An evaluation of whole gut lavage fluid for the detection of colorectal cancer using molecular techniques

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Dedication

To my wife and family

Declaration

I declare that this thesis has been composed by myself and that the work contained herein is my own except where specifically acknowledged in the text. This work was performed in the laboratories of the Gastrointestinal Unit and the Molecular Medicine Centre at the Western General Hospital, Edinburgh. These laboratories and their research groups were under the direction of the late Professor Anne Ferguson and Professor Andrew Wyllie FRS at the time. This work has not previously been submitted for a higher degree. Some of the work has been presented at national scientific and surgical meetings and published in peer reviewed journals as detailed in a separate section at the end of this thesis.

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Abbreviations

A	Adenine
AEMs	Attenuated Extracolonic Manifestations
APC	Adenomatous Polyposis Coli
C	Cytosine
Cdks	Cyclin dependent kinases
CHRPE	Congenital Hypertrophy of the Retinal Pigment Epithelium
CRC	Colorectal Cancer
DCBE	Double contrast barium enema
ELISA	Enzyme-Linked Immunosorbent Assay
FAP	Familial Adenomatous Polyposis Coli
FOBT	Faecal Occult Blood Test
FS	Flexible Sigmoidoscopy
G	Guanine
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
IGFIIR	Insulin-like growth factor II receptor
Ki-ras	Kirsten ras
MCR	Mutation Cluster Region
MIN	Microsatellite Instability
MMR	Mismatch Repair
N	Normal
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PTT	Protein Truncation Test
RER	Replication Error

RFLP	Restriction Fragment Length Polymorphism
SSCP	Single Strand Conformational Polymorphism
T	Thymine or Tumour
TGFß RII	Transforming Growth Factor Beta Receptor II
TRAP	Telomeric Repeat Amplification Protocol
3′ UTR	3′ Untranslated region
WGLF	Whole Gut Lavage Fluid

Abstract

Early colorectal cancers (CRC) can be cured with surgery. The five year survival for all stages of the disease remains around 40%. A survival improvement could be attained by increasing the detection of early cancers. Key genes are mutated at specific steps in colorectal carcinogenesis. Telomerase activity has been detected in 90% of CRC. The identification of cancer mutations, or telomerase activity in stool samples may be specific and non-invasive methods by which early colorectal cancers can be detected. All studies to date have encountered problems with impurities in stool inhibiting the polymerase chain reaction (PCR) amplification. Whole gut lavage fluid (WGLF) may provide a sample that is more amenable to molecular analysis. Some individuals have an increased risk of developing CRC, possibly because they have increased numbers of cells containing mutations in cancer genes in the colon. Methods developed for early CRC detection could be exploited to investigate increased cancer susceptibility. In this thesis I assess suitability of WGLF for PCR analysis. I also explore the feasibility of early CRC detection, by examining multiple mutation sites in DNA extracted from WGLF. Telomerase activity has been detected in saline washings from CRC pathology specimens. Thus, I also explore the possibility of detecting telomerase activity in WGLF samples. Finally, by examining synchronous colorectal cancers, I examine the possibility of mutational mosaicism in colorectal epithelium.

DNA was extracted from WGLF obtained pre-operatively from 40 patients undergoing cancer resections. This was analyzed for mutations in 4 genes commonly altered in CRC. Analysis consisted of a PCR/restriction enzyme enrichment strategy for *Ki-ras* and *p53* mutations, while *APC* and Transforming growth factor beta receptor II (*TGFβ RII*) mutations were analyzed by non-enriched single strand conformational polymorphism (SSCP). There were 26 mutations in 18/40 primary tumours (45%); 7 *Ki-ras*, 2 *p53*, 2 *TGFβ RII* and 15 *APC*. In the WGLF 2/7 *Ki-ras* mutations; 2/2 *p53*

mutations; 0/15 *APC* mutations; 0/2 *TGFß RII* mutations were detected. PCR inhibition was encountered and mutations were only detected with the enriched techniques. To assess the potential of detecting telomerase activity in cancer detection, WGLF from 6 patients was examined using the telomerase repeat amplification protocol (TRAP). Initial validation indicated telomerase activity could be detected in the protein extract equivalent to 60 cells per reaction. Analysis of WGLF samples indicated that polyethylene glycol (PEG) at high concentrations inhibited the PCR portion of the TRAP assay. Dilution of the WGLF samples, to give PEG concentrations that allowed PCR, still did not yield positive TRAP assay results. This is possibly due to the dilution of telomerase to concentrations below the sensitivity of the assay. Spiking diluted WGLF samples with 10^3 cells from telomerase positive cell lines still yielded negative results.

Identical *APC* mutations in synchronous CRC would strongly support the notion of mosaicism. In a related line of investigation to identify potential research uses for the molecular analysis of WGLF, I initially examined synchronous colorectal cancers for *APC* mutations. In 12 cases the mutation cluster region of *APC* was examined. No identical *APC* mutations were identified. However, a replication error phenotype was identified in 20% of cases, suggesting an underlying mismatch repair deficiency might predispose to synchronous cancers.

While DNA extracted from WGLF is suitable for PCR analysis, many samples are lost due to contamination or insufficient DNA. WGLF genetic diagnosis requires selective mutation enrichment, other methods used for mutation detection are too insensitive. TRAP inhibition has to be overcome to exploit the detection of telomerase activity in WGLF. Genetic moasicism was not identified as a mechanism for increased cancer risk by studying synchronous cancers.

Summary

The genetic mutations that drive carcinogenesis are known in great detail for the epithelium of the colon and rectum. The early detection of these tumour specific mutations in clinical material holds promise as a tool for early diagnosis and screening for colorectal cancer. This may be of particular use in people with a substantially increased risk of developing colorectal cancer, in whom there is a strong case for close surveillance. Traditionally the only certain way to achieve such surveillance has been repeated colonoscopy and biopsy. These are expensive procedures that carry their own morbidity and compliance cannot always be guaranteed. Recent work has demonstrated the value of orally administered solutions of polyethylene glycol (used for bowel preparation prior to surgery or radiology) in monitoring patients with inflammatory bowel disease. Similar samples may be of use in sampling exfoliated colonic cells and could provide an non-invasive method of providing material for molecular genetic surveillance. Such samples have already been shown to be potentially valuable targets for PCR techniques. Point mutations can be detected in stool and colonic washings but other mutation sites need to be exploited for population cancer screening. It is still not known just how powerful this type of molecular surveillance is likely to be. Initial validation of such molecular techniques for diagnosis must be undertaken before they can be applied to asymptomatic subjects in high risk categories.

In this thesis I have assessed the value of colonic washings (in the form of whole gut lavage fluid) obtained pre-operatively from patients undergoing cancer resections as a source of DNA for analysis of mutations in 4 genes commonly altered in colorectal cancer. DNA was extracted from whole gut lavage fluid from 40 patients undergoing cancer resections. Analysis of cancer specific mutations in whole gut lavage fluid consisted of a PCR/restriction enzyme enrichment strategy for *Ki-ras* and *p53* mutations, while *APC* and *TGFβ RII* mutations were analysed by non-enriched single

strand conformational polymorphism (SSCP). In this series, 18 patients (45%) had the potential for a genetic diagnosis of their cancer on the basis of analysis of 4 genes, yet only 4 (10%) were detected in the whole gut lavage fluid. My results demonstrate that DNA extracted from whole gut lavage fluid is suitable for PCR analysis but many samples are lost to further examination due to insufficient amounts of human DNA. These findings indicate that stool-based genetic diagnosis requires selective mutation enrichment before it will be useful in screening and that other methods commonly used for mutation detection (e.g. SSCP) are not sufficiently sensitive. Additional methods of mutation enrichment must be sought if these tests are to be used for screening. Moreover, multiple sites or new loci would need to be analysed before such tests could be comprehensive.

The identification of telomerase activity in up to 90% of colorectal tumours was published whilst my research was in progress. Telomerase activity offers an opportunity for a single molecular test for the detection of colorectal cancer, overcoming the limitations of multiple point mutation detection. A commercial kit that employed the Telomeric Repeat Amplification Protocol (TRAP) and an Enzyme linked immunosorbent assay (ELISA) was used to try and detect telomerase activity in previously stored unprocessed whole gut lavage fluid from 6 patients in the original study population. No telomerase activity could be detected in these samples. Spiking diluted whole gut lavage fluid samples with 10^3 cells from telomerase positive cell lines still yielded negative results suggesting the presence of a reaction inhibitor.

Previous work from Edinburgh examining the extra colonic manifestations of Familial polyposis coli (e.g. mandibular osteomas and congenital hypertrophy of the retinal pigment epithelium (CHRPE)) has shown a preponderance of such lesions in patients with replication error positive (RER+) cancers. The mismatch repair (MMR) deficiency implied by an RER+ phenotype may allow incorporation of initially silent mutations in key genes (e.g. *APC*) into apparently normal tissues at embryogenesis.

Such individuals would be mosaics for these mutations and thus may have an increased colorectal cancer risk. It had been hoped *APC* analysis of whole gut lavage fluid from individuals with a strong family history of colorectal cancer would enable elucidation of this hypothesis by allowing sampling of the entire colonic mucosa. My earlier work has shown that *APC* analysis in whole gut lavage fluid is not possible.

To further pursue the hypothesis of mosaicism I examined synchronous colorectal cancers for identical *APC* mutations. The discovery of identical *APC* mutations in synchronous cancers would strongly support the notion of *APC* mosaicism. In 12 cases the mutation cluster region of *APC* was examined. In no case was identical *APC* mutations identified. A RER+ phenotype was identified in 20% of cases suggesting an underlying MMR deficiency might predispose to synchronous cancers. This does not preclude the possibility of clonally different cancers arising from the same environmental insult.

1. Introduction

This thesis explores the possibility of exploiting the current knowledge of colorectal cancer genetics for use in the early detection of colorectal cancer and hence screening. In the last ten years much has been learned about the various genetic mutations that are frequently found in colorectal cancer. Although the picture of colorectal carcinogenesis is not complete, enough is known to allow us to start considering genetic techniques for potential screening applications.

It is the nature of colorectal cancer that has allowed such detailed genetic analysis. Colorectal cancer is a common Western disease and there is a wealth of pathological and clinical material available for examination. Furthermore there is unique access to cancers at different stages of progression providing information about all stages of the disease. The systematic stepwise progression of colorectal cancer from benign adenoma to invasive carcinoma, championed by Vogelstein, is now widely accepted. In accepting this theory it is recognised that although not present in every cancer certain specific mutations occur in pre-malignant adenomas. Detecting these mutations in clinically obtained samples such as whole gut lavage fluid offers a specific method of early colorectal cancer detection and thus obvious screening possibilities. False positive results and low specificity are a major failing of present faecal occult blood screening techniques.

Recent developments have focused on the rarer inherited forms of colorectal cancer, Familial Adenomatous Polyposis Coli (FAP) and Hereditary Non-Polyposis Colorectal cancer (HNPCC). Rigorous study of large kindreds with these conditions has highlighted two areas of pathogenesis involving the adenomatous polyposis coli (*APC*) gene in FAP and a family of genes involved in mismatch repair in HNPCC. These developments would suggest that even if genetic screening of the general population

proves to be impractical, targeted genetic screening for persons at risk of inherited colorectal cancer could prove fruitful and cost effective.

Telomerase is an enzyme that by employing an integral RNA template maintains telomere length in embryonic stem cells. It is not normally found in mature epithelial cells. Telomerase activity can be detected in 90% of colorectal cancers and colorectal cancer cell lines. The detection of telomerase activity in clinical material may offer a single target for which molecular techniques could be exploited for screening purposes.

It is thought that individuals with an inherited cancer susceptibility through a deficiency in DNA mismatch repair (e.g. in HNPCC) accumulate somatic mutations in genes critical to the development of colorectal cancer. It is conventional to regard these secondary mutations as events in the founder cells of neoplastic clones. However other possibilities exist such as mutational mosaicism acquired in cancer genes at an early stage of development. Successful development of genetic tests for the early detection of colorectal cancer may ultimately be used to elucidate the mechanisms of carcinogenesis in susceptible individuals.

In this thesis I discuss the implications of screening by conventional techniques and discuss the recent advances in our understanding of colorectal carcinogenesis. The potential for genetic tests to be used in early colorectal cancer detection has been recognised but remains to be fully evaluated. With this in mind I then discuss my research which focuses on three main areas. Firstly a proof of principle study to evaluate the systematic analysis of four cancer gene mutations frequently associated with colorectal cancer in whole gut lavage fluid obtained from sporadic colorectal cancer cases. Secondly a preliminary study validating an assay to detect telomerase activity in whole gut lavage fluid. Finally I shall attempt to address the possibility of mutational mosaicism as a cause for increased colorectal cancer susceptibility by analyzing a small subgroup of patients that have synchronous colorectal cancers.

1.1 Sporadic colorectal cancer

Colorectal cancer is the second most common cause of cancer death in the United Kingdom. It accounts for 22 deaths per 100,000 population in Scotland (Scottish Health Statistics 1996) and in 1994 there were 3267 new cases in Scotland (Scottish Intercollegiate Guideline Network (SIGN) 1997). The life time risk of sporadic colorectal cancer in the general population is 6% (Brown 1995; Bond 1997). The major form of treatment remains surgical resection with the addition of various adjuvant therapies for advanced disease. Despite many technological advances in medicine the overall five year survival for colorectal cancer has remained unchanged at around 30 - 40% (SIGN 1997). A patient's individual prognosis depends on the invasiveness of the lesion at the time of surgery. Dukes' A adenocarcinomas have a much better five year survival than Dukes' stage B and C (Mulcahy 1997). Unfortunately at present the majority of cancers are stages B and C at the time of surgery (Stower 1985). Improvements in diagnostic techniques that enable earlier diagnosis will result in more patients undergoing surgery for Dukes' A carcinomas and premalignant lesions allowing cure rather palliation.

High risk sub-populations

Certain sub-groups of the population have been shown to have an increased risk of colorectal cancer. Patients who have previously undergone surgical resection for colorectal cancer remain at an increased risk of developing a second primary (metachronous) cancer (Fante 1996). Parents and siblings of patients with adenomatous polyps also have an increased risk of developing colorectal cancer. The relative risk when compared to spouse controls was 1.78 rising to 2.59 if the patient had an adenoma diagnosed before the age of 60 (Winawer 1996). Similarly, first degree relatives of patients with colorectal cancer have an increased relative risk of 1.9 for

developing adenomatous polyps and hence, presumably, the potential to develop a carcinoma (Bazzoli 1995).

Due to the common nature of colorectal cancer chance family clusterings are recognised. However certain families have a strong family history of colorectal cancer and a genetic predisposition to colorectal cancer has been established in two conditions. Familial Adenomatous Polyposis (FAP) (Kinzler 1991) and Hereditary Non-polyposis Colorectal Cancer (HNPCC) (Lynch 1993). FAP is a dominantly inherited condition in which affected individuals develop numerous adenomatous polyps in the colon in their first or second decade; progression to colorectal cancer occurs in all affected individuals by the fourth decade if prophylactic surgery is not undertaken. In the west FAP cancers account for approximately 1% of all colorectal cancers (Cunningham 1996). HNPCC is characterized by an early age of onset of the disease, typically before the age of 50, with a tendency for lesions to be on the right side of the colon. Histologically the tumours tend to be mucinous or poorly differentiated, and there are more Dukes' stage A and B carcinomas than in colorectal cancer generally (Lynch 1993). The International Collaborative Group on HNPCC has proposed a series of clinical criteria (the Amsterdam criteria) by which to diagnose HNPCC (Vasen 1991). These are i) three or more relatives with histologically verified colorectal cancer, one of whom is a first degree relative of the other two; ii) colorectal cancer involving at least two generations; and iii) one or more colorectal cancer cases diagnosed before the age of 50. All three criteria are required for the diagnosis of HNPCC. The Amsterdam criteria are clinical criteria and as such are necessarily very stringent. They were established before the genes responsible for HNPCC had been identified. HNPCC may account for as much as 6% of colorectal cancer cases (Lynch 1993) although more recent studies suggest its incidence is lower at 0.3 - 1.4 % of all colorectal cancer (Evans 1997).

It is now evident from genetic analysis that mismatch repair mutations are identified in families that do not fulfill the Amsterdam criteria but have a strong family history of colorectal cancer. Similarly not all kindreds that fulfill the Amsterdam criteria harbour a detectable mutation in the mismatch repair (MMR) genes (Liu 1996b). There would appear to be a less definite distinction between a strong family history and true HNPCC (by the Amsterdam criteria) when the genetic information is taken into account. Families that fulfill the Amsterdam criteria may represent one end of a clinical spectrum which merges with families with a strong family history of colorectal cancer. Hence, when considered as a whole familial causes and patients with a positive family history represent around 23% of all colorectal cancer (CancerNet 1997).

These sub-populations and their first degree family relatives represent a group in whom surveillance and screening would offer a significant benefit.

1.2 Population Screening

Screening for a disease is justified when the disease is common and associated with serious morbidity and mortality, and when early detection and treatment of the disease results in improved prognosis relative to usual treatment. There must also be evidence that the potential benefits outweigh the potential harms and costs of screening (Winawer 1997). The natural history of colorectal cancer fulfills the disease criteria as a condition highly suitable for screening. Currently there are several candidate screening approaches. The ideal screening test itself should be simple, cheap and repeatable. It should be specific and have a high degree of patient compliance. No test currently meets these criteria, but the various methods and strategies available at the present time are discussed below.

General Population

i) Digital rectal examination

Digital rectal examination alone has generally been dismissed as a screening method since interpretation is subjective and the examination is limited to less than 10% of the large bowel (Bennett 1994). Routine digital rectal examination provided no significant reduction in mortality from distal rectal cancer (Herrington 1995).

ii) Faecal Occult Blood Testing

The simplest technique of screening for colorectal cancer is the faecal occult blood test (FOBT). It has been estimated that two thirds of cancers bleed to levels detectable in stool in the course of a week (Winawer 1997) and the aim of the FOBT is to identify this. The Haemoccult ® test (Smith Klein and Beecham) is commonly used and is based on a slide preparation of guaiac gum which exploits the pseudoperoxidase activity of haemoglobin to catalyse the phenolic oxidation of an indicator substrate. Certain foods exhibit peroxidase activity (red meat, turnips and horseradish) and dietary restrictions are therefore required to reduce the number of false positive tests. Vitamin C (by inhibition of the peroxidase reaction) can lead to a false negatives as can inaccurate interpretation of the slides. Because of the intermittent nature of bleeding from colorectal cancer a single FOBT obtained from digital rectal examination is of little use and repeated sampling of consecutive stools for three days is recommended (Winawer 1997).

Reported compliance for FOBT ranges from 30 - 90% and tends to decline with study progression. Younger subjects are less likely to comply and higher rates of compliance are found in individuals with a higher educational level and in subjects with a family history of the colorectal cancer (Winawer 1997). Two large studies in Europe have evaluated this test, the larger of these studies was from Nottingham (Hardcastle 1996). This was a prospective study of 152,850 subjects aged between 45 -74 years

using unrehydrated tests and 2 yearly rescreen. In the screened group 60% completed at least one FOBT screening with 38.2% completing all tests offered. Although the overall number of cancers in study and control groups were almost identical the screened group had a 15% reduction in cumulative colorectal cancer mortality and significantly higher percentage of Dukes' stage A cancers (20% compared with 11% in the control population). A second study from Denmark has confirmed these findings showing a similar number of cancers in each group but a significant reduction in the cumulative colorectal cancer mortality by 18% along with a significant increase in the number of early cancers when compared with control groups (Kronborg 1996).

One trial from Minnesota (Mandel 1993) has shown a significant reduction in mortality in patients screened annually compared to controls (cumulative annual mortality 5.88 per 1000 and 8.33 per 1000 respectively). This study employed rehydrated slides for FOBT which increased sensitivity but lead to a concomitant reduction in specificity (Winawer 1997). This study has met with some criticism because the annually screened group also had a 38% higher annual colonoscopy when rate compared with controls. The net result was that over one third of the participants in the study group underwent colonoscopy over a 13 year period. Thus the results may simply reflect the value of colonoscopic screening (Bennett 1994). Others would attach less significance to this potential bias. When a simple screening test is applied to a large population and is followed by a definitive diagnostic test there will be some random or accidental benefit from the diagnostic test itself. This effect for the Minnesota trial has been calculated at between 6-11%, not enough to account for the observed reduction in mortality (Bond 1997), thus supporting the use of FOBT in screening the general population.

A simple indirect screening test repeated at definite intervals does not have to be highly sensitive to be effective as long as it reliably detects developing neoplasms before they become incurable; application of the FOBT at one time may have a low sensitivity

but repeated use in a screening program over time maybe highly sensitive and effective (Bond 1997). Whilst mass population screening would inevitably lead to increased detection of colorectal cancers the poor specificity of the test would certainly lead to more colonoscopies being performed for other bleeding conditions of the bowel. This has serious resource implications. FOBT will also certainly miss a proportion of cancers. The impact of false positive and negative test results on the general population are also important considerations when contemplating mass population screening by FOBT. These considerations must be weighed against any potential benefits of FOBT. The case for regular FOBT for the general population appears compelling yet the Scottish National Clinical Guidelines concluded that the available evidence on FOBT did not warrant screening of the Scottish population at the present time (SIGN 1997).

A negative FOBT can lead to a false sense of security although the majority of cancers detected by FOBT are at Dukes' stage A and B (Hardcastle 1996) up to 62% of advanced stage cancers (Dukes' stage C and D) are missed by initial FOBT with 62-76% of these being in reach of a flexible sigmoidoscope (Schnell 1994). This suggests FOBT should be augmented in some way; indeed a recent prospective study has shown that the addition of flexible sigmoidoscopy (flexible sigmoidoscopy) to FOBT screening can yield a four fold increase in the detection of neoplastic lesions in the general asymptomatic population (Berry 1997).

iii) Flexible Sigmoidoscopy and Colonoscopy

The use of flexible sigmoidoscopy (and colonoscopy) as screening techniques requires significant medical input and they are invasive procedures with their own morbidity and mortality. Flexible sigmoidoscopy has several advantages over FOBT. The bowel can be visualised directly enabling biopsy or removal of suspected cancers or polyps. However it requires a hospital visit and preparatory enema, and the examination itself produces some abdominal discomfort. Around 40% of cancers will not be visualised with a 60 cm. flexible sigmoidoscope (Wu 1995). False positive examinations

are rare. Despite these limitations case control studies show that sigmoidoscopy can significantly reduce the risk of death from distal cancers over a 10 year period for a population at average risk of colorectal cancer. However patients dying from colorectal cancer also had fewer rectal examinations, FOB tests and heath check ups (Newcomb 1992; Selby 1992) . Screening the general population by flexible sigmoidoscopy can be expected to detect 3 carcinomas per 1000 subjects but compliance varies widely between 20 and 95% (Wherry 1994; Berry 1997).

The morbidity associated with screening endoscopy is 0.3% but the when compared with the decrease in colorectal cancer mortality attributable to screening this complication rate is acceptable (Kewenter 1996). The current recommendation of the American Cancer Society, for populations at average risk and a negative initial examination, is flexible sigmoidoscopy every 3 - 5 years from the age of 50 (Winawer 1997). Other data suggest that, following a negative examination, or polypectomy for early tubular adenomas, cancer development is rare. Hence the interval between examinations might be increased with safety. Even "once only" flexible sigmoidoscopy could be an effective screening strategy in these instances (Atkin 1992).

Colonoscopy is a more invasive procedure requiring thorough bowel cleansing, analgesia and sedation, necessitating day case admission to hospital. The morbidity associated with diagnostic colonoscopy is low (around 0.1%) but rises if polypectomy is performed (Kewenter 1996). Colonoscopy is currently the only available means of clearly identifying the 42% of neoplastic lesions that lie beyond the reach of the flexible sigmoidoscope and the 36% of lesions that are too small (less than 0.5 cm in diameter) to be visualised by double contrast barium enema. Colonoscopy has been advocated as a more appropriate initial screening procedure than FOBT (Wu 1995). There are no controlled clinical trials examining colonoscopy as a screening method (Winawer 1997). Mathematical models suggest flexible sigmoidoscopy every 5 years would reduce the incidence of colorectal cancer in a population of 100,000 by 1,976 cases from 4,988 to

3,013 and increased the life expectancy of the affected population by 8.6 years. The number of deaths would be expected to decrease by 967, but this would be at the cost of 3 deaths as a result of colonoscopy with 20 perforations and 49 major bleeding episodes (Winawer 1997).

iv) Double Contrast Barium Enema

Double contrast barium enema (DCBE) requires bowel preparation 24 hours prior to the procedure, the instillation of barium and air per rectum, fluoroscopic monitoring and exposure to x-rays. Around 5 - 10% of examinations are technically unsatisfactory requiring further examination by other means and the sensitivity of DCBE ranges from 70 - 90% for polyps greater than 1 cm and early carcinomas (summarised in Winawer 1997). There are no controlled trials examining the effectiveness of DCBE in screening for colorectal cancer but indirect calculations would suggest an efficacy similar to flexible sigmoidoscopy (Winawer 1997). A positive DCBE necessitates colonoscopy.

