/a	0.122516	0.106435	0.11483	0.1145937	2021	77