Genetic screening for inherited colorectal cancer syndromes

The rarity of FAP and HNPCC and the technical difficulties in identifying mutations makes genetic screening of the general population for these familial syndromes impractical. However if the mutation is identified in a proband case, screening of close family members is feasible in each syndrome. The fact that FAP and HNPCC are the result of germline mutations (Bailey-Wilson 1986) allows the possibility of family genetic screening to be considered. With FAP the dominant nature of its inheritance permits screening by simple restriction fragment length polymorphism(RFLP) or protein truncation tests (PTT) on blood samples once the germline mutation from the proband has been identified (Prosser 1994). The identification of the adenomatous polyposis coli (*APC*) gene has allowed predictive testing for FAP either by linkage analysis or mutational analysis. Linkage analysis is more restricted than mutational analysis. However mutational analysis requires characterisation of the mutation which is not

always possible. Family members who are non-gene carriers by mutational analysis can be reassured. Mutation carriers can be appropriately counselled and offered prophylactic surgery (Cunningham 1996)

The situation for HNPCC is more complex. While it is known a high proportion of HNPCC cases will have a germline mutation in one of the mismatch repair genes, not all cases do. This is either because the mutation lies in a non-coding portion of the gene or because not all the genes involved in MMR have been identified (Liu 1995; Liu 1996). Recent observations have emphasised the importance of constitutional mutations in mismatch repair genes in a high proportion of patients with HNPCC (Liu 1996b). The high cancer susceptibility of these patients probably derives from the fact that, because of deficient DNA mismatch repair, they more rapidly accumulate somatic mutations in genes critical to the development of colorectal cancer (Kinzler 1996). However, for HNPCC the incomplete penetrance, the number of genes which can potentially be mutated and the fact that not all potential target genes have been identified means that screening is a more complex issue. Unless the prevalence of the HNPCC genotype and classic syndrome is in the range of 50 cases per 10,000 or more people, very favourable assumptions must be made for population wide genetic testing to be cost-effective. If the prevalence of a homogeneous HNPCC syndrome is towards the lower end of current estimates (0.5 - 10%) or if the HNPCC syndrome were to be much more heterogeneous than observed in known HNPCC families, it appears unlikely that population wide genetic screening can achieve acceptable levels of cost-effectiveness (Brown 1995) . These variables of penetrance also pose difficult problems about screening the family members of HNPCC kindreds. The heterogeneity of MMR mutations (Weber 1997) and the number of genes to be examined have practical implications even when restricting screening to family members. The risk to related individuals in kindreds that fulfill the Amsterdam criteria is variable (Dunlop 1997). The

risk to family members of kindreds with a positive family history that do not fulfill the Amsterdam criteria is indeterminate.

Adenomas in HNPCC are often flat and have a greater tendency to progress to cancers than adenomas arising in the average risk population (Lynch 1993).This suggests a shorter surveillance interval is required than that for the average population. A period of 1-2 years between colonic examinations is currently recommended (Vasen 1995; Winawer 1997). Because of the proximal distribution of cancers in HNPCC the entire colon must be visualised preferably by colonoscopy (Winawer 1997). Due to the underlying DNA instability in patients with such germ line mutations they remain at risk of further metachronous colorectal cancer after operative removal of the first carcinoma. Moreover, their close relatives are also at risk.

There is good evidence that there is a group of people with an affected first degree relative that is at an increased risk of colorectal cancer, yet who do not fulfill the Amsterdam criteria for inherited genetic disease. It has been calculated that people in the general population with one affected first degree relative have a relative risk of 1.7 (Fuchs 1994) and it is recommended that these patients should undergo colonoscopic screening from the age of 40 particularly if the affected relative had the diagnosis made before the age of 55 (Levin 1992). Objective data would only tentatively support colonoscopic screening in asymptomatic patients if their life time risk were 1 in 2, however the psychological benefit of counseling and screening subjects with an affected first degree relative must also be considered (Mulcahy 1997).

These patients would therefore constitute a rewarding subset of the general population at which to direct appropriate screening tests. The Scottish Guidelines for the screening of subjects in high risk groups are that first degree relatives should be offered screening when FAP or HNPCC is diagnosed. Total colonoscopy or double contrast barium enema should be offered, starting in the teens for FAP and twenties for HNPCC. Colonoscopy should be performed every 2-3 years depending physical

condition and compliance. For those at risk of FAP more frequent 1-3 yearly flexible sigmoidoscopy should be considered (SIGN 1997). The cumulative morbidity of colonoscopy repeated every 1-2 years from the age of 20 could be unacceptable and any test that is less invasive with a comparable specificity would be an advantage. Genetic based tests may fulfill these criteria.

Cost effectiveness of potential screening strategies

Comparison of the various screening methods would require large multicentred studies to produce meaningful answers and so far has not proved practicable. One approach has been to devise a mathematical model based on published results to try to predict the cost-effectiveness of the various screening methods (Lieberman 1995). By making a number of assumptions regarding screening intervals, prevalence of colorectal cancer, cancer mortality and colonoscopy morbidity, Lieberman has shown that FOBT alone, although cheapest per life saved, prevents fewer cancer deaths than any other method of screening. Once-only colonoscopy would have the greatest impact on colorectal cancer morbidity, assuming that a polypectomy prevents subsequent cancer development in most patients. However, compliance was an important factor in determining the effectiveness of all the screening programs (Lieberman 1995). In a critical review of the current data Bond concluded that the combination of FOBT and flexible sigmoidoscopy has the potential to reduce colorectal cancer mortality in the general population by 50%, saving some 35,000 lives a year in the USA (Bond 1997). Further simulation studies showed that for a population of 100,000 the use of FOBT, followed by colonoscopy for patients testing positive , would reduce the incidence of colorectal cancer by 2,378 cases (from 4,988 to 2,610). The number of expected deaths would decrease by 1,330 with an increased life expectancy of 9.3 years. However it was estimated there would be 52 deaths from complications of colonoscopy, 304 colonoscopy perforations and 741 major post colonoscopy bleeding episodes (Winawer 1997).

Endoscopic screening is time consuming, expensive, and potentially hazardous but is justified in subjects at high risk because of its sensitivity and specificity for neoplasia (Mulcahy 1997). Identifying the point at which the benefits of endoscopy and radiology are outweighed by their disadvantages is difficult and most would offer such investigations if two or more first degree relatives were affected (Mulcahy 1997). The greatest cost of screening is the diagnostic evaluation prompted by a positive screen. Any test that reduces the number of false positive and hence subsequent investigations (i.e. improves the specificity if the screening test) will thus improve the cost-effectiveness of screening (Bond 1997). Again the use of genetic based tests may address this issue.

Summary of current screening issues

Although FOBT is the most cost effective method of screening, its lack of specificity means that additional tests (colonoscopy, flexible sigmoidoscopy or DCBE) are required to augment it. Such tests add significantly to the overall cost of colorectal cancer screening. Development of a simple stool or gut lavage based test that has a greater specificity than FOBT would constitute a substantial advance by reducing the number of additional tests performed.

1.3 Mutations in exfoliated cells

In the following sections various mutations specific to colorectal cancers will be discussed. Searching for these mutations in exfoliated cells contained in stool or colonic effluent may provide a test by which colorectal cancer may be detected.

Colonic epithelial cells migrate from the lower third to the top of a crypt in between 3 to 4 days. The epithelial cells proliferate (i.e. enter S phase) at a rate of 1.2 cells / 100 cells / hour. Although specific areas of cell extrusion at the tips of the villi cannot be

readily identified, in order to maintain a stable cell population, colonic epithelial cells are apparently lost from the lumenal surface at a similar rate (Lipkin 1963). Thus it is anticipated that stool and colonic effluent should provide a rich source of cells and DNA for analysis. One would expect colorectal cancers to contribute to these exfoliated cells.

Early evidence that cancer mutations can be detected in exfoliated cells was provided by the analysis of *p53* mutations in urine samples from patients with known bladder cancer (Sidransky 1991). Using PCR amplification techniques *p53* mutations were identified in DNA obtained the resected cancer. In three cases the *p53* mutation was then sought in DNA obtained from the sediment of pre-operatively voided urine. Phage hybridisation techniques were employed to increase the sensitivity of the urine tests. Identical mutations were found in the urine sediment DNA in each case. Mutant alleles accounted for 1-7% of the recombinant clones. No false positives were detected in six normal samples tested in a similar manner (Sidransky 1991). This study highlights a sensitive and specific technique for detecting cancer specific mutations in exfoliated cells. The disadvantage in this technique is that it requires prior identification of the mutation from the resected cancer to allow the use of hybridisation techniques. This is not possible when screening for an unknown cancer. Nonetheless the detection of cancer specific mutations in the stool by molecular techniques holds promise as a more specific screening test for colorectal cancer.

Certain key mutations have been implicated in colorectal carcinogenesis. By focusing on *Ki-ras*, *APC*, *p53*, and *TGFß RII* it may be possible to devise a strategy for screening for colorectal cancer. The clonal expansion of mutated cells in colorectal cancer means that analysis for these genes would be selective for colorectal cancer (Kinzler 1996). By choosing a mutation that occurs early in the adenoma / carcinoma progression early or even pre-malignant lesions could be detected. The cellular material exfoliated in faeces and other similar samples is likely to be degenerate. Any DNA

present is likely to be fragmented either by bacterial DNases or apoptosis. The polymerase chain reaction (PCR) can be used to specifically amplify small fragments of DNA and this could be usefully employed in a genetic test for colorectal cancer.

Cancer mutations in stool

The genetic mutations associated with sporadic and hereditary colorectal cancer are widely studied and are some of the best understood at the present time (Fearon 1990; Kinzler 1996) . The study of the rarer inherited colon cancer syndromes, FAP and HNPCC, has lead to important advances in the understanding of sporadic colorectal cancer. The principal concept for both hereditary and sporadic cancers is that the cancer develops by accumulating mutations in several genes in stepwise manner resulting in a progression from normal epithelium through adenoma to invasive carcinoma (Kinzler 1996). Mutations in *APC*, the gene associated with Familial Adenomatous Polyposis (FAP), initiate the neoplastic process (Kinzler 1996) and tumour progression results from accumulation of other mutations for example *Ki-ras, p53,* (Kinzler 1996) or Transforming Growth Factor Beta Receptor II (*TGFβ RII*) (Parsons 1995b). Oncogenes such as *Ki-ras* are activated by mutations, tumour suppressor genes (e.g. *p53*) are inactivated. Mutations in the mismatch repair genes produce an overall genetic instability and hypermutable state which leads to a more rapid accumulation of the other carcinogenic mutations (Kinzler 1996). These mutations and others, as yet unidentified, act in concert to produce a carcinoma.

Ki-ras mutations associated with colorectal cancer have been detected in stool and colonic washings of patients known to have *Ki-ras* mutations in the resected colorectal cancer. The successful detection rate varied widely from 16 - 81 % cases (Sidransky 1992; Tobi 1994; Hasegawa 1995; Smith-Ravin 1995; Cruickshank 1996; Nollau 1996; Villa 1996). *p53* mutations in stool have also been detected by single strand conformational polymorphism (SSCP) (Eguchi 1996). One study has even reported identifying *Ki-ras* mutations in colonic washings obtained four years before the

macroscopic lesion being detected (Tobi 1994) indicating a potential for pre-malignant detection.

The DNA obtained from stool samples has been shown to be heavily contaminated and can inhibit the polymerase chain reaction (PCR) used for genetic analysis (Smith-Ravin 1995; Cruickshank 1996). Colonic washings obtained at endoscopy however may be more suitable for genetic analysis by PCR techniques (Tobi 1994; Smith-Ravin 1995).

1.4 Whole gut lavage

Exfolilative cytology to aid the diagnosis of colorectal neoplasia was introduced several decades ago (Bader 1951) with good results. The specificity of the technique was compromised because the cytological characteristics of neoplasia could also be found in cells shed from adenomas with severe dysplasia and as a response to inflammation as in ulcerative colitis. (Rozen 1990; Wurerker 1993). The cumbersome and complex methods for obtaining the specimens along with the introduction of colonoscopy and the possibility of direct biopsy meant that the technique fell into decline (Rozen 1990). The introduction of bowel preparation with balanced electrolyte solutions has renewed interest in cytology, as it provided a simple and safe method for obtaining rectal fluid that can be used for cytological analysis (Rozen 1990; Handy 1995) . Whole gut lavage fluid is the clear effluent obtained per rectum following bowel preparation with polyethylene glycol balanced electrolyte solutions (Klean Prep ® Norgine Harefield Middlesex UK). High IgG concentrations in whole gut lavage fluid correlates well with disease activity and remission in patients with inflammatory bowel disease. Its use in diagnosis of these conditions is however limited (Choudari 1993). Whole gut lavage specimens have been shown to contain numerous inflammatory cells in patients with inflammatory bowel disease (Handy 1995). Normal colonocytes are

however an infrequent finding in whole gut lavage fluid (Brandt 1989; Wurerker 1993) and malignant cells have been detected with varying degrees of success in 30-94% of patients with adenocarcinoma (Brandt 1989; Rozen 1990; Gordon 1991; Tobi 1994). Tobi et al found that 70% of the lavage samples were satisfactory for analysis and identified 4/9 cancers and 8/19 adenomas (Tobi 1994). Brandt et al found 50% of the lavage samples contained insufficient epithelial cells for suitable analysis and were able to detect 4/14 (29%) of adenocarcinomas (Brandt 1989). However Gordon et al were able to identify 9/9 cancers on examining the lavage fluid with 1 false positive in 18 normal control samples (Gordon 1991).

1.5 Mutations associated with Colorectal cancer

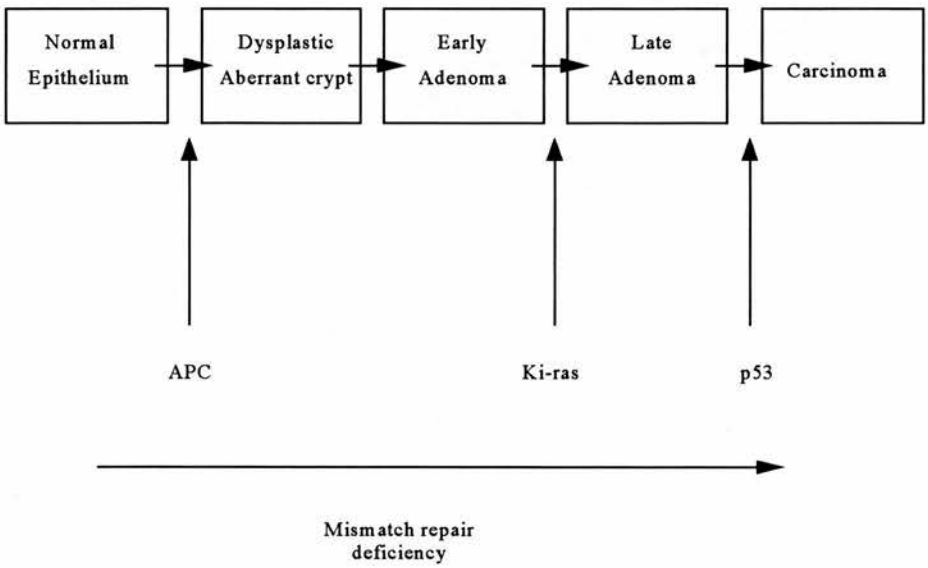
Systematic genetic analysis of colorectal cancers at different stages, ranging from early adenomas to invasive carcinomas, has suggested that the steps required for the development of colorectal cancer often involve the mutational activation of oncogenes or with the loss of tumour suppressor genes (Vogelstein 1988). Vogelstein *et al.* searched for *ras* mutations and allelic deletions in chromosomes 5, 17p and 18 in 192 colorectal cancers of varying stage. Mutations in *ras* were identified in 58% of adenomas greater than 1 cm in diameter, and in 47% of carcinomas, but only in 9% of adenomas less than 1 cm in diameter. Sequences on chromosome 5 linked to the gene for familial adenomatous polyposis (*APC*) were lost in 29-35% of sporadic adenomas and carcinomas (but interestingly not in tumours from patients with polyposis).

Chromosome 17p sequences were lost only in carcinomas but were found in 75% of all colorectal carcinomas. No such losses were identified in adenomas. A specific region in chromosome 18 was deleted in 13% of early adenomas, 47% of advanced adenomas and 73% of carcinomas. Hence it can be seen that molecular alterations parallel the development of adenoma to carcinoma, strongly suggesting that mutations in certain

key genes are required for the stepwise progression in the development of colorectal cancer (Vogelstein 1988). Advances in scientific techniques and knowledge have enhanced this theory supporting the original concept. Current theory (summarised in Kinzler 1996) suggests that *APC* acts as a gatekeeper to colonic epithelial cell proliferation and that mutations in *APC* are the rate-limiting step in the progression of colorectal cancer (Kinzler 1996). Other potential gatekeeper genes include *NF1* in Schwann cells, *Rb* in retinal epithelium. Normally wild type APC maintains a constant cell population in the colonic epithelium and mutation of *APC* leads to a permanent imbalance in cell proliferation promoting tumorigenesis. Similarly mutations in other genes associated with colorectal cancer in the presence of normal APC do not lead to a cancer formation implying the order of accumulation of mutations may be important (Kinzler 1996).

There is growing evidence to support these theories. Firstly, although FAP patients develop many tumours inactivation of the inherited wild type *APC* allele is required for adenoma formation (Ichii 1992). Also *APC* mutations have been found in the earliest stage of colorectal cancer, the aberrant crypt foci, which are foci of dysplastic epithelial cells in otherwise normal colon epithelial crypts (Jen 1994). These observations place mutations in *APC* at the start of the adenoma / carcinoma progression. Secondly, in contrast, cells with *ras* mutations are frequently found in aberrant crypt foci but if this mutation is the first event the result is a non-dysplastic focus which has little potential to progress (Jen 1994). Mutations in *p53* occur near the transition from benign to malignant lesion with an inactivating point mutation of one allele being the rate limiting step (Baker 1990). Although *p53* is mutated in over 80% of colorectal cancers patients with germ-line mutations in *p53* do not develop

Figure 1.1 The adenoma carcinoma progression



The genetic changes associated with colorectal carcinogenesis (modified from Kinzler 1996)

APC mutations initiate the neoplastic process with Ki-ras mutations enhancing adenoma formation and p53 mutations involved in the progression to carcinoma. Other mutations in as yet unidentified genes may influence other parts of the progression.

Mismatch repair deficiency confers a genomic instability allowing fast accumulation of relevant mutations and faster progression to carcinoma

polyposis and are not even at high risk of developing colorectal cancer (Kinzler 1996). These observations would supporting the theory that the order of mutations is important.

i) *Ki-ras*

The gene encoding *Ki-ras* spans between 35 - 40 kb on chromosome 12p with an open reading frame of 567 bp in 4 exons. The fourth exon shows alternative splicing with two alternative exons 4A and 4B. The gene with codon 4A encodes a membrane bound polypeptide with a molecular weight of 21,660 daltons with intrinsic GTPase activity. There is an associated pseudogene representing a processed mRNA version of the gene (McGrath 1983). Specific point mutations (e.g. codons 12 and 13) in the *ras* gene transform cultured cells (Land 1983) and confer neoplastic properties *in vivo*. With increasing understanding it is clear the ras signaling pathway is extremely complex (reviewed in Campbell 1998). Ras proteins are positioned at the inner surface of the plasma membrane where they act as binary molecular switches regulated by a GDP / GTP cycle. The proteins serve to transmit extracellular ligand mediated stimuli into the cytoplasm. Point mutations producing amino acid substitutions at positions 12, 13 and 61 lock it in the GTP bound active state and make it insensitive to negative modulating molecules. Activated ras proteins activate an ever increasing number of molecules in a complex cascade of which the main ras effector molecule is raf. Through a series of serine / threonine kinases this cascade ultimately influences nuclear transcription factors and gene expression.

Some 50% of colorectal cancers have such *ras* mutations (Bos 1987). Adenomas greater than 1 cm have a similar rate of mutation but fewer than 10% of adenomas less than 1 cm have *ras* mutations (Vogelstein 1988). *ras* mutations are thought to be weakly associated with malignant conversion because the frequency of *ras* mutations among advanced cancers is similar to that among larger adenomas (Vogelstein 1988). Analysis of the various dysplastic areas found in adenomas has shown *ras* mutations are

more frequently found in areas of moderate dysplasia when compared to areas of mild dysplasia. Thus *ras* mutations may contribute to a more advanced morphological phenotype during the late stages of adenoma progression but not directly to the malignant transformation from adenoma to carcinoma (Ohnishi 1997). Dukes' stage B and C cancers however show very significant differences in the type of *ras* mutation. The metastatic potential of these tumours could be related to the nature of the genetic alterations in *ras* rather than the hypothesis that Dukes C carcinomas eventually evolve from Dukes B. G→C and /or G→T transvertions at codon 12 appear to endow tumour cells with the capacity to metastasize whereas G→A substitutions do not (Moerkerk 1994).

ii) *p53*

p53 is a highly conserved vertebrate gene encoding a tumour suppressor protein which regulates the cell cycle in response to DNA damage. In normal cells p53 is an unstable nuclear protein. Following genomic DNA damage however, p53 is stabilised and activated as a transcription factor providing a crucial block to cell cycle progression leading to growth arrest or apoptosis (Cox 1995). Loss of the normal function of the p53 has been implicated in a wide variety of human cancers (Haffner 1995).

The gene encoding the p53 protein is located on chromosome 17p. The protein consists of 393 amino acids with four structural and functional domains. The N-terminal 42 amino acids constitute a transcription activation domain that interacts with basal transcriptional machinery positively regulating *p53* gene expression. However some of negative regulators of *p53* transcription (hMDM2 and adenovirus E1B-55Kd) also mediate their action via these same amino acids (Levine 1997). Located between amino acids 162-292 is a sequence specific DNA binding domain. This domain is protease resistant and contains a Zn^{2+} ion essential for binding activity (Levine 1997). In this domain residues lysine 120, serine 241, arginine 273, alanine 276 and arginine 283 make contact with the phosphate backbone of the DNA major groove. Residues lysine

120, cysteine 277 and arginine 280 interact via H bonds with the DNA bases and arginine 248 makes multiple H bonds in the minor groove (Cho 1994). An oligomerisation motif is harboured in the COOH terminal portion (residues 324 - 355). Tight dimers and dimer pairs form a remarkably symmetrical tetramer, held together through multiple molecular interactions (Haffner 1995; Levine 1997). The C-terminal 37 residues form an open domain which readily binds DNA and RNA and regulates the ability of p53 to bind to specific DNA sequences at its central core domain (Levine 1997).

Normally the concentration of p53 in the cell is low because of its relatively short half life of approximately 20 minutes. It is degraded by the action ubiqutin proteases (Levine 1997). One function of wild type p53 is to act as a sequence specific transcription factor which can activate genes possessing p53 consensus sites. p53 is activated by DNA damage, such as double strand breaks by γ radiation, UV irradiation or chemical cleavage. Following DNA damage p53 protein levels are significantly increased (Kastan 1991). The activation is proportional to the damage received however the actual mechanism is unclear (Levine 1997). Once activated the tetramer protein binds to the DNA sequence 5'-PuPuPuC(A/T); this sequence is repeated in two pairs each arranged as inverted repeats (Levine 1997). It is this sequence specific transactivation that mediates the growth-inhibitory action of p53. Growth inhibition is elicited through specific binding of p53 to target genes and their subsequent activation. (Pietenpol 1994). Products of some of the genes that are transcriptionally activated by p53 include p21, MDM2, GADD45, Cyclin G, Bax, IGF-BP3 (Levine 1997). Subtractive hybridisation techniques cloned the *WAF1* gene which was strongly induced by excess wild type p53. In addition over expression of *WAF1* alone reproduced the growth inhibitory effect of p53 (El-Deiry 1993). These observations suggest that some of the growth arrest properties of p53 are mediated by the *WAF1* gene product. Independently cloned by other researchers *WAF1* (or *CIP1/SDI*) encodes a protein

product p21. This is a potent inhibitor of many cyclin dependent kinases (Cdks). These enzymes are essential for transit through various stages of the cell cycle (Haffner 1995). p53 may arrest cells in G1 by activating *WAF1*. The resultant p21, by inhibiting the Cdks, in turn leads to hypophosphorylation of pRb, the protein product from the retinoblastoma gene, subsequently blocking cyclin E2F-dependent transcription causing cell cycle arrest (Haffner 1995). Normally pRb proteins are inhibitorily phosphorylated by active Cdks enabling transit through G1-S. Hence the growth inhibitory effect of p53 is mediated by induction of p21 and executed through under phosphorylation of pRb proteins. (Haffner 1995). The protein p21 can interfere directly with DNA synthesis by binding to proliferating cell nuclear antigen (PCNA) (Waga 1994). Attempts to isolate inhibitors of DNA synthesis in senescent cells also identified p21 (Haffner 1995). This suggests that p21 directly inhibits DNA synthesis. Thus induction of p21 by p53 may serve dual roles: preventing exit from G1 by inhibiting cyclin/Cdk complexes and in parallel blocking replicative DNA synthesis directly by binding to PCNA.

p53 also plays a role in apoptosis induced by DNA strand breaks. Radiation induced apoptosis in intestinal crypt cells of normal mice undergo extensive apoptosis. By contrast intestinal crypt cells in p53 null mice fail to exhibit enhanced apoptosis (Clarke 1994). The apoptosis modulating protein Bax is rapidly activated by p53 and this could promote p53 mediated apoptosis. (Selvakumaran 1994). In addition the transcription of Bcl-2 (another apoptosis modulating factor with death inhibitory activity) is repressed by p53. The Insulin like growth factor binding protein 3 (IGF-BP3) may also play a role in p53 mediated apoptosis being transactivated by p53 and inhibiting the growth promoting action of insulin like growth factor (Buckbinder 1995).

In the absence of functional pRb which is normally required for imposing a p53-mediated G1 arrest, instead of producing a growth arrest wild type p53 promotes apoptosis. Thus whether a given cell responds to the activation of wild type p53 by growth arrest or apoptosis is determined by the status of the pRb family. If these

proteins are functional the cell will arrest and may re-enter the cell cycle when p53 activity is normal. Conversely, in the absence of functional pRb the cell will fail to arrest and enter apoptosis (Haffner 1995).

A possible function of p53 may be to provide a reversible G1 growth arrest to allow for DNA repair prior to subsequent rounds of replicative DNA synthesis. This explanation is somewhat contradicted by the fact that some p53 blocked cells apoptose or senesce (Cox 1995). In addition over expression of p53 can lead to repression of many genes probably through interaction with components of the basal transcription machinery (Haffner 1995). Mitotic cells can proceed normally in the absence of p53 since mice that are p53 null develop fully (Cox 1995). The presence of functional p53 can restrict tumour development by promoting apoptosis of neoplastic cells. The same tumours in p53 null mice grow aggressively underscoring the tumour suppressing properties of p53 (Symonds 1994).

As described above wild type p53 is necessary for G1 arrest in response to certain types of DNA damage although this can be circumvented by activated oncogenes (e.g. *ras* and HPV E7) (Cox 1995). Humans, who are heterozygous for the wild type allele of *p53* develop cancer with a very high frequency, greater than 90% and often at an early age (Levine 1997). *p53* mutations are found in 50-55% of all human cancers (Hollstein 1994) The timing of *p53* somatic mutations in human cancers apparently depends on tumour type. In colorectal cancer *p53* mutation is a late event. In skin cancers, by contrast, *p53* mutation appears early in pre-malignant lesions (Levine 1997).

Tumours with allelic deletion of 17p frequently contain point mutations that results in amino acid substitutions in the retained allele. These mutations are not exclusively confined to tumours with allelic deletion (Lamb 1986). Some 98% of *p53* mutations occurring in human tumours represent somatically acquired mutations (Hollstein 1991) and 98% of substitution mutations occur in the region codons 110 - 307 contained in

exons 5 - 9 which is the most evolutionary conserved (Hollstein 1991). Analysis of *p53* mutations identifies several codons at which exceptionally high numbers of tumour mutations are clustered (hot spots). When mutations are examined separately by cancer type clear differences can be identified in the position of the hot spots and in the frequency of transitions and transversions. G:C to A:T transitions contribute the majority of colon cancer mutations (79%) and most occur at CpG dinucleotides. More than 50% of colon tumour transition mutations are at three CpG hot spot 175, 248 and 273 (Hollstein 1991). More rarely mutations produce deletions or chain terminations in the *p53* gene (Levine 1997).

These genetic changes in *p53* in cancer cells most commonly result in a missense mutation in one allele which produces a faulty protein. This faulty protein is then observed at high concentrations in these cells (Levine 1997). Mutations in *p53* gene occur during the process of tumourigenesis may cause tumour progression by a dominant negative effect . The dominant negative effect may be mediated by binding of the mutant *p53* product to the wild type product creating an inactive oligomeric complex. Further loss of control can be expected when the wild type allele is deleted leaving the cell with only the mutant allele (Lamb 1986).

More than 90% of missense mutations lie in the sequence specific DNA binding domain. Mutations in amino acids arginine 248 and arginine 273 are the most frequently altered residues and result in defective contacts with DNA and the loss of the ability of p53 to act as a transcription factor. Other sites for mutation alter the structural conformation of this domain and can be detected by the monoclonal antibodies (Levine 1997). Residues targeted by common hot spot mutations are either engaged directly in DNA contacts or else play pivotal roles in stabilising the proper structure of the binding domain. (Cho 1994) supporting the argument that the loss of sequence specific DNA binding is selected far more strongly in tumour cells. (Haffner 1995)

Tumours with the wild type p53 often respond well to chemotherapy e.g. teratocarcinomas via p53 mediated apoptosis. Other cancers which frequently contain *p53* mutations e.g. colorectal cancer respond poorly to chemotherapy. *p53* mutations have been reported to result in shorter disease free survival and lower total survival of patients (Aas 1996)

iii) *APC*

APC the gene associated with FAP was initially located by linkage analysis to chromosome 5q21-22 (Bodmer 1987) and subsequent cloning studies identified the gene (Groden 1991; Kinzler 1991). The coding region of the gene spans 8.5 Kb and has fifteen exons, the largest being the fifteenth which accounts for 3/4 of the coding region (Groden 1991; Kinzler 1991). Over 95% of mutations in *APC* lead to chain termination resulting in the expression of truncated proteins, the consistent deletion of the carboxy-terminal suggests that important growth suppressive activity resides in this portion of the protein. Germ line mutations are distributed in the 5′ portion of the gene with two hot spots at codons 1061 and 1309 accounting for 35% of the total (Polakis 1995). Somatic mutations exhibit definite grouping between codons 1286 and 1513 termed the mutation cluster region (MCR) (Miyoshi 1992). The finding that greater than 94% of somatic mutations found in FAP tumours are in the MCR suggests there is a selective pressure for at least one allele to be mutated in the MCR (Polakis 1995 Miyaki 1994). Further evidence to suggest that the position of the mutation in *APC* is important comes from the recognition that different mutations produce different FAP phenotypes, and the phenotype can be determined from the position of the mutation (reviewed in Cunningham 1996 and Polakis 1995). Individuals with mutations 5′ to codon 157 have an attenuated phenotype with few polyps. The presence of the extracolonic feature congenital hypertrophy of the retinal pigment epithelium (CHRPE) is also dependent on the position of the *APC* mutation, retinal lesions are almost always absent if the mutation is 5′ to exon 9 and present if 3′ to exon 9 (Olschwang 1993). This suggests

the expressed mutant protein has some residual interfering function that influences the presence of CHRPE and similar residual function could account for the neoplastic effects of expressed mutant protein (Polakis 1995).

The *APC* gene encodes for a 300 kilodalton cytoplasmic protein (Groden 1991) which can be divided into two domains, the amino terminal domain which represents a quarter of the protein is leucine rich and shows sequence similarities to myosins, intermediate filament proteins and *Drosophila* armadillo protein. The carboxy terminal three quarters is serine rich with no discernible homology to known proteins (Kinzler 1991). Two regions in the N-terminus show a coiled coil structure through which it homodimerizes (Kinzler 1991 Su 1993). APC interacts with several different proteins including ß-catenin, γ-catenin, tubulin, hDLG, and glycogen synthase kinase-3b (GSK-3b) (Morin 1997).

The function of APC remains unclear, although it has been implicated in cell adhesion. APC binds directly with ß and γ catenins, but not E-cadherin, which are all involved in adherins cell junctions (Su 1993; Rubinfeld 1993) and multiple ß-catenin binding sites have been identified (Polakis 1995; Morin 1997). Thus APC may serve as a down stream target for signaling by ß-catenin or may competitively bind ß-catenin regulating its action on another downstream target. Wild type APC has been shown to down regulate ß-catenin, mutant protein truncated at the MCR is also capable of binding ß-catenin but does not down regulate its action (Polakis 1995). Expression of $-NH_2$ deleted ß-catenin in epithelial cell models results in loss of cell morphology and the mutated ß-catenin can be shown to co-localise to APC protein clusters (Pollack 1997). ß-catenin also functions as a transcriptional activator when complexed with members of the T-cell transcription factor family (Tcf) (Molenaar 1996). Wild type APC can suppress signaling by the ß-catenin-Tcf complex (Korinek 1997) however the protein products of mutant *APC* genes present in colorectal cancer are defective in this activity suggesting regulation of ß-catenin is central to *APC*'s tumour suppressive effect

and this can be circumvented by mutations in *APC* (or in the ß-catenin gene) (Morin 1997). APC may, therefore, exist in equilibrium with ß-catenin and E-cadherin and mutations may alter this equilibrium causing loss of growth control resulting in adenoma formation.

The APC protein localises to the ends of the microtubules that extend in to actively migrating regions of epithelial cell membranes. APC levels increase at the crypt / villus boundary where migration is required for enterocytes to exit from the crypt, suggesting APC protein plays a role in directed cell migration (Nathke 1996). This function of APC would appear to regulated by binding of ß-catenin. Wild type ß-catenin binds transiently to APC at the microtubule complex, however mutant ß-catenin deficient in its N-terminus which contains the α-catenin and E-cadherin binding sites forms abnormally stable complexes with APC resulting in inhibition of cell migration. In the presence of normal cell proliferation stimuli this results in cell aggregates *in vitro* (i.e. polyps *in vivo*) (Pollack 1997). Given the size of APC and the number of proteins with which it interacts, it suggests that APC serves to integrate the signals from a variety of sources and transmit them to the nucleus by the ß-catenin / Tcf complexes (Kinzler 1996).

These observations would support the postulation that APC mutation is required for initiation of adenoma formation and subsequent carcinogenesis. When one functional copy of APC is present this can act as a gatekeeper maintaining a constant cell population, mutation in the gatekeeper gene leads to an imbalance in cell proliferation resulting in net proliferation (Kinzler 1996). *APC* mutations have been detected in very small adenomas and their precursors, aberrant crypt foci, suggesting initiation of adenoma formation requires an inactivating mutation of both *APC* alleles (Jen 1994). Also mutations in *p53* and *Ki-ras* without the presence of *APC* mutations do not appear to produce neoplastic cells (Kinzler 1996).

iv) Mismatch Repair Genes

The syndrome of Hereditary Non-Polyposis Colon Cancer (HNPCC) is characterised by young patients with right sided colonic cancers (Lynch 1993). Linkage analysis has proved a genetically determined form of HNPCC (Peltomaki 1993). With colorectal cancer being such a common disease establishing a true genetic link rather than chance clusterings for HNPCC required the analysis of large kindreds. Analysis of two large kindreds with the examination of over 300 microsatellite markers identified an HNPCC gene localising to chromosome 2p15-16 (four point lod scores of 6.47 and 6.01 for the two kindreds) (Peltomaki 1993). Other work identified a second HNPCC gene on chromosome 3p21 (Lindblom 1993). Loss of heterozygosity (LOH) analysis of the HNPCC gene on chromosome 2p16 surprisingly showed this gene was not deleted in any of 14 HNPCC cancers and only in one sporadic cancer. (Aaltonen 1993). Furthermore the majority of HNPCC tumours demonstrated shifts in the electrophoretic mobility of $(CA)_n$ and $(CAG)_n$ microsatellite repeat sequences (microsatellite instability).

Formal analysis of 5 loci in 14 HNPCC tumours revealed 11 (79%) cases in which two or more loci demonstrated mobility shifts (Aaltonen 1993). Such cancers are said to demonstrate a replication error (RER+) phenotype or microsatellite instability (MIN). This phenomenon is not noted in other normal tissues of patients with RER+ cancers (Thibodeau 1993) suggesting that it is this replication error occurs during the cancer development and that there is some underlying cause for the RER+ phenotype which might predispose cancer formation. This does not appear to be the case with sporadic colorectal cancer. Analysis of 46 sporadic cancers identified an RER+ phenotype in only 6 cases (13%) (Aaltonen 1993). In common with HNPCC tumours sporadic RER+ cancers tend to be right sided and are frequently diploid (Aaltonen 1993) suggesting either a common mechanism of tumourigenesis or that these sporadic cases represent previously undetected HNPCC kindreds.

Errors in replication were initially recognised as novel alleles in non-coding regions of DNA which contain dinucleotide repeats (microsatellites) (Ionov 1993). Mutations in the yeast genes *pms1, mlh1* and *msh2* involved in mismatch repair also lead to microsatellite instability similar to that identified in RER+ colorectal cancers (Strand 1993) suggesting mutations in human homologues of these genes could be involved in HNPCC tumourigenesis. The human homologue of the yeast mismatch repair gene *msh2, hMSH2* localises to chromosome 2p22-21 (Fishel 1993) close to the locus used for linkage analysis of the HNPCC kindreds confirming the involvement of human mismatch repair genes in HNPCC. To date five mismatch repair genes have been identified, by homology to bacterial and yeast mismatch repair genes, *hMSH2* (Fishel 1993; Leach 1993), *hMLH1* (Papadopoulos 1994), *hPMS1* and *hPMS2* (Nicolaides 1994) and *GTBP* (Palombo 1995). These gene products are responsible for DNA replication fidelity in humans. Mutations in these genes lead to an unstable genome in which errors in DNA replication frequently occur (Parsons 1993). Restoration of MMR function to cell lines deficient in MLH1 completely reverses replication error (Koi 1994). An RER+ phenotype has been demonstrated in 92% of HNPCC kindreds and MMR gene mutations can be identified in 70% of these (Liu 1996b). Of these mutations *hMSH2* mutations account for 31%, *hMLH1* 33%, *hPMS1* 2% , *hPMS2* 4% and *GTBP* 0%. hMSH2 and GTBP form a heterodimer but only hMSH2 is commonly mutated, similarly hMLH1 and hPMS2 heterodimerise yet only hMLH1 is commonly mutated (Liu 1996b). Some ethnic variation has also been noted. Over 90% of MMR mutations in Finnish families fulfilling the Amsterdam criteria were due to *hMLH1* suggesting a significant founder effect (Nystrom-Lahti 1996). Rather surprisingly *hMSH2* and *hMLH1* mutations were identified in only 25% of HNPCC kindreds in the Eastern United States (Weber 1997).

Some 86-92% of tumours from HNPCC patients are RER+ (Aaltonen 1994; Liu 1996b), contrast 16% of sporadic colorectal cancer. Additionally 57% of adenomas

from HNPCC patients are RER+ contrast 3% of sporadic adenomas suggesting the involvement of mismatch repair in the pre-malignant stage of HNPCC cancers. Interestingly in some HNPCC patients with multiple adenomata not all were RER+. This suggests the RER+ phenotype is not necessary for adenoma formation but that mismatch repair accelerates the adenoma - carcinoma progression (Aaltonen 1994), accounting for the younger age of HNPCC patients. Whilst the majority of HNPCC patients have germline mutations in one of the mismatch repair genes (Liu 1996b) only one in ten sporadic colorectal cancers demonstrating RER+ have germline mutations (Liu 1995). Furthermore, only 3/7 sporadic RER+ colorectal cancers had detectable mutations in the known MMR genes. This suggests a different mechanism of tumorigenesis in such cancers or that these cancers have mutations in previously undiscovered MMR genes. All tumours with identifiable MMR gene mutations in this study had mutations in both alleles fulfilling Knudsons criteria for a tumour suppressor gene (Liu 1995).

Of the 10-17% of sporadic cancers that demonstrate MIN (Bubb 1996; Ionov 1993) *hMSH2* alterations are found in 61% however only 4% have exonic mutations (Bubb 1996). Mutations in the mismatch repair genes are often indirectly detected by the identification of MIN. Replication error is not confined to non-coding regions of the genome and errors can conceivably occur in important oncogenes or tumour suppressor genes leading to tumour progression. Mutation rates in cells with MMR deficiency are two to three orders of magnitude higher than normal cells (Eshleman 1995). Hence the mutations in the HNPCC genes are not directly carcinogenic but act to accelerate the accumulation of other specific mutations required for colorectal carcinogenesis (Kinzler 1996).

Transforming Growth Factor Beta Receptor II Gene

Genes that contain repeated nucleotide sequences within their reading frame would be an obvious target for mutations due to mismatch repair. One such example is the

Transforming Growth Factor ß Receptor II (*TGFß RII*) gene which has a 10 bp polyadenine repeat (A_{10}) within exon 3 (Lu 1996). Transforming growth factor ß I (TGFßI) is a potent inhibitor of cell proliferation and many tumour cell lines have lost their response to TGFßI suggesting its loss of action may be important in neoplastic transformation (Alexandrow 1995). TGFßI acts through a receptor consisting of three components of which TGFß RII forms a part. Inactivation of either TGFß receptor I or TGFß RII results in TGFßI resistance (Wrana 1994). Frame shift mutations due to 1 or 2 bp insertions or deletions, suggestive of replication error, have been found within the A_{10} repeat in the *TGFß RII* gene in 90% of colorectal cancers or related cell lines with microsatellite instability (Parsons 1995b). This supports the idea that *TGFß RII* is an important target of inactivation in mismatch repair deficient tumours. Similar findings have been found in other genes with repeated sequences in their coding region for example, Insulin-like growth factor II receptor (*IGFIIR*) gene contains an 8 bp polyguanine repeat. IGFIIR binds TGFßI and is essential for TGFßI activation. IGFIIR also antagonises the growth stimulatory action of Insulin-like growth factor II; frame shift mutations in the polyguanine segment have been identified in 12.5% of HNPCC tumours and 9% of sporadic RER+ tumours. Interestingly there is an inverse relationship with *TGFß RII* mutations, RER+ tumours either have a *TGFß RII* mutation or an *IGFIIR* mutation but not both (Souza 1996). The *BAX* gene also contains an 8 bp polyguanine segment in which showed frame shift mutations in 50% of MIN colorectal cancers (Rampino 1997).

The practicality of a molecular screening approach will depend on the chromosomal sites chosen for analysis. Although it has been shown that any clonal expansion of genetically modified site is of value in screening for neoplasia (Mao 1994) in colorectal cancer there are additional advantages in studying changes in specific genes as these can have distinct biological significance (Hamilton 1992). As detailed in the preceding sections *APC* is the hallmark of almost all colorectal neoplasms, benign and malignant.

Ki-ras mutations are found in 45% of carcinomas and the larger dysplastic adenomas. Mutations in *p53* are found in 50% of carcinomas and very few adenomas. Finally *TGFβ RII* mutations are associated with the majority of cancers due to mismatch repair deficiency. Thus it is anticipated that analysis of these four important sites of mutation in colorectal cancer would be expected to identify the majority of cases.

1.6 Telomerase activity in neoplastic tissues

Most cells that can divide *in vivo* cannot do so indefinitely. The process that limits the proliferative potential of cells is termed cellular or replicative senescence. Replicative senescence is especially stringent in human cells and most proliferatively competent mammalian cells senesce. Somatic cells acquire this finite replication sometime during embryonic development through mechanisms which remain unclear (Campisi 1997). Senescence represents a phenotype in which cells although proliferatively arrested, remain biochemically active and viable for long periods of time (Wynford- Thomas 1997). There are 3 notable exceptions to senescence, germ cells, stem cells and malignant tumours. Replicative senescence can act as a tumour suppressive mechanism and tumour cells often acquire mutations that allow them to overcome the proliferative constraints of senescence. A number of well recognised proto-oncogenes and viral oncogenes appear to act by allowing cells to escape from replicative senescence (e.g. *C-MYC* and SV40 virus large T antigen gene). Also the tumour suppressor genes *p53* and *RB* are essential for maintaining senescence (summarised in Campisi 1997). In fibroblasts senescence can be induced by gene products known to inhibit cell proliferation such as $p21^{WAF1}$ and $p16^{INK4a}$. Senescence can be abrogated by mutations in *p53* and *pRb* (Wynford- Thomas 1997). Escape from cellular senescence predisposes a cell to neoplastic conversion (Oshimura 1997). It has been proposed that cellular senescence is controlled by genes which are activated or

have functions which become evident at the end of the proliferative life span of the cell (Sugaware 1990). Hybrids of normal and tumour cells senesce suggest the genetic information contained in the normal cell can correct defects in the tumour cell. Single chromosome transfer has shown putative senescence genes on at least 10 chromosomes suggesting a multiple pathway mechanism. A number of defects by which a cell can escape senescence and proceed to neoplasia are therefore possible (Oshimura 1997).

The mechanism by which cells sense the number of divisions they have completed and enter senescence depends in part on the length of the telomeres. Telomeres are the ends of linear chromosomes consisting in humans of the repetitive sequence TTAGGG. Telomeres serve to maintain the stability of the genome by protecting against chromosome fusions, translocations and non-dysjunctions.

DNA polymerases are unidirectional and require a labile primer, thus each round of replication leaves some 3′ bases at the telomere unreplicated hence for most cells telomeres shorten with each replication. Analysis of telomere length as a function of age or cell division number shows that telomeres gradually shorten with age or cell division number. It is thought that this gradual loss of telomeric DNA could lead to chromosomal instability and contribute to aging and senescence. In germ cells the average telomere length is maintained at around 5 - 10 kb (Blackburn 1991). Germ cells do not undergo replicative senescence and differ from most other cells in that they exhibit telomerase activity (Campisi 1997).

Telomerase is a multimeric ribonuclear enzyme that adds telomeric repeats to chromosome ends *de novo* (Campisi 1997) maintaining the length of the telomere. Telomerase synthesizes the G-rich strand of telomeres in a 5′ to 3′ direction. The reaction does require ATP and both the RNA and protein components are essential for the reaction. The telomerase RNAs of lower organisms have been shown to contain sequences that are complementary to their DNA telomeric repeats. It would appear that this complementary RNA acts as a template by which telomerase synthesizes the G-rich

strand. This is confirmed by the finding that mutated RNAs in the complementary sequence result in telomeres elongated by the new mutated sequence (Blackburn 1991). In cell lines and cancers exhibiting telomerase activity the telomere length is stabilised rather than continuing to elongate indefinitely. This suggests that a regulating mechanism exists for limiting telomere elongation by telomerase (Blackburn 1991; Cooper 1997; van Steensel 1997). Over expression of telomerase with mutated RNA in Tetrahymena can cause a dominant negative phenotype leading to telomerase shortening (Blackburn 1991). Human telomeric-repeat binding factor (TRF1) is a duplex telomeric TTAGGG repeat binding protein that is associated with human and mouse telomeres in interphase and mitosis. TRF1 over expression in a cell with telomerase activity and stable telomeres results in a gradual progressive shortening of the telomere. Mutant TRF1 that lacks a DNA binding motif demonstrates a dominant negative effect leading to telomere elongation. These findings suggest that TRF1 modulates telomere length through a negative feed back effect. Interestingly the DNA binding motif shows close homology to the proto-oncogene MYB (van Steensel 1997). In further support of a negative feedback regulation a telomere binding protein Taz1p (from the fission yeast *Schizosacchromyces pombe*) shares homology with the MYB DNA binding domain of TRF1. Disruption of its gene *taz1*$^{+}$ leads to massive increase in telomere length (Cooper 1997) suggesting these telomere binding proteins may be negative regulators of telomerase or may protect against telomerase independent pathways of telomere elongation. In *S. cerevisiae* the telomere binding protein Rap1p also shares homology with the MYB domain of TRF1. Telomere length in *S. cerevisiae* depends on the number of Rap1p molecules bound to the telomere (Marcand 1997). Peak and average telomere length have been shown to be shorter in cancers when compared adjacent normal tissue (Engelhardt 1997) suggesting the telomerase / TRF1 feed back mechanism may have some influence in tumourigenesis.

The G rich strand (G-strand) is always located at the 3′ end of the chromosome and it is thought that the G-strand overhangs its complementary strand and is thus single stranded. There are a number of proteins that avidly bind to single stranded G-strands, which itself can also form complex secondary structures *in vitro*, whether such structures exist *in vivo* and their possible function remain to be elucidated (Wellinger 1997).

Telomerase activity is not found in somatic cells (Engelhardt 1997) but its activity has been noted in cancer cell lines and tumours suggesting it may play a role in tumourigenesis (Campisi 1997). Whether telomerase activity in cancers represents a real return to telomerase expression by the cancer cells or is simply due to an enrichment in stem-like cells that are known to have telomerase activity is not understood. However, semiquantitative studies have shown that the amount of telomerase activity depends on the proportion of tumour cells in the examined specimen (Engelhardt 1997). A survey of 3500 cancers and cancer cell lines has shown telomerase activity is wide spread in neoplastic tissue. In colorectal cancers and cell lines telomerase activity can be detected in 89% of carcinomas and 45% of adenomas (Shay 1997). Other research would suggest this proportion is even higher at around 95% (Odogwu 1997). Telomerase activity has been identified in histologically normal tissue adjacent to cancers and in premalignant lesions suggesting telomerase activity in these tissues is due to the presence of previously undetected cells (Kim 1997). The detection of telomerase activity may therefore represent a ubiquitous and early tumour marker that could be used for screening.

1.7 Mutational mosaicism

As described in section 1.5.4 individuals with mutations in the mismatch repair genes have an increased cancer susceptibility. Recent work has shown that greater than 50%

of individuals who develop colorectal cancer at an atypically early age (less than 35 years of age) have germ-line mutations in the mismatch repair genes (Liu 1996). It is widely assumed that these individuals accumulate mutations in genes essential to colorectal cancer development exclusively in the founder cells of tumours or their neoplastic clones (Kinzler 1996). Certain individuals from HNPCC families with apparently heterozygous MMR gene mutations show constitutionally deficient MMR which can induce alterations at repeat sequences in non-neoplastic tissues (Parsons 1995). This raises the possibility that individuals bearing germ-line mutations in mismatch repair genes may undergo somatic mutations in cancer genes at an early stage in embryonic development and that cells bearing these mutations may become distributed into a variety of tissues. Such individuals would thus be genetic mosaics with some cells having an enhanced cancer susceptibility scattered amongst normal cells (Dunlop 1996b). Support for this theory comes from the fact that certain patients with attenuated extracolonic manifestations of FAP, such as mandibular osteomas and congenital hypertrophy of the retinal pigment epithelium (CHRPE), but with non-polyposis cancer, are significantly more likely to have right sided RER+ cancers. This suggests an underlying MMR defect in such individuals (Dunlop 1996). One interpretation of this observation is that such individuals have a germline deficiency in MMR and an early acquired moasicism in *APC*. *APC* mosaicism in humans has been reported (Mandl 1994). Cancers associated with mismatch repair gene defects tend to display a constellation of clinical and molecular pathological features including MIN (Lothe 1993; Kim 1994). RER+ cancers account for 15 - 20% of conventional late onset colorectal cancer (Lothe 1993; Thibodeau 1993; Aaltonen 1994). Interestingly germline defects in the mismatch repair genes are seldom found in patients with late onset disease (Liu 1995; Liu 1996b). At present it is not clear whether such patients carry constitutional mutations in hitherto undiscovered MMR genes, or, bear mutations exclusively in the founder cells of their cancers or are limited mosaics for certain cancer

predisposing mutations such as *APC*. whole gut lavage fluid may provide a method by which the entire colonic mucosa can be sampled and may be useful in determining the possibility of *APC* mosaicism in macroscopically normal mucosa.

1.8 Aims

This thesis had several aims. Firstly in recognising the difficulties in using stool for DNA mutational analysis the suitability of whole gut lavage fluid for DNA extraction and PCR analysis was evaluated. Secondly where all previous studies examining cancer mutations in stool samples had confined their search to one gene it is clear that several mutations sites must be interrogated to improve overall sensitivity. As a proof of principle the systematic analysis of 4 genes commonly mutated in colorectal cancer (namely *Ki-ras* codon 12, *p53* codon 248, *APC* mutation cluster region and *TGFß RII*) was initially undertaken.

In a related line of work while telomerase activity can be detected in laboratory lavage samples from colorectal cancer specimens, this activity has not been detected in clinically obtained samples. Preliminary work to try and detect telomerase activity in whole gut lavage fluid was undertaken.

Successful development of techniques to detect mutations in whole gut lavage fluid would provide a useful research method by which to examine mechanisms of colorectal carcinogenesis particularly in high risk individuals. In high risk individuals with a macroscopically normal bowel random colonic biopsy for genetic analysis at colonoscopy is of little value. Whole gut lavage may provide a method by which the whole colonic mucosa could be sampled. In this way it may be possible to identify pre-malignant mutations in high risk individuals in whole gut lavage. One obvious candidate would be *APC*. With successful detection of *APC* mutations in whole gut lavage fluid in known cancer patients the final aim of this thesis was to apply these tests to whole gut

lavage fluid obtained from asymptomatic high risk individuals to try and further elucidate the mechanism of tumorigenesis in these subjects.

2. Materials and methods

2.1 Whole gut lavage fluid

Whole gut lavage fluid was obtained preoperatively by standard methods, developed in the GI laboratory Western General Hospital Edinburgh (Choudari 1993) from patients with known colorectal cancer. Local ethical approval was obtained and each patient gave informed consent. Polyethylene glycol in the form of a balanced electrolyte solution "Klean Prep" (Norgine Ltd., Harefield, Middlesex, UK.) was administered orally at a rate of 250 ml every 15 minutes until the effluent passed per rectum was clear of particulate matter. A 100 ml sample was then collected for DNA extraction. The majority of samples were obtained from hospital inpatients, but in six patients sample collection was performed as an out patient procedure. Here additional samples were processed and stored according to laboratory protocol. Lavage fluid was clarified in a centrifuge at 2500 rpm for 5 min. To 3 x 1.5 ml aliquots of unfiltred and unprocessed lavage fluid 30 µl of 2% sodium azide was added and then stored at -70°C. A 10 ml aliquot of lavage fluid was filtered through Whatman GF/A 12.5 filter paper. A second 10 ml aliquot was removed from the unfiltred lavage fluid. To 5 ml of each aliquot 0.5 ml of 0.1% of soya bean trypsin inhibitor, 0.28 ml of 0.1M disodium ethylene diamine tetraacetic acid (pH 8.0), 0.12 ml of 0.3M phenylmethylsulphonylfluiride and 0.06 ml of 2% sodium azide were added. The mixtures were incubated at room temperature for two minutes before the addition of 0.3 ml of new born calf serum. For each processed sample (filtered and unfiltered) 1.5 ml aliquots were dispensed and stored at -70°C.

2.2 Cellular analysis of whole gut lavage fluid

Cell separation was undertaken by density gradient centrifugation adapted from methods developed in the G I laboratory at the Western General Hospital Edinburgh (Handy 1995).

Discontinuous Elution gradients

Twenty-five ml of whole gut lavage fluid was coarsely filtered using surgical gauze then centrifuged at 850 g for 10 minutes. The supernatant was discarded and the cell pellet resuspended in 5 ml of Hanks' balanced salt solution (Sigma Chemical Co. Ltd., Poole, UK). This was layered on top of a discontinuous density gradient using Histopaque 1119 and 1077 (Sigma) and centrifuged at 700 g for 30 minutes. Resulting cell layers were harvested separately washed with Hanks' balanced salt solution and used for cell count or preparation of cytospin slides.

Continuous elution gradients

The discontinuous gradient was further refined by preparing a continuous density gradient using a gradient mixer with 5 ml of Histopaque 1119 and 1077. The mixture was siphoned into the centrifuge tube at a constant rate (2 ml / min.) using a peristaltic pump. (The refractive index RI of samples taken at intervals from a control gradient when prepared as outlined above demonstrated a linear relationship to the proportions of Histopaque 1119 and 1077. This confirmed even and satisfactory mixing of the buffers to produce a continuous elution gradient.) Whole gut lavage samples were layered on the top of continuous gradients and processed in an identical manner to the discontinuous gradient. After cell separation the gradient was siphoned off again using the peristaltic pump at the same rate in to 1 ml aliquots. Cell counts were performed and the density of the gradient for each aliquot determined by measurement of the refractive index.

Cell counts

These were made using a haemocytometer in the standard way. A 5µl drop of each sample was placed on the haemocytometer slide and covered with a cover slip to produce an even film. The slide was then examined under x 100 magnification and cells counted. For the discontinuous elution gradient cell counts were made for each cell layer. For the continuous gradients each 1 ml aliquot was analysed. Average cell counts were recorded from 4 observations for each layer or aliquot.

Cytospin preparation

Duplicate slides were made using 50µl and 100µl aliquots of the two separate cell layers for samples using a discontinuous gradient and for each aliquot that contained cells when the continuous gradient was used. The samples were centrifuged at 90 g for 10 minutes, air dried and fixed in 10% formalin before Haematoxylin and Eosin staining. Histological examination was performed under the supervision of a Consultant Histopathologist (Dr. M. J. Arends).

2.3 DNA extraction

From paraffin sections

Normal and tumour control DNA samples were obtained from paraffin blocks of the surgically resected specimen. Three 10 µm sections were cut using a microtome, placed in a sterile 1.5 ml eppendorf tube and stored at 4°C until required. DNA was extracted using standard methods (Levi 1991). Briefly 400 µl of lysis buffer (10 mM Tris, pH 8.3, 50 mM KCl, 0.45% Tween 20, 2.5 mM $MgCl_2$) was added to each eppendorf tube along with Proteinase K (0.5 mg/ml) and incubated at 55°C for twenty-four hours then

boiled for 20 minutes, 100µl aliquots of the resulting supernatant were stored at -20°C until required and 3-5µl aliquots were used for PCR analysis.

From frozen tissue

In a dedicated class II biological safety cabinet a small portion of the tissue (0.3 mm^3) was diced and place in a sterile 1.5 ml. eppendorf tube to which 500 µl of TE-9 SDS (500 mM Tris pH 8.0, 20 mM EDTA, 10 mM NaCl, 1% SDS) was added along with proteinase K (0.5 mg/ml) and incubated for 48 hours at 48°C vortexing intermittently. An equal volume of TE (10 mM Tris pH 8.0, 1 mM EDTA) saturated phenol was added, mixed thoroughly and the phases separated using a microfuge (13,000 rpm for 2 minutes). The aqueous phase was then removed to a clean tube and another separation with TE saturated phenol undertaken. The aqueous phase was then mixed with an equal volume of chloroform / iso-amyl alcohol (ratio 24:1) and further separation in the microfuge at 13,000 rpm for 2 minutes. Once again the aqueous phase was removed to a clean tube and the DNA precipitated by adding 0.25 ml of 7.5M ammonium acetate and 1 ml of cold absolute ethanol and left over night at -20°C. The precipitate was pelleted using the microfuge and vacuum dried before resuspension in 0.25 ml of TE buffer.

From whole gut lavage fluid

Several methods of DNA extraction from whole gut lavage fluid were evaluated. Successful DNA extraction was determined by subsequent PCR amplification of the mitochondrial gene cytochrome b (Smith-Ravin 1995). The best method was adapted from the method used by Smith-Ravin *et al.* (Smith-Ravin 1995) to extract DNA from stool samples. To 100 ml whole gut lavage fluid, in 4 aliquots of 25 ml, lysis buffer was added to a final concentration of 10 mM Tris, 1 mM EDTA, 0.5% SDS, pH 7.5 within 15 minutes of sample collection and incubated at room temperature for 30 minutes. The samples were clarified by centrifugation at 1500 rpm using a Sorvall RT 6000D

centrifuge at 5°C. The supernatant was removed from each sample and extracted with an equal volume of TE saturated phenol in a clean tube. The supernatant was again removed to a clean tube and the DNA was precipitated with an equal volume of cold isopropanol. This precipitate was pelleted in the centrifuge at 3000 rpm for 30 minutes and the supernatant discarded. The resulting pellet was dried before resuspension in 500 µl - 1 ml of TE and digested with proteinase K (0.5 mg/ml) at 48°C for 24 hours. Two to three phenol / chloroform extractions as required followed by ethanol precipitation were then undertaken. Finally the resulting pellet was dissolved in 50 - 100 µl of TE followed by further purification in GeneClean Spin Columns (Bio 101, Inc., Vista, CA, USA.) according to the manufacturer's protocol.

DNA concentration measurement by spectroscopy

The concentration of DNA samples was determined by measuring the absorption of the sample at 260 nm and calculated using the following formula.

$[DNA] = 50Dx$

Where 50 represents a standard correction factor for double stranded DNA

D = the adsorption at 260 nm

x represents a correction for dilution of the sample (usually 500)

DNA concentration measurement by UV fluorescence

Aliquots (1µl) of standard DNA concentrations (10 µg/ml to 500 µg/ml) where placed on to gels containing ethidium bromide (0.5 mg/ml). Close to these where placed 1µl aliquots of DNA solutions from whole gut lavage fluid samples. These were left for 10 minutes to be absorbed into the gel. The gels were then visualised using video equipment under UV light in a light tight chamber. The standard DNA concentrations were used to construct a standard curve and the whole gut lavage fluid DNA concentrations were then estimated using quantitative software (Biorad Molecular Analyst) and by eye.

2.4 Polymerase Chain Reaction

Primers

Unless otherwise stated all primer sequences used were as published in the literature and are referenced in the relevant section. Stocks were obtained from commercial companies (Cruachem Ltd., Glasgow, Scotland, UK.; Oswell DNA Service, University of Southampton, Southampton, UK.) and stored at -20°C. Aliquots for PCR and sequencing were diluted to a concentration of 10 mM and stored separately from the stock solutions at -20°C. Additional primers were designed from known sequences using the "Primer" shareware program available on the Internet (Whitehead Institute, Cambridge, Massachucetts, USA.)

Optimisation of reaction conditions

All reactions were performed using *Taq* DNA polymerase (Gibco BRL, Life Technologies, Paisley, Scotland, UK.). Reactions were set up in a separate dedicated Class II biological safety cabinet and any dilution was performed with autoclaved deionised water. To avoid cross contamination DNA was added to prepared reaction mixtures in a second separate dedicated horizontal laminar flow hood. All reactions (unless otherwise stated) were prepared according to the manufacturer's protocol and contained 10x reaction buffer diluted to a final concentration of 20 mM Tris-HCl, (pH 8.4) and 50 mM KCl . A 10 mM nucleotide mixture was diluted to give a final concentration of 0.2 mM of each nucleotide. For reactions using DNA from paraffin sections a 1% W-1 detergent was added to a final concentration of 0.05% (v/v). Each reaction contained 2.5 units of *Taq* DNA polymerase. Initial PCR optimisation was performed by varying Mg^{2+} and primer concentrations. A 50 mM $MgCl_2$ solution was added in 1µl aliquots to vary the final concentration from 1.0 to 2.5 mM. Each primer

was made up to a 10μM concentration and added in 2.5μl aliquots to vary the final concentration between 0.25 and 1 μM. Between 5 and 200 ng of template DNA (in a volumes of 1 - 5μl were added. The resultant mixtures were diluted using autoclaved distilled water to a final reaction volume of 50 μl. Evaporation was prevented by a 25 μl mineral oil overlay. Reactions were performed in a Hybaid Omnigene thermocycler. For reactions that required further optimisation the annealing temperature (Tm) was also varied by ± 1°C increments. The end point for optimisation was a single PCR product seen on agarose gel electrophoresis with ethidium bromide staining. Negative control reactions containing no DNA were included in every reaction. Individual reaction conditions are detailed in the appendix. To confirm successful amplification all PCR products were routinely resolved on 1-3% agarose gels, depending on product size. A 1 Kb molecular weight marker was added to one lane and each gel run at constant voltage (125V) with 1x TBE buffer (Tris Borate EDTA). Ethidium Bromide (1 mg/ml) was added to each gel and products were visualised under UV light.

***Ki-ras* codon 12 enriched PCR**

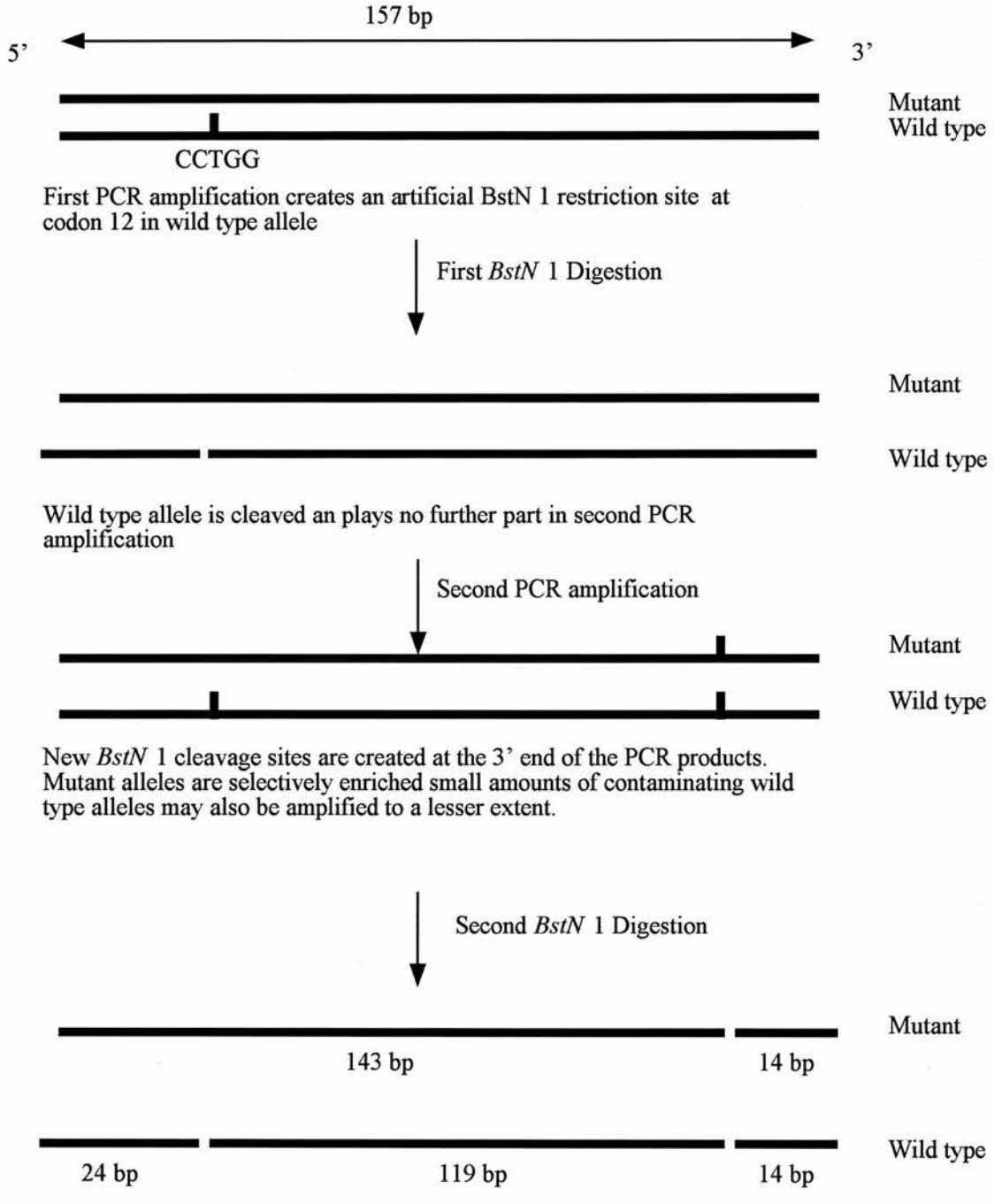
The point mutations at *Ki-ras* Codon 12 and p53 codon 248 were analysed using an enriched PCR strategy (Kahn 1991; Tobi 1994). For the detection of *Ki-ras* codon 12 mutations the 5′ primer was modified to contain a G to C substitution at the first base pair position of codon 11 which in turn produces *BstN I* restriction enzyme site (5′..CCTGG..3′) overlapping the first two base pairs of codon 12 (Fig 2.1). Wild type product will therefore be cut by *BstN I*, where as mutant product will not, enabling activating mutations of the *Ki-ras* oncogene to be detected by restriction fragment length polymorphism (RFLP). To selectively enrich for mutated DNA two rounds of PCR amplification are used each followed by restriction enzyme digestion with *BstN I*. The first PCR contains the 5′ modified primer along with an unmodified 3′ intronic primer. After *BstN I* digestion, in which the 5′ portion of any wild type product is

cleaved, an aliquot of the digest is used as the template for a second PCR. Because the wild type product has lost its 5′ portion it takes no further part in the reaction allowing the mutant product to be selectively amplified. As an internal control for enzyme fidelity a second *BstN I* site can be incorporated in the 3′ primer used in the second round of PCR amplification along with the 5′ modified primer. A second *BstN I* digestion of this reaction product will remove any wild type product which may have been carried through from the first reaction allowing easy identification of any mutations by polyacrylamide gel electrophoresis (PAGE). Mutations detected in the whole gut lavage fluid samples were confirmed by direct sequencing with a 3′ nested primer.

In detail a protocol with modification from Tobi and Kahn (Kahn 1991; Tobi 1994). was used as follows. The first PCR reaction was performed in a 100µl volume containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 10 ng of each primer and 2.5 units of *Taq* DNA polymerase for 15 cycles with an annealing of temperature 55°C. (Primers: sense, with a *BstN I* restriction site: 5′ACTGAATATAAACTTGTGGTAGTTGGACCT3′, antisense, wild type: 5'TCAAAGAATGGTCCTGCACC3′.). Using 10µl of the first PCR product a *BstN I* enzyme digestion was undertaken with 20 units of enzyme in a final reaction volume 20µl incubated at 60°C for 3 hours. Next a second PCR using 1µl of the digestion product as template was carried out in a 50µl volume. The reaction conditions were identical to the first PCR except 150 ng of each primer was used and 35 cycles were performed. (Primers sense: *BstN I* restriction site, antisense: *BstN I* restriction site 5′TCAAAGAATGGTCCTGGACC3′).

Finally a second *BstN I* restriction enzyme digestion was undertaken using 20µl of the second PCR product and 10 units of enzyme in final reaction volume 30µl incubated at 60°C for 2 hours. The products of digestion were analysed by PAGE

Figure 2.1 Enriched PCR for *Ki-ras* codon 12 mutations exploiting a modified *BstN 1* restriction site



using a 15% non-denaturing acrylamide gel in the Miniprotean apparatus (Bio-Rad Laboratories Ltd., Bio-Rad House, Hemel Hempstead, Hertfordshire, UK), 10µl of PCR product were mixed with 5µl of loading buffer and all 15µl were loaded into each lane. The gels were run at constant voltage (125v) until the Bromophenol blue dye ran off the end of the gel. The gels were then stained with ethidium bromide (0.5 mg/ml in 500 ml 1x TBE) for 20 minutes and destained for 10 minutes in 1x TBE buffer and the products visualised under UV light. Any uncut PCR product was seen on the gels as a 157 bp product. Cleavage of mutant DNA produced fragments of 143 bp and 14 bp in length whilst any contaminating wild type DNA would produce fragments of 119 bp, 24 bp and 14 bp in length (figure 2.1).

The mutant band on the 15% polyacrylamide gel was cut out and the DNA extracted by boiling in DDW for 20 minutes. The extracted DNA (10µl) was then subjected to a further *Ki-ras* PCR amplification using the original 5'-primer and a new nested 3'-primer (5′-CTC TAT TGT TGG ATC ATA TT). The resultant PCR product was then directly sequenced and compared with a similar PCR product derived from DNA extracted from the tumour.

***p53* codon 248 enriched PCR**

The analysis of *p53* codon 248 was undertaken in a similar manner using the *Msp I* restriction enzyme. In this assay the primers were not modified to produce artificial restriction sites, as the wild type sequence at codon 248 produces a *Msp I* restriction site (CCGG) and mutation of any of the base pairs in this codon destroys the restriction site. An initial PCR amplification was undertaken using 5 µl of DNA from whole gut lavage fluid in a 100 µl reaction containing 10 ng of each primer, 1.5 mM Mg^{2+} , for 15 cycles, annealing temperature 58°C. (Primers: sense: 5′-TGT GTT ATC TCC TAG GTT GG, antisense 5′-TGG CAA GTG GCT CCT GAC.) A 10 µl aliquot of the first PCR reaction was digested with 2U of *Msp I* in a 20 µl volume at 37°C for 1 hour. A 1 µl aliquot of the digest was then used as a template for a second PCR reaction using

150 ng of the same primers in a 50 µl reaction for 30 cycles. A second *Msp I* digest was performed using 10 µl of the second PCR product. Because the primers were not cut by the restriction enzyme, to ensure complete enzyme digestion, at each stage positive and negative controls were included in each assay. This eliminated the possibility of false positive results by incomplete enzyme digestion. Mutant alleles remained uncut and appeared as a single 143 bp band, whilst wild type alleles were cut into 85 and 58 bp products. In tumour samples where mutations were detected, the corresponding whole gut lavage fluid samples were analysed in a similar manner. All mutations detected were confirmed by direct sequencing of gel purified products as described previously using a 5′ nested primer (5′- TAG GTT GGC TCT GAC TGT) and the original 3' primer. Reaction conditions were identical to the those for the second PCR of the enriched strategy.

2.5 Sequencing of PCR products

PCR products were sequenced directly using the dideoxy chain termination reaction as outlined in the manufacturers protocol (Sequenase version II enzyme (Amersham International, UK)). The sequencing products were radio-labeled by the incorporation of α^{35}S dATP and run on preheated 40 cm x 30 cm 6% polyacrylamide denaturing gels with 7M urea. Initially gels were run at 70 watts until the bromphenol blue of the loading buffer had just run off the end of the gel. The resultant gels were dried and autoradiographs produced. Longer runs were undertaken as determined by the sequence information obtained.

2.6 End Labeling protocol

To allow analysis of PCR products by autoradiography some PCR products were end labeled with γ^{33}P ATP. First one primer (of the primer pair) was end labeled in a 20 µl reaction containing the primer (5 mM), γ^{33}P ATP (0.3 mM) (ICN Oxon England) and 20 units of T4 DNA polynucleotide kinase (Gibco BRL, Life Technologies, Paisley, Scotland) buffering conditions (70 mM Tris, pH 7.6, 0.1M KCl, 10 mM $MgCl_2$ 5 mM Dithiothreitol (DTT), 0.5 mg/ml Bovine Serum Albumin (BSA)). The reaction mixture was then incubated at 37°C for 1 hour.

In a 50µl reaction 10µl of the appropriate PCR product was labeled using 1.5µl of the previously labeled primer; the reverse primer, additional buffer, magnesium, nucleotides and *Taq* DNA polymerase were added to achieve standard PCR concentrations and two cycles of 94°C for 3 minutes, 55°C for 2 minutes and 72°C for 10 minutes undertaken.

2.7 Single strand conformational polymorphism.

Both radiolabeled and silver staining techniques were employed to detect polymorphisms.

For PCR products end labeled with γ^{33}P ATP, 12µl of the PCR product was added to an equal volume of denaturing buffer (95% Formamide, 20 mM EDTA, 0.05% Bromophenol Blue and 0.05% Xylene Cyanol) and then resolved on 50% MDE (FMC Bioproducts Rockland ME USA) gels containing 10% glycerol. Electrophoresis was performed at 10 watts and at room temperature overnight. The resulting gels were dried and autoradiographs taken. Samples were run in duplicate on separate gels.

For unlabelled products silver staining was performed. The protocol adopted was identical to that described above except one of the electrophoresis plates was covered with a bind solution (99.2% absolute ethanol, 0.5% glacial acetic acid and 0.3% γ-methacryloxypropyltrimethoxysilane). The gel on the bind plate was fixed in 10% ethanol for 10 minutes followed by 1% nitric acid for 10 minutes. It was then rinsed with DDW for 5 minutes before staining with 12 mM silver nitrate solution for 30 minutes. The gel was then rinsed quickly (10 seconds) with DDW and 100 ml of chilled developer added (0.3M sodium carbonate decahydrate, 0.019% formaldehyde). As soon as a precipitate appeared the developer was poured off and 1 litre of fresh developer added and agitated until bands appeared. The developer was then discarded and the gel was then fixed with 0.1M citric acid.

2.8 Microsatellite analysis

To determine the replication error (RER) phenotype of the cancers examined in this thesis five microsatellites were examined by PCR. Two poly A repeats BAT 26 and BAT 40 (Liu 1996b) and three CA repeat D2S123, D5S346 and D13S60 (Susan Farrington personal communication) were analysed. (For PCR conditions see appendix). The PCR products of normal and tumour pairs were end labeled with γ^{33}P ATP and resolved denaturing PAGE (6% polyacrylamide, 7M urea, 0.5x TBE buffer). The resultant gel were transferred to 3 mm chromatography paper, dried and autoradiographs taken. Novel alleles were identified by band shifts of the tumour sample in relation to the paired normal. Gels were run for both forward and reverse end labeled primers. Tumours were described as RER+ if shifts were seen at two or more loci.

2.9 Protein Truncation Test

Some of the analysis of APC mutations was undertaken using the protein truncation test (PTT) as detailed below.

For PCR products to be used as templates for in vitro transcription and translation the 5′ primer must be modified to incorporate four functional regions (Roest 1993). There is a T7 DNA dependent RNA polymerase transcription initiation sequence, which is preceded by some spacer nucleotides at the 5′ end so that the T7 promoter region is at least 20 nucleotides long, in order to enhance transcriptional activity. These spacer nucleotides can incorporate a restriction site (e.g. *Bam HI*) to aid cloning amplification. The T7 promoter is linked to a ribosome binding sequence immediately followed by an ATG translation initiation codon coupled, in an in frame manner, to the relevant gene specific primer . The antisense primer was not modified. PCR amplification using these modified primers was undertaken in the manner previously described. Cycling conditions were different because of the long length of the 5′ primer and the PCR product. These were typically a denaturation time of 60 s., an annealing time of 60 s., and an extension time of 90 s.

The PCR products were then used as a template for a single tube in vitro transcription / translation reaction (TNT Quick ® , Promega Biotech, Southampton, UK). Reactions were performed as described in the manufacturers protocol using 5μl of PCR product as a template. The protein products were radiolabeled by the incorporation of α^{35}S Methionine (ICN Oxon England). To ensure successful translation a leuciferase control reaction was performed in each reaction which produced a 61 kDa protein product. Protein analysis was performed by SDS-PAGE as described in the manufacturers protocol. In each case the samples were run on 10% 12.5% and 15% gels. The gels were dried on 3 mm chromatography paper and

autoradiographs produced. Truncated proteins were identified as abnormally fast migrating bands on the autoradiogram.

2.10 Telomerase PCR ELISA

Telomerase activity in whole gut lavage fluid samples was assessed using a PCR ELISA technique (commercially available from Boehringer Mannheim) based on the Telomeric repeat amplification protocol (TRAP) (Kim 1994). All experiments were conducted as recommended by the manufacturer's protocol with the following modifications. The protein concentration of the cell lysates and whole gut lavage fluid samples were calculated as described below (section 2.11) and divided into aliquots containing 50 mg of total protein, snap frozen in liquid nitrogen and stored at -70°C. The TRAP reaction was performed using 25 or 50 mg of lysate or whole gut lavage fluid protein in a 50µl reaction. The optimum cycling conditions were modified until a characteristic ladder pattern consisting of multiple PCR products increasing by 6 bp increments was produced when resolved on MDE gels as for microsatellite analysis. These were an initial incubation at 27°C for 30 minutes then telomerase inactivation at 94°C for 5 minutes followed by PCR amplification of 35 cycles, 94°C denaturation for 45 seconds, 50°C annealing for 45 seconds, 72°C elongation for 90 seconds, with one final elongation of 10 minutes at 72°C. Cells known to have telomerase activity (either Kidney 293 or Colon cancer HT29) were used as positive controls with the negative control being heat inactivated cells (15 minutes at 94°C). PCR products were transferred to streptavidin coated microtitre plates and the ELISA performed using kit reagents according to the manufacturers protocol. Microtitre plates were analysed on an MRX Microplate reader using MRX Revelation software (version 3.02). Absorbency was measured at 450 nm with a reference wavelength of 630 nm. For successful interpretation of the ELISA absorption readings of greater than 1.0 $A_{450\ nm}$ -

$A_{630\ nm}$ for the positive control and less than 0.2 $A_{450\ nm}$ - $A_{630\ nm}$ for the negative control were required for each experiment.

2.11 Protein concentration assay

Protein concentrations of cell lysates and whole gut lavage fluid were measured using a Bicinhoninic Acid (BCA) assay (Pierce Chemical Company). The manufacturers protocol was followed and ELISA plates read at 405 nm. Using the absorption data a standard curve for known protein concentrations was produced and the equation of the line calculated using a computer spread sheet (Microsoft Excel ® Version 6.0). This equation was then used to calculate protein concentrations for unknown samples from their absorption readings.

2.12 Nuclear Ploidy

Nuclear DNA ploidy was assessed by Mr. Robert Morris (Chief MLSO) using published methods (Vindelov 1983). Briefly for fresh tissue small tissue fragments were suspended in a citrate buffer and then treated with trypsin for 10 minutes. A trypsin inhibitor was then added before staining the cells with propidium iodide. For paraffin embedded tissue 50μm sections were cut, placed on a glass slide and rehydrated. The rehydrated sections were incubated with 1 ml of pepsin solution (0.5% pepsin, 0.9% saline pH 1.5) at 37^{o}C for 60 minutes. Samples were then washed with Phosphate buffered solution (PBS). The resulting solution was filtered through a 70μm sieve and the supernatant removed after centrifugation. Nuclei were stained with propidium iodide.

Flow cytometry analysis was then undertaken using EPICS (Coulter Ltd, Beaconsfield, Bucks, UK). Solutions were diluted to 10^6 cells per ml and 5000 nuclei were counted for each analysis.

2.13 Loss of Heterozygosity analysis of APC

Polymorphic sequences in the 3' untranslated region of APC and APC exon 11 were analysed by restriction fragment length polymorphism (Curtis 1994). PCR reactions were performed in 100 µl volumes. Each reaction contained 200 ng of genomic DNA and 50 pmol of the appropriate primer (Appendix 1). Cycling conditions consisted of an initial denaturation step of a 5 minutes at 94 °C. Followed by 30 cycles of denaturation for 30 seconds at 94 °C, 30 seconds at a specific annealing temperature and 60 seconds at 72 °C to allow extension, with a final extension step of nine minutes at 72 °C. PCR products were analysed by gel electrophoresis following restriction enzymes digestion as required.

For restriction enzymes digestion, PCR reaction products were ethanol precipitated, resuspended, and the entire sample incubated overnight with five units of the appropriate enzyme, according to the manufacturer's recommendations. The 3' untranslated *APC* region contained an *Ssp I* polymorphism, cleavage product sizes were: allele 1 270 bp and allele 2 135 bp, this reaction also contained a constant band at 580 bp which plays no part in the determination of LOH. Products were resolved on 3% agarose gels. *APC* exon 11 contains a *Rsa I* polymorphism with product sizes of 132 bp for allele 1 and 87/45 bp for allele 2. These products were resolved on 12% polyacrylamide gels. Gels were stained for 30 minutes in 0.5 mg/ml ethidium bromide and DNA fragments visualised by UV illumination. Allele loss or retention was evaluated by eye.

2.14 Study Population

Whole gut lavage samples were collected pre-operatively from 40 patients with confirmed colorectal cancer and these patients form the basis of the study population. For each patient normal and tumour DNA were obtained for comparison from representative sections from the paraffin sections of the surgically resected specimen. The median age of the study population was 71 years (range 39 - 90) with a male : female ratio of 24 : 16. There were 7 Dukes A carcinomas, 14 Dukes B, 17 Dukes C and 2 metastatic lesions; 7 right-sided tumours, 16 left and 17 rectal (See table in Appendix 2). In addition whole gut lavage fluid samples were obtained from 15 patients undergoing bowel preparation for diagnostic colonoscopy (for alteration in bowel habit) which was ultimately normal.

3. Cytology of whole gut lavage fluid

In order to optimize subsequent sample analysis potential methods of cell isolation from whole gut lavage fluid were undertaken. Expected benefits of cell isolation were firstly a reduction in potential PCR contamination when using DNA obtained from whole gut lavage fluid samples. Secondly, a relative concentration of mutant DNA with respect to the vast excess of normal DNA from inflammatory cells in whole gut lavage fluid samples if inflammatory cells could be separated from epithelial cells. Thirdly the concentration of any mutant cells may also be allow the use of immunohistochemical techniques to detect p53 stabilization by flow cytometry.

Methods

Discontinuous density elution gradients familiar to the GI Laboratories and continuous density gradients (detailed in section 2.2) were employed. For each sample visible cell layers were harvested cell counts were performed and cytospin slides were made using aliquots of 50 and 100µl from each sample. These slides were then stained with haematoxylin and eosin prior to microscopic examination.

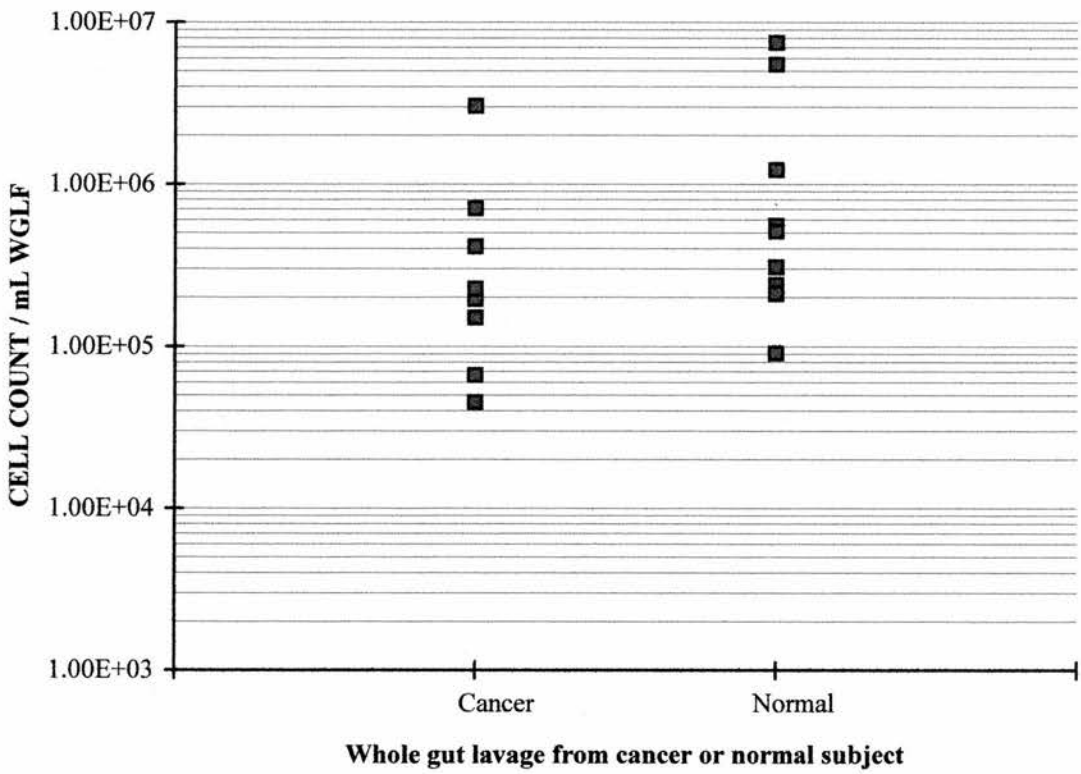
Whole gut lavage fluid samples from 8 patients were processed on a discontinuous density gradient and 10 were processed using a continuous density gradient. There were 8 samples taken from cancer patients and 10 from normal controls. These control patients were patients whom had a diagnostic colonoscopy for positive family history which ultimately was normal. In a further 5 whole gut lavage fluid samples from cancer patients only cytospin slides were made.

Results

The mean cell counts per ml for each sample are shown for cancer samples and normal samples in figure 3.1. There was no difference in the mean cell counts per ml for cancer patients when compared to normal subjects (Mann-Whitney U Statistic = 55.0, p=0.453; Students t-test p=0.313).

A qualitative assessment of the purification of each sample was made by examining the H & E slides with a Consultant Histopathologist specializing in cytometry. Bacteria were ubiquitous in all the samples examined as was particulate debris. Despite the attempted cell separation all slides examined had severe and persistent bacterial and debris contamination. Where cells were identified white blood cells particularly neutrophils and macrophages were most frequently identified followed by squamous epithelial cells, red blood cells and occasionally lymphocytes. Cells representing colonic epithelium or neoplastic cells were seldom identified. Of the 52 slides examined from 13 cancer patients no samples showed overtly neoplastic cells and only 4 slides (in 4 different patients) showed cells that were regarded as suspicious of neoplasia. It was clear from the above analysis of these initial samples that purification did not confer any potential benefit for subsequent DNA extraction and later samples were not processed in this way.

Figure 3.1 Mean cell counts for whole gut lavage samples from cancer patients and normal controls.



Discussion

Cell count alone did not discriminate between cancer and normal samples. Since many neoplasms frequently bleed and may be sources of substantial numbers of exfoliated cells they might have been expected to significantly increase cell counts in whole gut lavage fluid. Increased cell counts in whole gut lavage fluid are noted in patients with active inflammatory bowel disease this is principally due to inflammatory cells (Handy 1995). The mean cell counts for my samples were an order of magnitude greater than that described in previous work (Handy 1995). However cell recovery from stool samples varies over a wide range (Gordon 1991) and the difference between my results and others could represent inter sample variation or a difference in early processing of the samples. The particulate debris widely noted in the samples may also artificially elevate cell counts. Colonic epithelial cells and neoplastic cells were a rare finding which is in agreement with other studies (Gordon 1991; Handy 1995). Inflammatory cells and squamous cell were frequent findings. This is probably because inflammatory cells are designed to migrate in to different environments to confer host immunity and as such are likely to be robust and capable of surviving in the colonic lumen. Conversely colonic epithelial cells are only sloughed into the colonic lumen when in a state of degradation, undergoing apoptosis, and so would not be expected to last long in the colonic lumen. Squamous cell represent unavoidable contamination of the sample from the anal margin, or possibly from the nasopharynx and oesophagus.

Purifying the whole gut lavage fluid samples by density gradients proved unsatisfactory. It had been hoped that the use of these density gradients would help separate cells in the whole gut lavage fluid sample from the bacteria and particulate debris. Two main problems were encountered. Firstly, the passage of particulate debris through the density gradient destroyed it. For the discontinuous gradients most of the cells remained on the top of the gradient with only small proportion passing through the

gradient to the second layer. There was also no separation of the cells into different colonies at these two layers. These findings were also noted in previous work (Handy 1995). For the continuous gradients no discrete layer of cells suitable for extraction was formed. Subjectively, particulate debris could be seen throughout the continuous gradient contaminating it at every level. Microscopically the same problem was noted with bacteria. Secondly, the amount of bacteria and particulate debris was far in excess of the cellular material. Although many bacteria were removed by the gradients, they still far out numbered any cells. Preparations using spin gradients removed a lot of the faecal debris but also removed a substantial proportion of the cellular material. Removal of this contamination lead to major loss of the cellular material available for analysis. In all samples persisting debris contamination meant that flow cytometry analysis of whole gut lavage fluid for p53 stabilization would not be possible.

These cytological investigations permit the following conclusions. Firstly cells with recognizable morphological features are recovered in large numbers from the whole gut lavage. Presumably these samples will contain many cells with pyknotic nuclei and other non-viable cell degradation products. It is therefore reasonable to expect that genomic DNA to be available for PCR amplification albeit, heavily admixed with bacterial DNA. Secondly there is no significant alteration in cell number recovered from cancer patients and those with macroscopically normal colons. The differences in recovery of mutant versus normal DNA is unlikely to reflect trivial reasons such as greater availability of cells in cancer patients. Finally, epithelial cells are always in the great minority and well preserved tumour cells were never seen. This confirms that this method is limited for cancer diagnostic purposes by conventional cytology. The absence of colonic epithelial cells and the infrequent finding of suspicious or malignant cells tends to favour a DNA based approach to colon cancer screening. Small fragments of DNA from apoptotic colonocytes or neoplastic cells may persist in whole gut lavage fluid where cells can no

longer be identified. The question thus remains whether the enhanced sensitivity afforded by PCR has the capacity to generate diagnostic information.

4. PCR amplification of DNA extracted from whole gut lavage fluid

In order to identify the best method of DNA extraction several methods were evaluated. The end points for this evaluation were the total DNA yield and successful PCR amplification of the extracted DNA. Much of the previous work on PCR amplification of DNA obtained from stool has been hampered by inhibition of the PCR (Sidransky 1992; Tobi 1994; Hasegawa 1995; Smith-Ravin 1995; Nollau 1996; Villa 1996). This has been attributed to faecal contaminants in the PCR which can be partially removed by the use of spin column purification (Smith-Ravin 1995). For this reason, spin column purification was included as a final step in each of the DNA extraction protocols. In addition, it has been estimated that human DNA accounts for only 10% of the total DNA extracted from stool (Hasegawa 1995). It would be reasonable to assume this proportion is similar in DNA extracted from whole gut lavage fluid. PCR amplification of the mitochondrial sub-units cytochrome b and cytochrome oxidase has been used to confirm the presence of human DNA in DNA extracted from stool. These mitochondrial DNA fragments are present in high copy number in eukaryotic cells. Their PCR products are 300 bp in size for cytochrome b and 1.4 kb for cytochrome oxidase (Smith-Ravin 1995). The PCR amplification of these mitochondrial subunits was therefore used to determine the best DNA extraction method and confirm the presence of human DNA in the DNA extracted from whole gut lavage fluid.

Because cytochrome subunits are present in high copy numbers in each cell it is expected that they will amplify easily. The amplification of single copy genes might not be expected to be achieved so easily. This was investigated by amplifying exon 1 of the *Ki-ras* oncogene.

Methods

One patient undergoing an ultimately normal diagnostic colonoscopy for altered bowel habit was able to provide in excess of 500 ml of whole gut lavage fluid. This was divided into 25 ml aliquots and one aliquot was used for each of three previously published methods of DNA extraction (Tobi 1994; Smith-Ravin 1995; Eguchi 1996). Various elements of each of the individual methods were also combined and evaluated using the same whole gut lavage fluid sample as detailed above. Final end points measured were the total DNA yield as measured by spectroscopy (section 2.3). PCR amplification of cytochromes b and oxidase was undertaken using 50 ng of whole gut lavage fluid DNA (for reaction conditions see appendix 1). PCR products were resolved on agarose gels, stained with ethidium bromide and the intensity of the product bands compared subjectively by eye. The DNA extraction method that ultimately gave the most intensely stained PCR product was adopted as the preferred method of DNA extraction. This proved to be a method based on the extraction method published by Smith-Ravin *et al.* (Smith-Ravin 1995) and modified as described in section 2.3.

There was some concern about the accuracy of the DNA concentration estimation by spectroscopy. The DNA concentrations were therefore re-measured at a later date by UV fluorescence estimation. Even here technical problems were encountered. In samples where the DNA concentration was less than 10 ng/µl the image on the BioRad viewer was of insufficient quality to allow the software to calculate a DNA concentration. Visual estimation on a standard UV lightbox had to be undertaken for these samples. Some 10 samples had such a low intensity fluorescence image that even estimation by eye was impossible. These samples were given a concentration and yield value of zero.

Using the optimal method DNA was extracted from 100 ml of the 40 whole gut lavage fluid samples forming the study population. The DNA yield for each sample was initially estimated using spectroscopy measurements this was later repeated using UV fluorescence estimates. Each of the 40 samples of whole gut lavage fluid DNA was subjected to analysis of cytochromes b and oxidase using published methods (Smith-Ravin 1995). In addition the single copy gene *Ki-ras* was amplified for each sample. (Reaction conditions are detailed on appendix 1.)

Results

The first attempts to extract the DNA from whole gut lavage fluid resulted in brown pigmented solution even after passage through spin columns. Electrophoresis of a 5µl aliquot of this solution on to ethidium bromide stained gels did however produce a smeared band confirming the presence of DNA. Further DNA extractions on the control sample were evaluated to try and remove this pigmentation. Repeated phenol / chloroform extractions eventually resulted in unacceptable losses of DNA with each successive extraction producing only a minor reduction in the pigmentation. Subjective analysis of DNA smears on agarose gels suggested the optimal extraction method proved to be two phenol / chloroform extractions followed by passage through a commercial spin column. This still allowed amplification of the cytochrome b mitochondrial subunit (appendix 1) using 50 ng of the DNA obtained from the whole gut lavage fluid. This method was therefore employed to extract DNA from the whole gut lavage fluid samples used to make the study population.

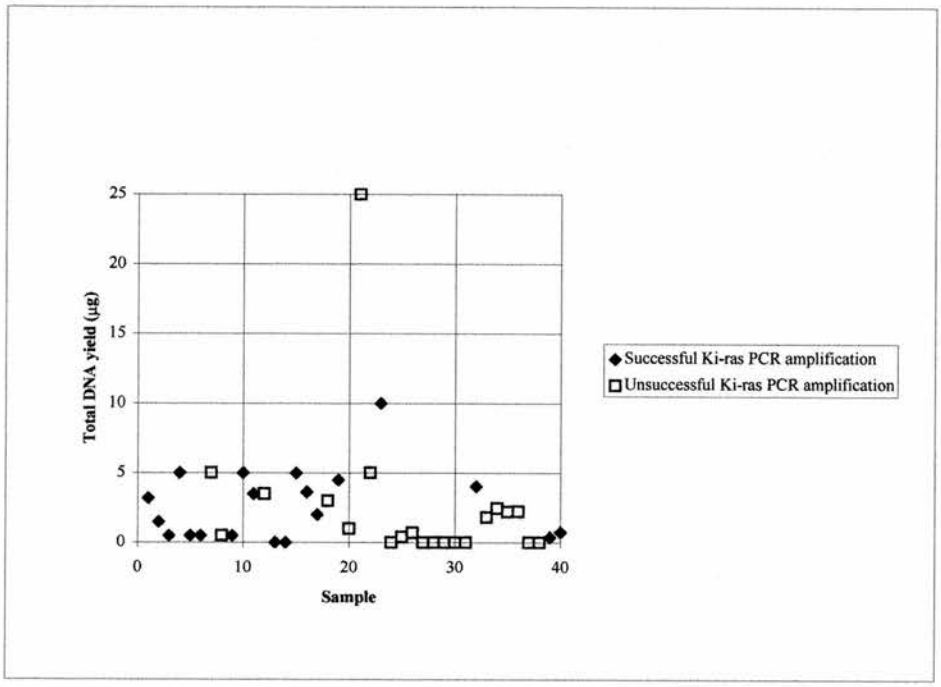
For the 40 samples which made up the study population the mean total amount of DNA per 100 ml sample (measured by spectroscopy) was 2.35 µg (SD 3.75 µg) however the median value was 1 µg (interquartile range 0.005 to 3.15µg). However the majority of the samples lie between 0 and 7 ng/µl with 2 samples producing high yields

skewing the mean figure. When the samples were re-examined using UV fluorescence measurements the mean total amount of DNA was 2.58 μg (SD 4.2 μg) with a median value of 1.25 μg (interquartile range 0.28 to 3.53 μg). (Figure 4.1)

Cytochrome b and oxidase analysis confirmed the presence of non-bacterial DNA in 37/40 (93%) of whole gut lavage fluid samples. Despite the pigmentation of some samples Cytochrome b was amplified in 37/40 samples however cytochrome oxidase with a larger amplification product could only be amplified in 16/40 (40%) of whole gut lavage fluid DNA samples. There were no cases where cytochrome oxidase could be amplified and cytochrome b could not. (Appendix 2)

The analysis of the single copy gene *Ki-ras* was less successful with only 19/37 (51%) of samples from colorectal cancer patients amplifying successfully. The mean total DNA (using spectroscopy measurements) for these 19 samples was 3.42 mg (SD 4.92 mg). There was no difference in the total DNA of this group when compared to those in which cytochrome b was amplified (SE diff. = 1.626, p=0.12). The mean total DNA for these same samples when measured using UV fluorescence was 2.66 μg (SD 2.52 μg). The scatter diagram of those samples which did successfully PCR amplify *Ki-ras* and those that did not would suggest a significant difference in the total DNA yields. However the difference in the median values for DNA yield are not statistically different. The median DNA yield for samples which amplified Ki-ras was 2.00 μg (interquartile range 0.50 - 4.26) compared with a median value of 0.71 μg (interquartile range 0.00 - 2.46) Mann-Whitney U statistic = 146 (p = 0.146).

Figure 4.1 Scatter diagram comparing the DNA yield (from 100 ml of whole gut lavage fluid) of samples that successfully amplified *Ki-ras* to those that did not.



	n	µg	sd
Mean DNA yield for samples that successfully amplified Ki-ras	19	2.66	2.52
Mean DNA yield for samples that unsuccessfully amplified Ki-ras	21	2.52	5.28

Standard error of difference of means 0.82 (Not significant p >0.5)

	µg	interquartile range
Median DNA yield for samples that successfully amplified Ki-ras	2.00	0.50 - 4.26
Median DNA yield for samples that unsuccessfully amplified Ki-ras	0.71	0.00 - 2.46

Mann - Whitney U test Statistic = 146, p = 0.1473

Discussion

DNA extraction from whole gut lavage fluid proved troublesome. Few of the papers looking at mutational analysis in stool samples discuss the amount or quality of DNA obtained from stool. Smith-Ravin and colleagues showed that DNA samples extracted by simple phenol / chloroform extraction failed to amplify by PCR even when the samples were spiked with high quality DNA concluding that some PCR inhibitors persist even after phenol / chloroform extraction. They also demonstrated that these inhibitors could be removed to some extent by using spin columns designed for purification of plasmid DNA (Smith-Ravin 1995). Using spin column preparation the total DNA yield varied between 0 and 7 μg (with two exceptions). Villa *et al.* obtained a mean of 7.3 μg of DNA from 100 mg stool samples from patients with colorectal cancer (Villa 1996). (No other study documents the DNA yield.) This yield is double the mean of this study and some seven times greater than the median value. This difference may represent differences in sample type or more likely differences in DNA extraction. Villa employing a glass milk extraction stage rather than spin column preparation. Glass milk purification has been shown to be less effective than spin column purification (Smith-Ravin 1995).

The persisting pigmentation in some samples would suggest persisting contamination that hampers DNA concentration estimation and possibly PCR amplification. The problems in estimating the DNA concentration were overcome using UV fluorescence measurements but in several cases the ultimate DNA yield was so low that it was not possible to accurately estimate the DNA concentration even using UV fluorescence techniques. It is also possible that the pigmentation lead to some quenching of the UV fluorescence leading to an under-estimation of the DNA yield. Previous work in the colorectal cancer laboratories in Edinburgh has shown that DNA extraction from lung

cancers can result in a persistent black colouration following phenol / chloroform extraction yet these samples still amplified by PCR (L. Curtis personal communication).

PCR amplification of cytochrome b was possible in 93% of the study population. This PCR product is 300 bp in length. Amplification of the cytochrome oxidase subunit with a product size of 1.4 kb was less successful. Microscopy of whole gut lavage fluid samples demonstrated rather degenerate cells many of which are presumably undergoing apoptosis which is known to degrade DNA into fragments of ~ 200 bp (Wyllie 1980). This along with any DNase secreted by bacteria and digestive juices would lead to degraded DNA when extracted as illustrated by smears on electrophoresis. Any free DNA in the whole gut lavage fluid will be in a similar state of degradation. DNA smears do show some high molecular weight DNA this most certainly represents bacterial DNA. In contrast it has been noted that low molecular weight DNA fragments can persist in the aqueous phase of DNA solutions even after precipitation and pelleting the DNA in a centrifuge at high *g* (Wyllie 1980). Hence it is possible that some of the tumour DNA useful for analysis may be lost in the discarded supernatant.

Amplification of the *Ki-ras* gene fragment (143 bp) was successful in only 51% cases available for analysis. There is a tendency although not statistically significant for whole gut lavage fluid samples that do amplify *Ki-ras* to yield more DNA. This has also been noted in stool samples (Cruickshank 1996). The difficulty in amplifying single copy genes probably reflects the culmination of all the above problems namely reduced DNA yield due to repeated purification, persisting PCR contaminants, and degraded DNA.

In this section I have demonstrated that DNA can be obtained from whole gut lavage fluid but in a rather degraded and impure form. This would suggest that for successful mutational analysis by PCR techniques product sizes should be kept small and highly sensitive detection methods are required.

5 Development of Transforming Growth Factor Beta Receptor II assay

The extensive interrogation of many microsatellites to determine RER status is labour intensive and not ideally suited to the screening situation. The close correlation between *TGFβ RII* mutations and the RER+ phenotype (Parsons 1995b) would suggest RER status can be evaluated using a single test. Other work would suggest similar information can be obtained by interrogating the BAT 26A locus (Hoang 1997). In order to assess whether such a high correlation was applicable to the Scottish population and to develop a robust assay that could be applied to whole gut lavage fluid DNA a separate series of 18 archived cancers with previously determined RER status was initially assessed for *TGFβ RII* mutations.

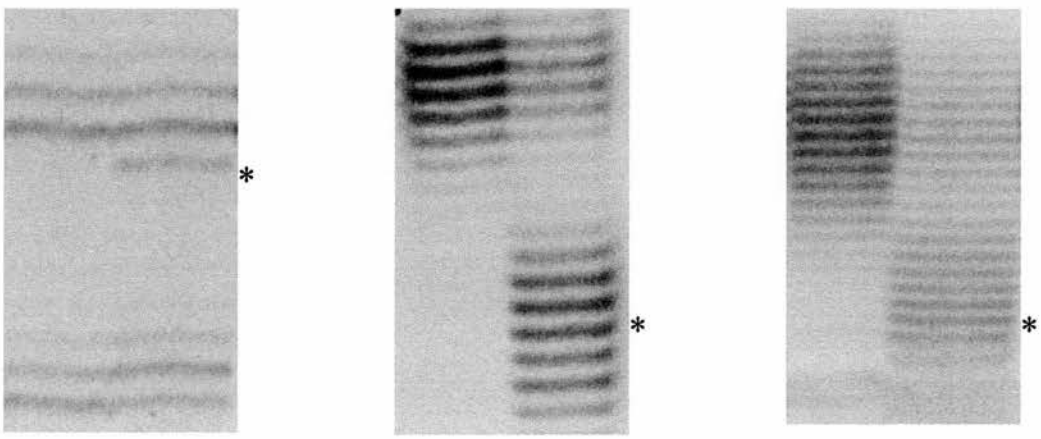
The RER phenotype for each control patient was determined by examining microsatellites as published (Parsons 1995b; Liu 1996b). DNA from paraffin sections of the tumour and normal colonic mucosa were amplified by PCR and the resulting products end labeled with γ^{33}P ATP. The labeled products were analysed in normal/tumour pairs by electrophoresis through denaturing polyacrylamide gels (6% acrylamide, 7M urea run at 50^{o}C for one hour). Novel alleles were detected as shifts in the electrophoresis band pattern between normal and tumour. An RER+ phenotype was assigned if shifts were noted in at least two microsatellites. The microsatellites studied were BAT26A a 26 polyadenine repeat in an intron of the MSH2 gene on chromosome 2, BAT40A a 40 polyadenine repeat in the 3 Beta hydroxylase gene (Parsons 1995b), and the CA repeats D2S123, D5S346, and D13S60 (S. Farrington personal communication). as these were known to exhibit microsatellite instability in 98% of RER+ tumours (Liu 1996b, Parsons 1995b). The RER status was then compared to the presence or absence of *TGFβ RII* mutations (*vide infra*) in the control DNA. Finally

TGFβ RII mutations were sought in whole gut lavage fluid by the same method and compared to that detected in the control tumour DNA.

The genetic defect responsible for the increased cancer susceptibility for patients with HNPCC has been attributed to germ-line mutations in the family of genes encoding proteins responsible for mismatch repair of DNA (Kinzler 1996). To date mutations in four genes in this family have been identified (MSH2, MLH1, PMS1 and PMS2) (Cunningham 1996). Mutations in any one of these genes confer a genomic instability characterised by microsatellite instability or replication error (RER+). The genes themselves are not oncogenes or tumour suppresser genes. They are thought, through the inherent genomic instability, to predispose to mutations in other oncogenes or tumour suppresser genes (Kinzler 1996). The characteristic lesion in RER+ tumours are short insertions or deletions in repeated sequences e.g. Poly A or CA repeat sequences (Aaltonen 1994). The majority of such sequences are either intronic or intragenic and as such mutations in these regions are unlikely to produce neoplastic mutations.

Transforming growth factor beta 1 (TGFß) is an important inhibitory growth regulatory protein in embryogenesis and in epithelial cells throughout life. It has been shown that TGFß acts through three membrane receptors and that mutations in TGFß RII block cell signaling of TGFß and confer neoplastic properties. The RII gene contains a 10 bp poly A (A_{10}) sequence which may be susceptible to mutation in MMR deficient individuals. Indeed it has been shown that this gene contains a mutation in this region in 90% of RER+ tumours and cell lines (Parsons 1995b). Thus it would make a suitable target for genetic screening of HNPCC families.

Figure 5.1 Microsatellite instability analysis at loci D2S123, BAT26 and BAT 40



Three representative gel photos to show microsatellite instability

For each sample normal and tumour DNA was amplified and the PCR products were end labelled with γ^{33}P ATP. These radiolabelled products were resolved on polyacrylamide gels. The gels were dried and contact autoradiographs produced by overnight exposure in cassettes with intensifying screens.

Examples here show normal and tumour pairs for the CA repeat D2S123, and the polyA sites BAT 26 and BAT 40. In each case the tumour sample shows microsatellite instability indicated by presence of novel alleles (*).

Development of assay

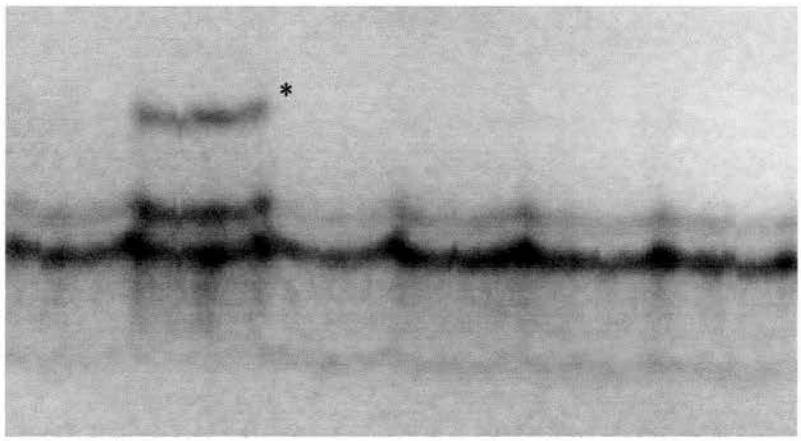
The work by Parsons *et al.* identifying *TGFβ RII* mutations in RER+ cancers and cell lines (Parsons 1995b) was performed on cDNA. Their analysis used a 73 bp PCR fragment that spanned the A_{10} section. The products were resolved on denaturing polyacrylamide gels allowing resolution of 1 - 2 bp shifts. Using their published primers and reaction conditions and despite rigorous optimisation I was unable to obtain sufficiently clean reaction products to perform a similar analysis on genomic DNA. At this time the genomic sequence for TGFß RII was not in the public domain. Attempts at redesigning the primers were unsuccessful because of an intervening intron whose location was at that time unknown. Publication of the genomic sequence of TGFß RII localized the A_{10} segment to exon 3 and the use of their published intronic primers enabled successful amplification of DNA from all sources. Mutational analysis was then performed by SSCP (Lu 1996).

To assess the assay firstly cell lines with known mutations in the TGFß RII gene were analysed. The cell lines HCT 116 and LoVo are known to harbour deletion / insertion mutations in the A_{10} repeat (Parsons 1995b). Radiolabelled SSCP on MDE gels (as outlined in the materials and methods section) confirmed a suitable assay, clear mobility shifts were seen when compared to a normal control SW 480. Next archive DNA from 18 cancers known to exhibit MIN at one or more loci was analysed in a similar manner.

Results

These are summarised in Table 5.1. Of the 18 samples 12 exhibited an RER+ phenotype, 6 showed MIN at only one locus. Of the 12 RER+ cancers 7 (58%) had a mutation in *TGFβ RII* as did 1/6 (16.7%) cancers with MIN at one locus.

Figure 5.2 SSCP analysis of *TGFß RII* mutations in RER+ cancers with normal tissue controls.



Using PCR techniques, exon 3 of the *TGF*β *RII* gene was amplified for each sample in normal and tumour pairs. The PCR products were end labelled with γ^{33}P ATP and resolved on non denaturing polyacrylamide gels. The gels were dried and contact autoradiographs produced by overnight exposure in cassettes with intensifying screens.

The above image shows representative samples in normal tumour pairs. A *TGF*β *RII* mutation can be clearly seen in lane 2 (*).

Table 5.1 Transforming growth factor beta receptor II mutations in 18 cancers known to exhibit microsatellite instability

Sample	MIN (no. of Loci)	RER Phenotype	TGFBRII exon 3 SSCP
1	4	Pos	Shift
2	2	Pos	Shift
3	4	Pos	Shift
4	2	Pos	No shift
5	4	Pos	Shift
6	3	Pos	Shift
7	2	Pos	No Shift
8	2	Pos	No Shift
9	2	Pos	No Shift
10	4	Pos	No Shift
11	1	Neg	No Shift
12	1	Neg	No Shift
13	1	Neg	Shift
14	1	Neg	No shift
15	1	Neg	No shift
16	1	Neg	No shift
17	4	Pos	Shift
18	4	Pos	Shift

MIN Microsatellite instability.
(Loci examined BAT 26, BAT40, D2S123, D5S346, D13,S60)

RER Replication error

12 of 18 samples (67%) exhibited an RER+ phenotype

7 of 12 samples (58%) with an RER+ phenotype had a TGF Beta Receptor II mutation in exon 3 when analysed by SSCP

Discussion

This small series has shown *TGFß RII* mutations in 58% of RER+ cancers. This is less than the 90% described by Parsons *et al.* (Parsons 1995b). This may reflect the use of SSCP for mutation detection. SSCP is known to be less sensitive that other methods of mutation detection. One other series that employed SSCP identified *TGFß RII* mutations in 85% of RER+ or HNPCC cancers (Akiyama 1997). Whereas another study that used direct sequencing identified *TGFß RII* mutations in 58% of RER+ cancers (Togo 1996). The study by Parsons analysed many cell lines rather than just DNA from cancers and the high rate of *TGFß RII* mutations may reflect the fact that cell lines can undergo additional mutations in the process of immortalisation.

Parsons examined cDNA looking for resultant 1 or 2 bp shifts by electrophoresis on large polyacrylamide gels. However *Taq* polymerase slippage at the A_{10} site means that artificial mutations can easily be generated due to poor fidelity of the polymerase enzyme. The method used by Parsons to control for this still relied on the subjective interpretation of several bands for each sample which may account for the higher estimates of *TGFß RII* mutations (Parsons 1995b). The mutation pattern produced by SSCP is clearer and open to less subjective interpretation.

In a study of sporadic colorectal cancers Akiyama *et al.* demonstrated a RER + phenotype in 22 /69 (32%) carcinomas Yet only 7/69 (10%) showed a 1 or 2 bp deletion mutation at the A_{10} site (Akiyama 1996). The 18 cancers in my study were chosen on the basis of MIN and RER + phenotype but were chosen from a large archive of sporadic cancers. This study population probably comprises of a mixture of sporadic RER + cancers and true HNPCC cancers. This would account for the low rate of *TGFß RII* mutations when compared to Parsons series but high rate when compared to Akiyama.

It is interesting to note in the main study population of 40 patients, from whom whole gut lavage fluid was obtained, 2 of the cancers showed a RER+ phenotype and both of these cancers had a *TGFβRII* mutation (Appendix 2).

6 Mutational analysis of whole gut lavage fluid DNA

Point mutations in *Ki-ras* codons 12 and 13 occur in 40 - 50% of cases of colorectal cancer (Bos 1987); *p53* mutations occur in 50 -75% of colorectal cancers with half of the transition mutations occurring at codons 175, 248 and 273 (Nigro 1989; Hollstein 1991). Therefore, examination of a range of mutation sites associated with colorectal cancer will be required to achieve an acceptable level of sensitivity for genetically based diagnostic tests (Sidransky 1994). Some 80% of patients with colorectal cancer have *APC* mutations that occur throughout its coding region with no particular hot spot, but mutational clustering is noted in exon 15 (Miyoshi 1992). For the small subset of cancers with a replication error phenotype (RER+), 90% are reported to have a frameshift mutation in a 10 base pair polyadenine sequence in exon 3 of *TGFß RII* (Parsons 1995b; Lu 1996). Although a few cancers will contain mutations in more than one gene, the cumulative effect of systematic analysis at these sites in addition to the point mutations at *Ki -ras* codon 12 and *p53* codon 248 would be expected to identify mutations in virtually all colorectal cancer and hence might provide a comprehensive screening method, if applied to stool or colonic washings. No systematic study of this array of genes in stool or colonic washings has been reported although there have been opportunistic reports in which one or two sites were interrogated.

The studies examining *Ki-ras* mutation detection in stool (Sidransky 1992; Tobi 1994; Hasegawa 1995; Smith-Ravin 1995; Cruickshank 1996; Nollau 1996; Villa 1996) have employed a variety of detection methods with varying success. They all however make use of techniques which selectively enrich for a particular point mutation. A similar technique to examine *p53* codon 175 mutations has been used with limited success (Carpenter 1995).

For the analysis of point mutations I employed the technique of enriched PCR which has been shown to be one of the most sensitive techniques capable of detecting 1

mutant allele in 10^4 normal alleles (Kahn 1991). In addition this technique avoids the use of radioactive isotopes making it an attractive method for a potential screening test.

The nature of the mutations in *APC* and *TGFβ RII* means that there are no suitable point mutations to allow interrogation by an enriched PCR technique and SSCP was employed to examine these genes. SSCP has also been used to examine exons 5-9 of p53 in the stool of cancer patients (Eguchi 1996).

Methods

As a proof of principle I initially interrogated DNA samples from the study population for mutations at codon 12 of the *Ki-ras* gene, *p53* codon 248, exon 3 of *TGFβ RII* and in the MCR of *APC* (as described in the materials and methods section). Ki-ras mutations in whole gut lavage fluid DNA were also sought in 15 control patients. Examination of *Ki-ras* and *p53* was performed as described in the materials and methods section.

The majority of *APC* mutations produce premature stop codons and as such the protein truncation test (PTT) is an ideal technique for their detection. For a successful assay a protein product of at least 300 amino acids (i.e. a PCR template of at least 1 Kb) is required to produce a product containing sufficient radiolabelled methionine for detection by autoradiography. Previous analysis of the DNA from whole gut lavage fluid using mitochondrial fragments had shown that products of approximately 1 Kb cannot be readily amplified. It was therefore elected not to analyze for APC mutations in whole gut lavage fluid using the PTT. The MCR region of the *APC* gene was therefore divided into 4 overlapping sections (Cooper 1996). These sections were then amplified by PCR and the resulting products analysed for mutations by SSCP on MDE gels. A technique with which this lab is fully conversant.

Table 6.1 Mutations detected in the cancers and whole gut lavage fluid samples of the study population

			CYTOCHROME		Ki ras		p53		APC		RER		PATHOLOGY		
STUDY NO	SEX	AGE	b	OXIDASE	TUMOUR	LAVAGE	TUMOUR	LAVAGE	TUMOUR	LAVAGE	MIN	TGFβ RII	SITE	DUKES	STAGE
1	F	60	+	+	+	+			SECTION 2				RECTUM	A	pT2 G2 N0
2	M	85	+										DESCENDING	B	
3	M	81	+	+									SIGMOID	C	
4	M	66	+	+			+	+					RECTUM	A	pT2 G2 N0
5	M	73	+	+					SECTION 1		1		SIGMOID	A	pT1 G2
6	M	63	+										RECTUM	C	
7	M	86	+	+					SECTION 3				SIGMOID	B	
8	F	83	+		+	NO PCR			SECTION 1				RIGHT	C	pT3b N0
9	M	62	+	+					SECTION 3				RECTUM	B	pT3b N0
10	M	71	+	+	-	+							TRANSVERSE	C	pT3c G2 N1
11	M	79	+	+					SECTION 1		1		RECTUM	A	pT2 N0
12	M	47											DESCENDING	D	pT3c G3 N0
13	F	74	+		+	+			SECTION 2				RECTUM	B	
14	F	75	+	+	+	-			SECTION 4				SIGMOID	C	pT3b G2 N0
15	F	71	+	+									SIGMOID	C	pT3b G2 N0
16	F	62	+										DESCENDING	C	
17	F	66	+										RECTUM	A	pT1 G2
18	F	39	+	+									DESCENDING	B	
19	F	72	+	+					SECTION 1				RECTUM	B	pT4 N0
20	F	90	+						SECTION 1				RECTUM	C	pT3b G2 N0
21	M	61	+										SIGMOID	D	pT4 G3 N1 M1
22	M	57	+	+									RECTUM	C	pT3 G3 N0
23	F	55	+		+	NO PCR			SECTION 1		1		RECTUM	C	pT3C G3
24	M	73	+	+					SECTION 1				RECTUM	B	pT3a G2 N0
25	M	88	+										SPLENIC FLEXURE	B	pT3b N0
26	F	90	+						SECTION 1				RECTUM	C	
27	M	71	+						SECTION 4		4	+	RIGHT	B	pT3A N0
28	M	72	+				+	+					TRANSVERSE	C	pT3b N1
29	M	64	+										SIGMOID	A	pT2 G2 N0
30	M	78	+								1		RECTUM	B	
31	M	78	+										SPLENIC FLEXURE	B	pT4
32	F	81	+										DESCENDING	B	

			CYTOCHROME		Ki ras		p53		APC		RER		PATHOLOGY		
STUDY NO	SEX	AGE	b	OXIDASE	TUMOUR	LAVAGE	TUMOUR	LAVAGE	TUMOUR	LAVAGE	MIN	TGFβ RII	SITE	DUKES	STAGE
33	F	78	+	+	+	NO PCR							SIGMOID	C	pT3a G2 N0
34	F	67	+										RECTUM	C	pT1
35	M	59	+		+	NO PCR							RECTUM	A	pT2 G2 N0
36	M	69	+	+									SIGMOID	B	
37	M	68	+										SIGMOID	C	
38	M	53											RECTUM	C	pT3 G2 N1
39	M	80	+										DESCENDING	B	pT3 G2 N0
40	F	62							SECTION 3		3	+	CAECUM	C	pT3a N1 G3

For Cytomchrome Data

+	Successful PCR amplification
-	Unsuccessful PCR amplification

For *Ki-ras* and *p53*

+	Mutation detectected
-	Mutation not detected
No PCR	No PCR amplifcation possible of DNA from whole gut lavage fluid

For *APC*

Section x	Section of MCR in which APC was identified

For RER (Analysis of tumour DNA)

MIN	Number of unstable of loci
+	TGFβ RII mutation detected in tumour DNA

Results

These are summarised in Table 6.1. There were 26 mutations in 18/40 primary tumours (45%); 7 *Ki-ras* mutations, 2 *p53* mutations, 2 *TGFß RII* mutations and 15 *APC* mutations. In the DNA extracted from whole gut lavage fluid 2/7 *Ki-ras*; 2/2 *p53* mutations; 0/2 *TGFß RII* mutations and 0/15 *APC* mutations were detected.

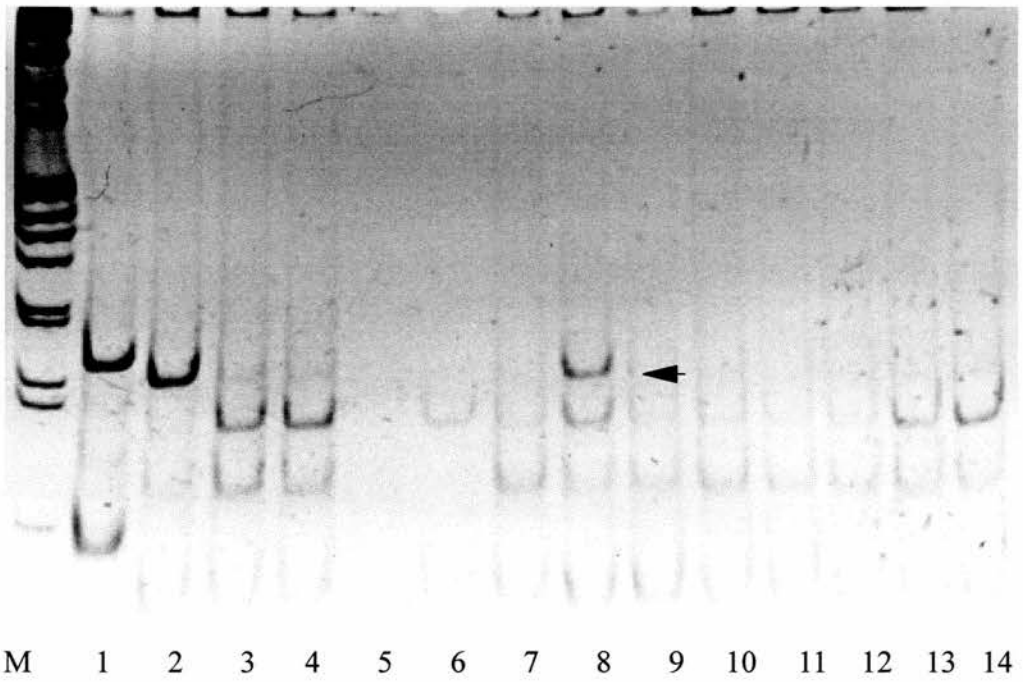
Direct sequencing of the PCR products from control DNA from the resected tumours detected 7 *Ki-ras* codon 12 mutations (Table 6.1). It was possible to amplify the *Ki-ras* gene from DNA extracted from the whole gut lavage fluid in 19/40 samples. Two of the known *Ki-ras* mutations were detected by enriched PCR of the DNA extracted from whole gut lavage fluid (figure 6.1). In 5 cases the known mutation was not detected. In one sample whole gut lavage fluid PCR amplification was achieved but a wild type electrophoresis pattern was produced and in the remaining 4 no amplification was achieved. In addition one whole gut lavage fluid sample showed a mutation which was not detected in the tumour DNA by direct sequencing despite repeated investigation. No mutations were detected in the 15 control whole gut lavage fluid samples. Of these 15 samples 9 amplified successfully and produced negative assays and 6 would not PCR amplify.

Restriction enzyme digestion of tumour DNA identified 2 *p53* codon 248 mutations and both of these were detected in the corresponding whole gut lavage fluid DNA (Figure 6.2).

The SSCP analysis of *APC* on control samples was shown to be sufficiently sensitive to identify 1 mutant allele in 50 wild type. Similar analysis of *TGFß RII* showed a sensitivity of 1 in 20.

There were 2 *TGFß RII* mutations detected by SSCP in the paired normal and tumour control samples. Neither of these mutations were detected by SSCP analysis of the corresponding whole gut lavage fluid DNA. In one case no PCR product was

Figure 6.1 Enriched PCR for *Ki-ras* codon 12 on DNA obtained from whole gut lavage fluid from patients with known colorectal cancer



Enriched PCR products for Ki-ras codon 12. PCR products were resolved on the 12% polyacrylamide gel then stained with ethidium bromide and viewed under ultraviolet light. The image has been captured on the BioRad gel documentation system and inverted to produce a negative image. No other computer enhancement has been used.

M: one kilobase marker

1: uncut control PCR product

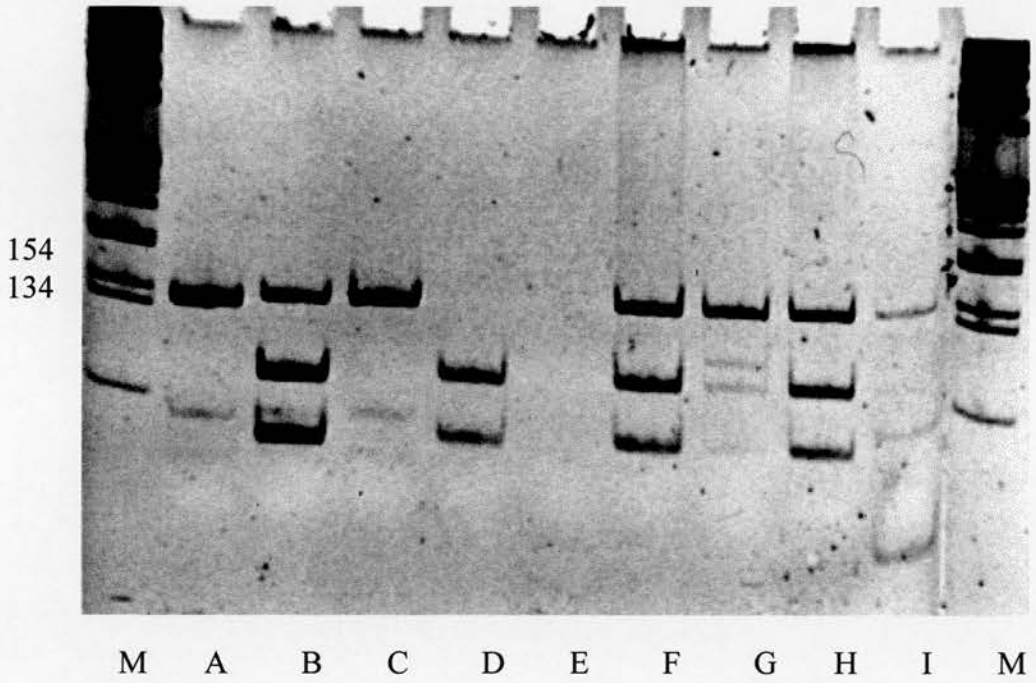
2: positive control cut with *Bst N1*. In the second PCR reaction the 3' primer contains a modified *Bst N1* restriction site and hence mutant PCR products were cleaved into two fragments 143 and 14 bp in size.

3 - 13: Enriched PCR of WGLF DNA.

14: negative control cut with *Bst N1*.

A Ki-ras codon 12 mutation can clearly be seen in lane 8 (arrowed).

Figure 6.2 Polyacrylamide gel electrophoresis of enriched PCR for the detection of *p53* codon 248 mutations



Lanes: M: Marker DNA (1 Kilobase ladder; Life Technologies Inc. [Gibco BRL])
A: uncut PCR product
B: *Msp I* digested positive control
C: mutant-enriched positive control
D: *Msp I* digested negative control
E: mutant-enriched negative control
F and H: *Msp I* digested tumour DNA from paraffin sections
G and I: mutant-enriched PCR of corresponding WGLF showing mutations.

Products of amplification and digestion have been resolved on 15% polyacrylamide gel. Mutant alleles remain undigested as a single 143 bp product. Wild type alleles are cleaved into 58- and 85 bp products. Enriched-PCR control reactions show how mutant alleles can be selectively amplified. This example shows the two p53 mutations successfully detected in WGLF DNA.

amplified for further analysis and in the second despite PCR amplification only the wild type signal was detected on SSCP.

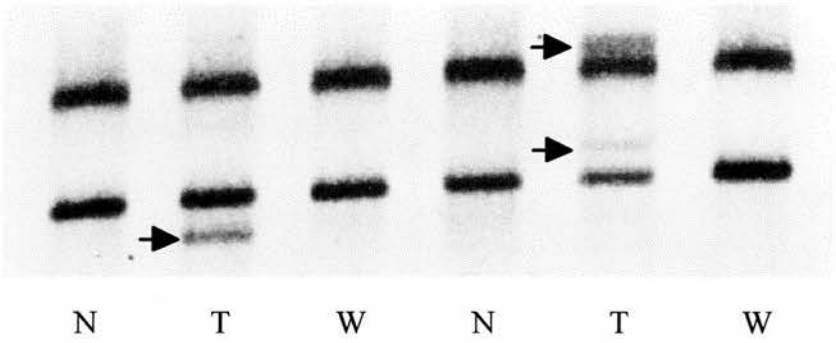
For *APC* 15 mutations in the MCR were detected in the normal / tumour pairs however none of these could be detected by SSCP analysis of the whole gut lavage fluid DNA. In 10 cases no PCR amplification occurred in 5 no SSCP was identified despite PCR amplification (figure 6.3).

6.4 Discussion

By the analysis of the four mutation sites described, 18 (45%) patients had the potential for a genetic diagnosis of their malignancy. This is lower than expected even allowing for the inefficiencies of SSCP and the fact that only *Ki-ras* codon 12, *p53* codon 248 and the MCR of *APC* were analysed. This low number of mutations can be explained by the following reasons. The study population is small and as such there may be a degree of sample bias reducing the overall number of mutations. Regional variation may also produce differences in the number of mutations when our Scottish population is compared to other published series. Projection from one population base to another may not be entirely accurate. I have already shown the frequency of *TGFß RII* mutations in the Scottish population is lower than that published for other populations.

When extracting DNA from paraffin sections the cancer was not microdissected and so the samples will inevitably contain some normal DNA. The sensitivity of mutation detection is reduced by not microdissecting and thus some mutations will fail to be detected. It has been shown that the more sensitive the technique employed the more mutations are detected (Carpenter 1996). SSCP is regarded as less sensitive than other established mutation detection techniques and the use of SSCP may have lead to an underestimation of the number of *APC* MCR mutations. The overall low number of

Figure 6.3 An autoradiograph of the SSCP analysis of *APC* in two whole gut lavage fluid samples.



Autoradiograph of γ^{33}P ATP labelled PCR products resolved on 50% MDE gel by SSCP.

N: normal DNA from paraffin section

T: tumour DNA from paraffin section

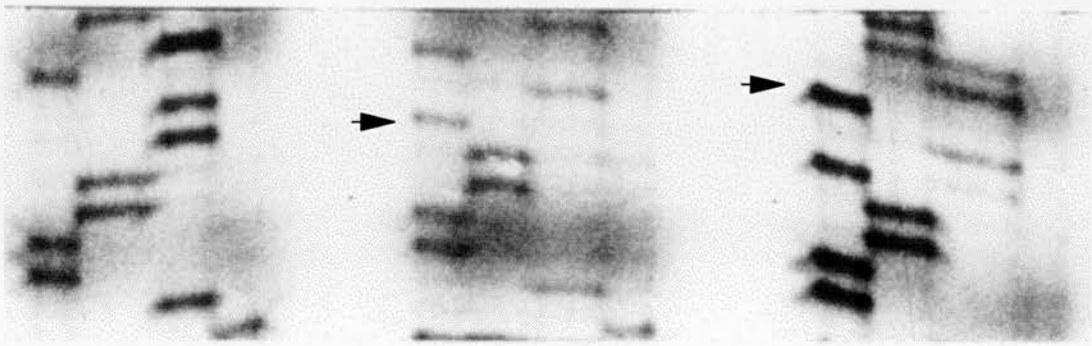
W: WGLF DNA from corresponding cancer patient

Extra bands representing APC MCR mutations (arrowed) can be seen in the tumour DNA in each of these two examples. The corresponding WGLF DNA samples only show the normal pattern.

mutations that were detected in cancer DNA from paraffin sections does, however, highlight potential limitations imposed on any ultimate screening strategy. By confining our interrogation to known mutations for each site examined a proportion of mutations will not be identified thus limiting the overall effectiveness of the screening strategy. Notwithstanding, these limitations, as a proof of principle, further examination of whole gut lavage fluid DNA is still valid. Interrogation of cancer DNA from paraffin sections determines the denominator by which whole gut lavage fluid DNA analysis can then be assessed.

Only 4 mutations were detected in the whole gut lavage fluid DNA, and so a genetic diagnosis was only possible in 10% of the study population by whole gut lavage fluid examination. For *Ki-ras* and *p53*, failure to detect mutations was largely attributable to the failure in DNA amplification from the whole gut lavage fluid sample. Previous studies examining *Ki-ras* and *p53* in stool have all experienced PCR inhibition reducing the number of samples available for genetic examination (Sidransky 1992; Tobi 1994; Hasegawa 1995; Smith-Ravin 1995; Cruickshank 1996; Nollau 1996; Villa 1996) . In this study, although high copy number DNA suitable for amplification was identified in 93% of the whole gut lavage fluid samples, single copy genes could be amplified by PCR in only half of these samples. There was no significant difference in the amount of DNA in those samples that amplified and those samples that would not. This suggests that PCR inhibition is not a significant problem with whole gut lavage fluid samples when amplifying genes of high-copy number, but may be problematic when studying single-copy genes. In addition, although human DNA is estimated to account for 10% of the total DNA extracted from stool (Hasegawa 1995) many samples in this study were lost to further examination presumably because there was insufficient human DNA available for satisfactory amplification of single copy genes. In those that did amplify, identical mutations were found in both tumour and whole gut lavage fluid samples when compared by direct sequencing (figure 6.4).

Figure 6. 4 Sequence reaction for whole gut lavage fluid sample in which *p53* mutation was detected by enriched PCR



N: normal control DNA from paraffin section showing normal sequence.

W: WGLF DNA. Enriched PCR of WGLF DNA showing mutant sequence. The DNA used for this sequencing reaction was obtained from an enriched PCR amplification. The mutant band was cut from a polyacrylamide gel and the DNA extracted by boiling it in deionised water. The extracted DNA was then amplified using a nested 3' primer and this product was then sequenced.

T: tumour control DNA from paraffin section of tumour resected from the same patient from which sample W was obtained.

Sample T shows a G to A substitution mutation at the third base pair of codon 248 (arrowed). Some residual normal sequence remains because of normal stromal tissue contaminating the tumour DNA.
Sample W shows the identical mutation in DNA obtained from WGLF taken preoperatively from the same patient. This mutation was successfully isolated by enriched PCR techniques.

PCR amplification of whole gut lavage fluid DNA for *APC* was less successful than for *Ki-ras* possibly because the product size was larger (approx. 220 bp compared to 143 bp). For *APC* and *TGFß RII* it was not possible to identify any mutations even when PCR amplification was successful (fig 6.3). Mutations in the whole gut lavage fluid samples were only detected after selective enrichment permitting detection of 1 mutant in 10^4 wild type alleles (Kahn 1991). All the previous studies examining Ki-ras mutations in stool have employed an enrichment strategy (Sidransky 1992; Tobi 1994; Hasegawa 1995; Smith-Ravin 1995; Cruickshank 1996; Nollau 1996; Villa 1996) . One study had limited success employing SSCP to identify *p53* mutations in the stool of known colorectal cancer patients. However these were all advanced cancers and FOB testing on the same patients was four times more accurate (Eguchi 1996). I found SSCP to be insensitive when examining whole gut lavage fluid samples. On control DNA I was able to detect only 1 mutant in 50 wild type alleles for *APC* and 1 in 20 for *TGFß RII*. The sensitivity of the *TGFß RII* assay is compromised by *Taq* slippage which produces artificial deletions in the polyadenine repeat sequence in wild type cell lines reducing the fidelity of the PCR amplification (Parsons 1995b).

Since the experimental work for this thesis was undertaken researchers have demonstrated that *APC* mutations have been detected in stool samples of colorectal cancer patients (Deuter 1998). Once again an enrichment technique was required. In a sample of 23 patients, 21 *Ki-ras*, *p53* and *APC* mutations were detected in 13 different cancers. Thus 56 % of patients in this study had the potential for the genetic diagnosis of their colorectal cancer. Some 11 tumours showed *Ki-ras* and *p53* mutations by direct sequencing. Direct sequencing of the stool samples detected three point mutations in two stool samples (compare to work presented in this thesis). Examination of the MCR of *APC* in tumour DNA detected only five mutations in 23 cancers using a heteroduplex technique (i.e. 22 % which is much less than might expected). Initial heteroduplex analysis of the corresponding stool DNA failed to detect mutations due to

a high a background contamination of wild type DNA. However when the region of the gel that contained the putative heteroduplex DNA was excised and this DNA subjected to further PCR amplification, subsequent heteroduplex analysis successfully demonstrated the expected mutation in all five cases. This technique relied on prior knowledge of the positions on mutant heteroduplex DNA by analysis of the resected tumour DNA, and hence is not practicable for screening purposes. The authors suggest this problem could be overcome by exercising larger portions of the gel to encompass all possible mutation harbouring DNA fragments. DNA extracted from this excised segment of gel could then be subjected to a second mutant enriching PCR amplification and further heteroduplex analysis.

The identification of a *Ki-ras* mutation in one whole gut lavage fluid sample but not the tumour may represent detection of neoplastic cells not represented in the paraffin section. Repeated analysis of DNA from the paraffin section failed to show any *Ki-ras* mutation. Multiple *Ki-ras* mutations are found in tissues of nearly half of patients with colorectal cancer containing *Ki-ras* mutations indicating that distinct evolutionary subclones may be involved in the development of tumour in some patients (Zhu 1997). Other studies have shown the heterogeneous nature of *Ki-ras* mutations in adenomas sampling an adenoma at only one site revealed a mutation in 38% of cases but this increases to 57% if two site from the same adenoma were examined (Saraga 1997). In 15% of cases the two sites examined contained different *Ki-ras* mutations. It is presumed that the paraffin section in my study did not contain the subclone of cells with the *Ki-ras* mutation detected in the whole gut lavage fluid DNA. This demonstrates the potential of such a sampling technique to contain DNA that represents the entire colonic mucosa rather than an small macroscopically abnormal section of colonic mucosa. Subsequent colonoscopy in this individual did not identify any synchronous lesions. A *Ki-ras* mutation in colonic effluent from a macroscopically normal colon has been noted previously (Tobi 1994) and was felt to highlight the potential of

premalignant detection by genetic tests. With the advent of more sensitive techniques for mutation detection it is now clear that *Ki-ras* mutations occur at high frequency in the microscopically normal tissue adjacent to carcinomas and adenomas containing *Ki-ras* mutations suggesting these mutations might be useful markers for early detection of colorectal cancer (Zhu 1997).

These findings show that whole gut lavage fluid is suitable for PCR analysis but its usefulness as a screening resource is still limited because in many samples examination of single copy genes is not possible. The finding that enriched techniques are required highlights the fact that wild type DNA is vastly in excess of any mutant DNA. This reduces the overall sensitivity of any method that is employed for mutation detection and will undoubtedly mean mutations that are present will be underestimated by being lost in a background "noise" of wild type DNA. For successful identification of mutations commonly associated with colorectal cancer in the whole gut lavage (or stool) from colorectal cancer patients, a method that selectively enriches for the mutation must be employed. At present, this limits detection to point mutations at fixed known sites, for example *Ki-ras* codons 12 and 13 and *p53* codons 175 and 248. By using increasingly sensitive techniques such as radiolabelled hybridisation or fluorescence PCR (Carpenter 1996) and improving sample collection to reduce PCR inhibition, the detection of such point mutations could approach 100%. However, successful analysis at these four sites would still only have the potential to detect approximately 60% of colorectal cancers (Bos 1987; Nigro 1989; Hollstein 1991). This detection rate is no better than present FOB testing and, as such, severely limits the potential for early colorectal cancer diagnosis by genetic tests. Given current technology and knowledge of the mutations associated with colorectal cancer, it seems unlikely that genetic techniques offer any advantage over existing screening methods.

For such tests to be usefully employed as screening tests, either a new point mutation that occurs at high frequency in colorectal cancer must be identified, or other

methods that enrich for unknown mutations must be developed. Possible alternative strategies include the detection of cancer specific mutations in the plasma or serum of patients with colorectal cancer. *Ki-ras* mutations have been detected in DNA extracted from the plasma of patients with colorectal cancer, in a small study of 14 patients with colorectal cancer. Anker and colleagues demonstrated that using mutant allele specific primers 7 cancers had *Ki-ras* codon 12 mutations and the same mutation could be detected in 6 (86%) of the plasma samples. However these included only one Dukes' stage A cancer and 5 Dukes' stages C and D suggesting mutant DNA in the plasma is a late phenomenon limiting its diagnostic and screening use (Anker 1997). This is also borne out by two other studies in which mutant DNA was detected in the serum of patients with head and neck cancer and the plasma of patients with lung cancer but only in patients with advanced stage disease (Chen 1996; Nawroz 1996). Splicing variants of the mRNA for the transmembrane glycoprotein CD44 have also been detected in stool sample of patients with cancer. Variant 6 and variant 10 mRNA was detected in 17 of 25 (68%) of stool samples obtained preoperatively from colorectal cancer patients. Successful detection was not dependent on stage of the cancer but was influenced by position of the lesion. Rectal and sigmoid lesions were more easily detected than right sided lesions. CD44 variant expression occurs in a variety of tissues and is also seen in ulcerative colitis and Crohns disease. The source of the variant mRNA in this study was unknown (as colonic cancer cells were not cytologically identified) and may have originated other sources such as from activated lymphocytes (Yamao 1998). Another possibly more promising area of progress lies in the determination that almost 95% of colorectal cancers exhibit telomerase activity (Odogwu 1997). This allows potential screening tests to focus on a single molecular abnormality for examination with the potential for increases in sensitivity and specificity.

7. Telomerase

As discussed in the introduction the detection of telomerase activity may represent a single molecular test which could be used for the early detection of colorectal cancer. The telomeric repeat amplification protocol (TRAP) (Kim 1994) can detect telomerase activity in as few as 10 - 100 cells. (An oligonucleotide primer acts as a substrate for telomerase extension, the products then serve as templates for PCR amplification.) Combined results on the detection of telomerase activity indicates a specificity of 91%, sensitivity of 85%, a positive predictive value of 93% and a negative predictive value of 81% (Kim 1997). Telomerase activity has been detected in 55% of voided urine samples from patients with bladder cancer and 84% of bladder washouts from the same patients (Kinoshita 1997) and also in 60% of lumenal washings from resected colorectal cancers (Yoshida 1997) although these were obtained artificially by perfusing the resected specimen with saline until it ran clear before obtaining the sample for analysis. This finding does however highlight a potential use for whole gut lavage fluid in detecting telomerase activity. There are no studies detecting telomerase activity in stool.

Six patients from the original study group had whole gut lavage fluid stored -70^{o}C. These samples were used to see if telomerase activity could be detected in whole gut lavage fluid obtained from patients with colorectal cancer.

Methods and Results

All experiments utilised the PCR-ELISA TRAP assay (Beohringer-Manheim), (see materials and methods). All assays contained a manufacturer provided positive control the kidney cell line (kidney 293). A negative control was produced by heat inactivating

the positive control. Using frozen normal and tumour tissue from archived colorectal cancer cases protein extraction was performed as described in the manufacturers protocol. These protein extracts were then subjected to the PCR-ELISA TRAP assay. Several experiments were required to optimize the reaction conditions, particularly the PCR portion of the assay. Optimum conditions are detailed in the materials and methods. These reaction conditions were then used for all subsequent reactions. Each reaction was performed in duplicate as were absorption readings. Mean absorption readings with standard errors have been plotted in the histograms.

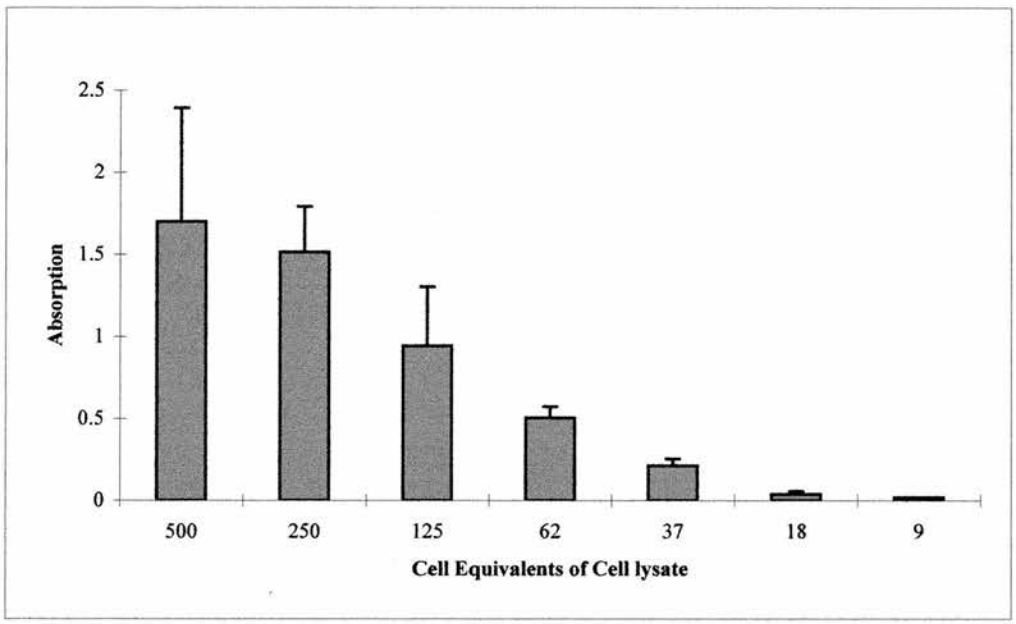
Next, the limit of sensitivity of the assay was assessed. Telomerase extracted from the cancer cell line HT 29 which exhibits telomerase activity was compared to the kit control assay. Doubling dilutions were performed from an initial protein concentration equivalent to 500 cells. Two sets of doubling dilutions were performed firstly with DDW and secondly with PEG at a concentration (59 g/L). A doubling dilution effect was noted with DDW dilution. It was possible to detect telomerase activity at protein concentrations equivalent to 62 cells (fig 7.1). However, all the PEG diluted reactions were negative.

To evaluate the effect of PEG on the PCR a Ki-ras PCR as previously described was performed on control DNA. To a series of reactions a doubling dilution of PEG was added starting from a concentration 59 g/L. The PCR products were resolved on a 2% agarose gel. The results showed that PEG inhibited any PCR amplification down to a 16 fold dilution (3.69 g/L) (figure 7.2). A second PCR assay was then performed focusing on PEG concentrations around 3.69 g/L). PCR amplification was achieved with a PEG concentration of 4.72 g/L or less which represents a 12.5 fold dilution of whole gut lavage fluid. Subsequent TRAP assays containing HT 29 cells and PEG concentrations of 4.7 g/L produced satisfactory ladder patterns on silver stained MDE gels when the PCR products were resolved and positive ELISA results again down to protein extract equivalent to 62 cells.

Next the whole gut lavage fluid from six patients with known colorectal cancer was analysed. In each case unfiltered processed samples had been snap frozen and stored at -70^{o}C and were analysed following initial protein concentration determination. With telomerase being an intracellular protein, unfiltered whole gut lavage fluid samples were therefore required for analysis. It was elected to perform the initial analysis on unfiltered, unprocessed whole gut lavage samples. TRAP assays were performed on aliquots containing 50 mg and 25 mg total protein from each lavage sample. Each sample was diluted to adjust the final PEG concentration in the PCR to 4.7 g/L and then subjected to TRAP ELISA analysis. No telomerase activity was detected in any of the samples (figure 7.3).

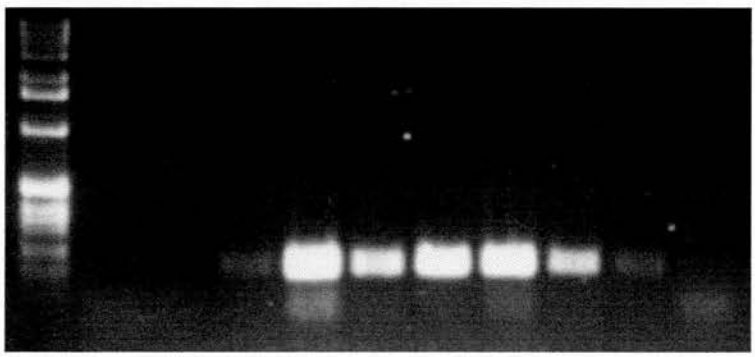
Finally one of the whole gut lavage fluid samples was spiked with HT 29 protein extract in doubling dilutions from a protein extract with an initial concentration equivalent to 1000 cells. PCR-ELISA analysis of this series of samples was also negative in all cases (figure 7.3).

Figure 7.1 Telomerase PCR ELISA absorption readings for doubling dilutions of positive control cell line kidney 293



Absorption: A450nm - A630nm
Error bars represent standard errors
A positive assay was assumed if an absorption reading of
> 0.5 A450 - A630 was obtained
Hence it can be seen that this assay can reliably detect telomerase activity in the protein cell lysate equivalent to 62 cells.

Figure 7.2 PEG inhibition of a *Ki-ras* PCR on an unrelated positive control DNA



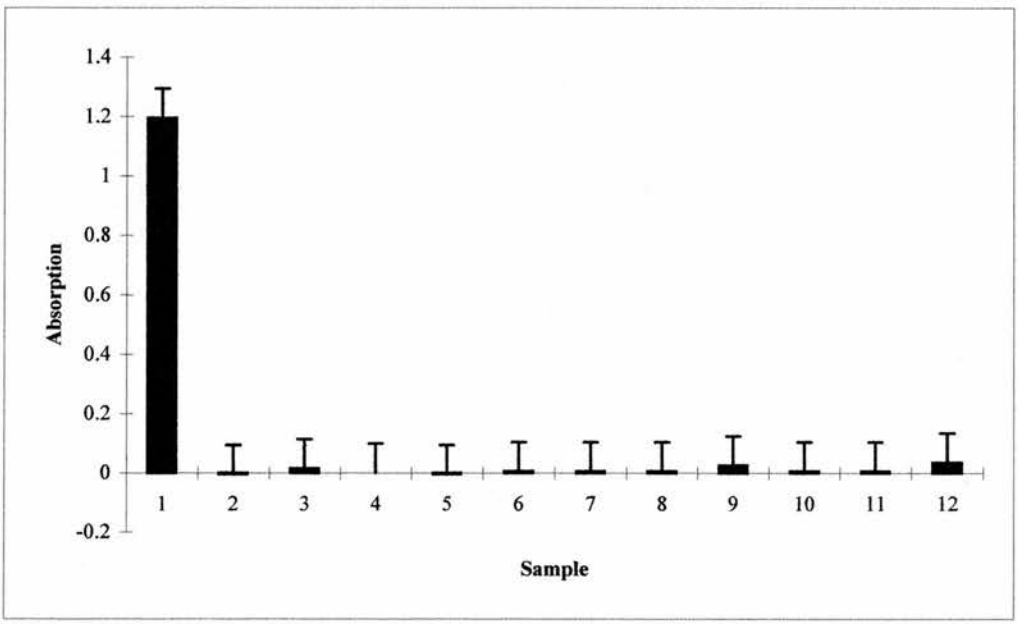
M 1 2 3 4 5 6 7 8 9 10

Ki-ras PCR amplification with doubling dilutions of PEG (initial concentration 59 g/l) in reaction mixture. Products have been resolved on a 2% agarose gel, stained with ethidium bromide and viewed under ultraviolet light.

M: 1 Kilobase marker
1: 50% PEG
2: 25% PEG
3: 12.5% PEG
4: 6.25% PEG
5: 3.75% PEG
6: 1.87% PEG
7: 0.9% PEG
8: 0.45% PEG
9: No PEG
10: Blank

It can be seen that concentrations of PEG >12.5% completely inhibit PCR amplification. A further experiment showed that PEG concentration of 10% or less allowed successful PCR amplification.

Figure 7.3 Telomerase PCR ELISA absorption readings for whole gut lavage fluid from a cancer patient and whole gut lavage fluid with 1000 cell equivalents of positive cell line HT29 added.



Error bars represent standard errors
Absorption: A450nm - A630nm
Sample

1	Positive Control
2	50mg WGLF Protein
3	25mg WGLF Protein
4	50mg WGLF+ 1000 Cell equivalents HT29
5	50mg WGLF + 500 Cell equivalents HT29
6	50mg WGLF + 250 Cell equivalents HT29
7	50mg WGLF + 125 Cell equivalents HT29
8	50mg WGLF + 62 Cell equivalents HT29
9	50mg WGLF + 37 Cell equivalents HT29
10	50mg WGLF + 18 Cell equivalents HT29
11	50mg WGLF + 9 Cell equivalents HT29
12	Neg Control

Discussion

In this small series no telomerase activity was detected in the whole gut lavage fluid samples. Unfortunately, no frozen tumour tissue was available for analysis from these samples to confirm the presence of telomerase activity in the original tumour. Given the fact that at least 90% of colorectal cancer exhibit telomerase activity (Odogwu 1997; Shay 1997) one could reasonably assume 5 of the 6 samples studied would exhibit telomerase activity.

The concentration of PEG in the PCR is important with concentrations above 4.7g/L significantly inhibiting the PCR. It is possible that despite dilution there was still an inhibitory concentration of PEG in our samples. PEG solutions have been shown to interfere with ELISA detection of tumour associated antigens in whole gut lavage fluid. This inhibitory effect is thought to be due to PEG interfering with antibody binding to the plastic ELISA plates. This inhibitory effect shows an inverse exponential correlation with increasing PEG concentrations and PEG concentrations below 12 g/L do not significantly affect results (Tobi 1991). The PEG concentration in my samples was diluted to below 4.7 g/L and so will have minimal influence on the results. In our laboratories when assaying for human immunogloblins in PEG derived whole gut lavage fluid, pre-coating the microtitre plates with antiserum to human immunoglobulin prior to adding the lavage avoids this PEG interference (O'Mahony 1990). Development of an antiserum to telomerase that could be used in a similar manner may improve the assay sensitivity and could prove to be useful avenue of further research.

The proportion of cancer cells compared to normal stromal cells within a cancer specimen affects the telomerase activity detected when compared to pure cancer cell lines as a control. This relationship is linear. Prostate cancers (that only contained 5% of cancer cells when examined histologically) demonstrated significantly less telomerase activity when compared to colorectal cancers and sarcomas which showed 30% and

65% tumour cell infiltration (Engelhardt 1997). The findings in section 3 have demonstrated that cancer cells are an infrequent finding in whole gut lavage fluid and it may be that telomerase activity in whole gut lavage fluid samples is beyond the limit of the TRAP assay sensitivity either because of the dilution required for optimal PEG concentration or because the proportion of cancer cells to normal cell is too low.

While dilutional factors may play a role even whole gut lavage fluid samples spiked with cells known to exhibit telomerase activity, at a concentration that should be detected, also produced a negative assay. This suggests some other confounding factor. Telomerase is known to be extremely sensitive to inactivation by heat and RNase (Morin 1989). It is possible that heat inactivation occurred before the samples were frozen at -70^{o}C. This is however require the samples to be heated to greater than 50^{o}C and is therefore unlikely. The whole gut lavage fluid samples used in this study were unfiltered and unprocessed and telomerase inactivation by bacterial or endogenous RNase could have occurred. Intestinal secretions contain proteases which can effect the analysis of immunoglobulins and the addition of protease inhibitors may reduce immunoglobulin loss from gut lavage samples (O'Mahony 1990). These proteases could also lead to telomerase degradation. This suggests that modification of the collection of whole gut lavage fluid to include an RNase inhibitor and possibly trypsin inhibitors, as used in processed whole gut lavage samples for immunoglobulin assays, might enhance telomerase activity detection in these samples.

One study has detected telomerase activity in lumenal washings from cancer specimens (Yoshida 1997). These samples were artificially obtained in the laboratory. My work described above has shown the fragile nature of telomerase and the fastidious assay required for its detection limits the detection of telomerase activity in the clinical samples presently available. Further developments as indicated above will be required before a reliable clinical test of telomerase activity is available.

8. Mutational analysis of synchronous colorectal cancers

It was hoped that the hypothesis of *APC* mosaicism could be explored by using whole gut lavage fluid to obtain cytological samples from the entire colonic epithelium in individuals known to be at high risk for colorectal cancer. Unfortunately, I have shown present methods of *APC* mutation detection are not sensitive enough to detect mutations in DNA obtained from whole gut lavage fluid.

A proportion of patients with colorectal cancer present with two primary colonic cancers (synchronous cancers). In these, individuals one could reasonably expect an increased cancer risk. Individuals with an increased risk of colorectal cancer may be mutational mosaics for *APC* which predisposes them to cancer (Dunlop 1996b). It has also been noted that the overall prognosis for patients with synchronous lesions is very poor when compared to sporadic and metachronous cases (Bekdash 1997). Also the frequency of *Ki-ras* mutations is lower in patients with synchronous cancers than in sporadic cancers (Hayakumo 1995). These findings suggest tumourigenesis for synchronous cancers may be different to sporadic lesions. Assuming *APC* acts as a gatekeeper for the development colorectal cancer (Kinzler 1996), patients with *APC* mosaicism may be more likely to acquire multiple cancers (either synchronous or metachronous). Colonic epithelial *APC* mosaicism may therefore play a role in the tumourigenesis of synchronous cancers. The broad spectrum of known *APC* mutations in colorectal cancer (summarised in Cunningham 1996) and the large size of the gene (Groden 1991) mean that identical *APC* mutations in synchronous colorectal cancers would be highly suggestive of *APC* mutational mosaicism rather than a chance finding. The demonstration of identical *APC* mutations in synchronous tumours would thus provide strong support for the mosaicism theory. To further pursue this hypothesis I have analysed *APC* mutations and RER status in 12 cases of synchronous colorectal cancer.

Methods

The pathology reports of all individuals undergoing colonic resection for cancer in one teaching hospital for the period 1989 to 1996 were scrutinized. Patients with a report indicating synchronous cancers were identified. A search of the histological archive, tissue and DNA stores was then undertaken to retrieve material suitable for DNA analysis.

DNA was prepared from either frozen tissue stored at -70°C or extracted from paraffin blocks. (Section 2.3).

APC mutations were detected using the protein truncation test (figure 8.1) for DNA extracted from frozen tissue (Prosser 1994) or single strand conformational polymorphism for DNA extracted from paraffin sections (Cooper 1996). Microsatellite instability analysis was undertaken by exploring the microsatellite sequences at D2S123, D5S346, D13S160, and BAT40 (Liu 1995; Dunlop 1996). These sites were chosen because the have been used in other published work to determine RER status and because they are sites with which our laboratory is particularly familiar. Evidence of chromosomal instability rather than microsatellite instability was sought by loss of heterozygosity (LOH) for *APC* and tumour DNA ploidy. For LOH of *APC* two known polymorphic sites were analysed by restriction fragment polymorphism; the 3′ untranslated region and exon 11 (Curtis 1994) (figure 8.2). An assessment of tumour ploidy was performed by Mr. Robert Morris. This was performed on either fresh tissue stored at -70°C or tissue taken from 10 μm paraffin sections (Vindelov 1983). The nuclei were stained with propidium iodide and analysed by flow cytometry (EPICS, Coulter Ltd. Beaconsfield, Bucks UK).

Results

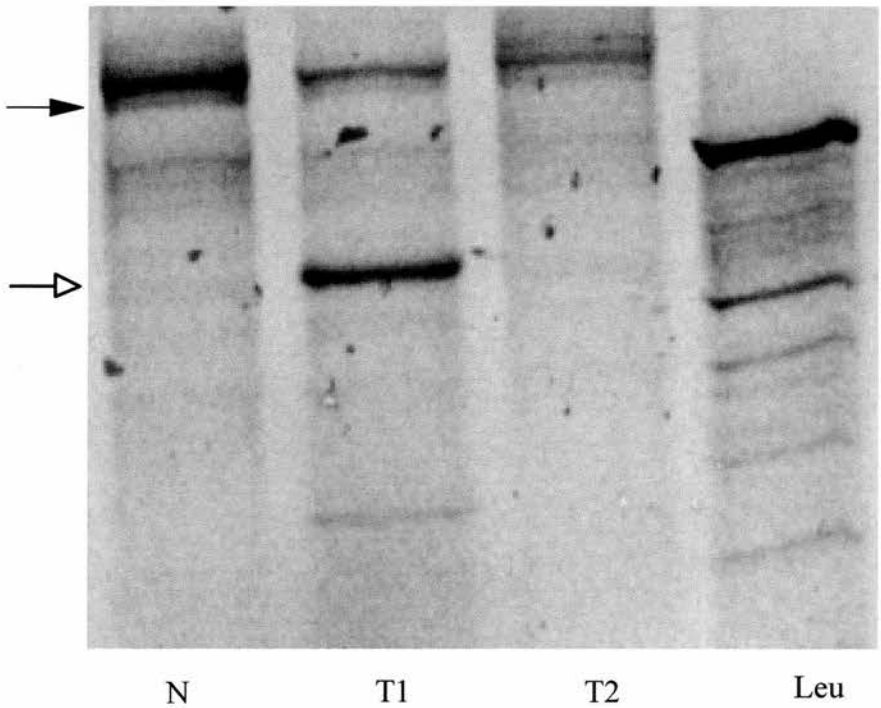
A total of 17 (4 %) patients were identified from the records of 424 patients who underwent surgical resection for cancer in the study period. Of these 17 patients only 12 had archive material available that was suitable for DNA analysis. These 12 formed the study population. There were 11 cases with two synchronous tumours and 1 with three. In all 25 tumours were analysed. The mutations that were detected are summarised in table 8.1.

Of the 25 tumours 10 demonstrated an *APC* mutation. Two individuals had an *APC* mutation in both of their cancers, 6 had an *APC* mutation in only one of the tumours examined and 4 had no mutations detected in any of their cancers. In no case however were identical APC mutations detected in the same individual.

There were 5 cancers that demonstrated an RER+ phenotype, and a further 4 which demonstrated MIN at only one locus of the 4 tested. Thus 20% of cancers demonstrated an RER+ phenotype and 36% of cancers demonstrated MIN at one or more locus. All the RER+ cancers occurred in 2/12 patients (16.7%). MIN in at least one locus was seen in 4/12 patients (33%). One individual with 3 cancers showed RER+ phenotype in two cancers, the third cancer was unstable at one locus only. In a second individual both cancers were RER+. In a third patient both cancers demonstrated MIN at one locus only. One individual had one cancer which was RER+ and one which did not show any MIN. In two other patients one cancer showed MIN at one locus and the other did not demonstrate MIN. Two cancers demonstrating MIN at one locus also had an *APC* mutation identified in the MCR. (Table 8.1)

Cancers exhibiting RER+ phenotype did not have any *APC* mutations in the MCR. Similarly only two cancers that showed MIN at one locus had an *APC* mutation in the MCR (Tables 8.2 and 8.3). These differences did not reach statistical significance (Fishers exact test).

Figure 8.1 Protein truncation test for *APC* mutations in normal and cancer tissue from a patient with synchronous colonic cancers



In all three lanes (N, T1 and T2) the normal protein product can be easily identified (solid arrow). In sample T1 there is also an additional faster migrating band indicating a truncated mutated protein product. This product is not seen in lane T2 (open arrow). A leuciferase control reaction was included in all protein truncation experiments to confirm successful translation and transcription. The smaller protein fragments in Leu represent protein degradation as a result of heating before loading the sample on the gel, this can also be seen the other sample lanes.

Table 8.1 ***APC*** **mutations,** ***APC*** **loss of heterozygosity, RER phenotype and DNA ploidy for 25 synchronous cancers in 12 patients.**

Case Number	Cancer	APC Mutations		RER		APC LOH		Ploidy
		PTT	SSCP MCR	Status	No of Loci	3' UTR	Exon 11	
1	T1	Seg 2/G2	-	Neg	0	Retained	Retained	Diploid
	T2	Seg 3/J2	Section 4	Neg	0	LOH	Retained	Diploid
2	T1	-	-	Pos	4	NI	NI	Diploid
	T2	-	-	Neg	0	NI	NI	Diploid
3	T1	-	-	Neg	0	Retained	NI	Aneuploid
	T2	-	-	Neg	0	Retained	NI	Aneuploid
4	T1	-	-	Pos	2	Retained	NI	Diploid
	T2	-	-	Pos	2	Retained	NI	Diploid
5	T1	-	-	Pos	3	NI	Retained	Diploid
	T2	-	-	Pos	2	NI	Retained	Diploid
	T3	Seg 3/J2	Section 3	Neg	1	NI	Retained	Diploid
6	T1	-	-	Neg	0	Retained	NI	Diploid
	T2	-	-	Neg	0	Retained	NI	Diploid
7	T1	Seg 3/J2	Section 3	Neg	1	Retained	Retained	Diploid
	T2	::	-	Neg	1	Retained	Retained	Diploid
8	T1	Seg 3/J2	Section 4	Neg	0	Retained	Retained	Aneuploid
	T2	::	-	Neg	0	LOH	LOH	Diploid
9	T1	Seg 3/J2	Section 1	Neg	0	Retained	NI	Aneuploid
	T2	::	-	Neg	1	Retained	NI	Diploid
10	T1	Seg 3/J2	Section 3	Neg	0	NI	NI	Diploid
	T2	-	-	Neg	0	NI	NI	Diploid
11	T1	Seg 3/J2	Section 4	Neg	0	NI	NI	Diploid
	T2	::	Section 3	Neg	0	NI	NI	Diploid
12	T1	Seg 3/J2	Section 3	Neg	0	NI	Retained	Diploid
	T2	::	-	Neg	0	NI	Retained	Diploid

PTT: Protein truncation test
Seg 3/J2 (Segment of *APC* exon 15 which produced truncated protein)
Pos: Positive
Neg: Negative
LOH: Loss of Heterozygosity

Section x Section of MCR which contains *APC* mutation
NI: Non-informative
:: No DNA suitable for PTT
- No mutation detected

Table 8.2 Relationship between RER phenotype and *APC* mutations in 25 cancers from 12 patients with synchronous cancers.

	APC mutation present	APC mutation absent
RER +	0	5
RER -	10	10
Not significant p = 0.0613 Fisher's exact test		

Table 8.3 Relationship between MIN phenotype and *APC* mutations in 25 cancers from 12 patients with synchronous cancers

	APC mutation Present	APC mutation absent
MIN +	2	7
MIN -	8	8
Not significant p = 0.2290 Fisher's exact test		

Aneuploid cancers were identified in 4 cancers in 3 patients. In one patient both cancers were aneuploid. In this patient no *APC* mutations were identified, there was no microsatellite instability or loss of heterozygosity. In the second patient one cancer exhibited aneuploidy. This cancer also had an *APC* mutation detected and exhibited LOH at the 3' UTR and exon 11. No MIN was detected. The second cancer in this patient showed no mutations, LOH or MIN. In the third patient the aneuploid cancer also had a mutation identified in *APC* but did not show any LOH or MIN. The diploid cancer showed LOH at the 3' UTR but no MIN or *APC* mutation.

No cancers exhibiting an RER+ phenotype displayed LOH or aneuploidy. These differences were not statistically significant (Fishers exact test $p > 0.99$).

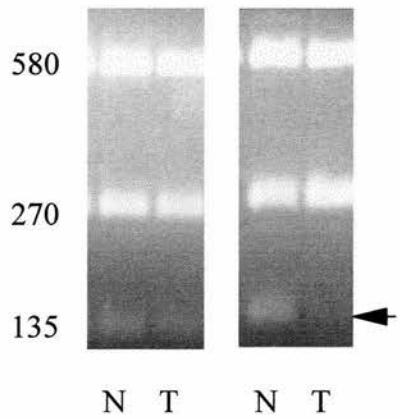
Discussion

In this series 4% of patients with colorectal cancer had a synchronous cancer which is in keeping with other reports where synchronous cancers account for 2.5 - 3.9% of patients with colorectal cancer (Fante 1996; Bekdash 1997). In our series *APC* mutations show no concordance, strongly suggesting synchronous colorectal cancers occur as individual events with regard to *APC*. Only 6% of synchronous colorectal cancers show concordant *Ki-ras* and *p53* mutations in both lesions (Koness 1996), supporting the conjecture that synchronous colorectal cancers are the result of independent events. *Ki-ras* mutations are confined primarily to two codons (Bos 1987) and the majority of *p53* mutations are clustered around mutation hot spots (Hollstein 1991). It is therefore conceivable that concordant *Ki-ras* and *p53* mutations in synchronous cancers could result in independent cancers by chance. The low incidence of concordant *Ki-ras* and *p53* mutations merely strengthens the support for synchronous cancers arising independently. In *APC* the large range of reported mutations and the size of the gene preclude the possibility of the same mutations

occurring by chance. Patients with advanced rectosigmoid adenomas are more likely to have advanced proximal adenomas or cancers than patients with early recto-sigmoid lesions (Papatheodoridis 1996) implying independent cancers with differing mutational profiles could arise from a common environmental insult. Analysis of *Ki-ras* and *p53* mutations in six primary cancers occurring in one individual showed each cancer to manifest the genetic characteristics typical of the tissue of origin suggesting the synchronous primary cancers arose independently and progressed along different carcinogenic pathways (Herring 1996). The evidence from multiple gastric cancers would appear to be conflicting. In one study multiple gastric cancers show discordance for *APC*, *MCC*, and *p53* mutations indicating independent origin for synchronous gastric cancers (Kang 1997). Other work analyzing synchronous gastric cancers has shown identical *p53* and *c-erbB2* status in 66% of patients with multiple synchronous gastric cancers suggesting precancerous lesions arise from conditions leading to similar genetic alterations (Wittekind 1997).

Patients with HNPCC are four times more likely to acquire a metachronous cancer than patients with sporadic colorectal cancer. The second malignancy occurring on average 8.7 years after the first (Fante 1996). HNPCC patients have a defect in their DNA mismatch repair system (Lynch 1993) and are thought to acquire mutations in key genes related to carcinogenesis at an increased frequency (Kinzler 1996). They may therefore also be at an increased risk of synchronous cancers (Dunlop 1996). In the reported series an RER+ phenotype was present in 16.7% of patients. MIN was demonstrated at 2 or more loci in 20% of cancers and 1 or more locus in 36% of cancers. Bubb *et al.* in a large series of 219 sporadic cancers that included 4 synchronous cancers demonstrated MIN at 2 or more loci in 10.5% of cancers and MIN at 1 or more loci in 17.8%. (Bubb 1996). This difference, when compared to the series reported here, could represent sample bias in the small population considered. It could equally indicate a predisposition to an RER+ phenotype similar to that noted in

Figure 8.2 Loss of Heterozygosity for the 3′ untranslated region of *APC*.



The 3' untranslated region of the *APC* gene contains an *Ssp1* polymorphism.

For each sample normal and tumour pairs were amplified by PCR. These products were then subjected to *Ssp I* enzyme digestion. The products were then resolved on 3% agarose gels. For each sample after enzyme digestion there is a constant 580 bp band. The two alleles are 270 bp and 135 bp in size.

The above gels shows a representative samples.

The left hand normal/tumour pair shows an informative sample with no LOH. The right hand normal/tumour pair shows an informative sample with the tumour sample showing loss of the 135 bp allele (arrowed).

patients with attenuated extracolonic manifestations (Dunlop 1996). This in turn could indicate that patients with synchronous colorectal cancers have an underlying germline MMR deficiency. The small sample size in my study limits the power of any analysis and differences in mutational profiles failed to reach significance. The small sample size when analyzing synchronous cancers is principally limited by the availability of cases. In my study based in a centre with a strong interest in colorectal cancer and molecular genetics with a large archive of DNA, there was still only 12 cases available for detailed analysis. Pooling of samples from other centres would seem to be the only feasible method by which to get a suitable sample size.

Consequently, there are few studies examining RER phenotype in synchronous cancers. Multiple primary cancers (cancers occurring in different organs at the same time) have been studied. An RER+ phenotype can be detected in at least one cancer in 89% of patients with multiple primary cancers (however only one patient had multiple colorectal cancers) but only 11% of patients with a single primary cancer (Horii 1994). Patients with independent multiple gastric cancers also have a higher prevalence of MIN than solitary cancers 65% versus 24% (Shinmura 1995). The proportion of RER+ cancers in our study is not as high as the aforementioned studies. In patients with multiple primary cancers the cancers analysed were in a wide range of tissues and this population is more in keeping with the characteristics of Lynch's Syndrome type II which is known to have a high incidence of RER+ phenotype (Lynch 1993). Thus these populations are very different from our own purely synchronous colorectal cancer population. As such, these studies may have study populations biased towards an RER+ phenotype. Metachronous tumours have a greater frequency of microsatellite instability than sporadic colorectal cancer (Sengupta 1997) suggesting an underlying defect in their mismatch repair system. RER screening may be potentially useful in identifying patients at increased risk of additional primary cancers (Horii 1994).

Although not statistically significant there is a tendency for the cancers in this study to have either an *APC* mutation or an RER + phenotype (Table 8.2). This suggests there might be two mutually exclusive pathways for colorectal carcinogenesis. If *APC* mutations are the initiating event in the pathogenesis of both RER - and RER + colon cancers then, the pattern and type of mutation should be the same in both types of cancer. If, however, genomic instability is the initiating event in the pathogenesis of RER + cancers, differences in their *APC* mutations when compared to those in RER - tumours would be expected. One might expect a decreased frequency of a allele loss at *APC* might occur in RER + tumours. Similarly more frame shift deletions than nonsense mutations would be expected. Also, mutations in *APC* in RER + tumours should tend to occur in stretches of simple repeat sequences. Previous experiments have shown that the spectrum of *APC* mutations differs between RER + and RER - cancers. Huang *et al.* showed that in 19/52 RER+ cancers that came from kindreds fulfilling the Amsterdam criteria for HNPCC RER+ cancers had a significant increase in frameshift mutations when compared to non-RER tumours suggesting the RER phenotype precedes and is responsible for the *APC* mutation (Huang 1996). In a separate study examining sporadic cancers there was no evidence that sporadic RER + and RER - colon cancers differed in their frequency of allele loss at *APC*, the overall frequency of *APC* mutations, or their frequency of framed shift *APC* mutations (Homfray 1998). Homfray concluded that in sporadic RER + and RER - colon cancers, *APC* mutations initiate tumourigenesis; mismatch repair becomes defective in sporadic RER + tumours at a later stage. It remains possible that MMR mutations initiate the growth of some tumours, but that its effect at such a stage would be to confer a selective growth advantage; an RER + phenotype and *APC* mutations would then occur later (Homfray 1998).

Although there were no identical *APC* mutations in this series I have shown there is a predisposition to a RER+ phenotype when compared to another series examining

sporadic colorectal cancer (Bubb 1996). It is possible that the actual mutations in *APC* might arise from a common mechanism (e.g. defective MMR) albeit in different parts of the gene. Attempts were made to characterise the MCR mutations detected in the study population by fluorescence sequencing but this was limited by the available time. Four mutations were confidently identified and two were indeed 2 base pair deletions from an AG_5 repeat in the MCR (nucleotides 4388 to 4395 (Groden 1991)). This mutation has been previously described in association with RER+ cancers and HNPCC (Huang 1996).

In the reported series the majority of cancers were diploid which is a known feature of HNPCC (Lynch 1993). In a small series examining cancers with synchronous adenomas, evidence of cytogenetically related clones in both the cancer and the adenoma was detected in 5/7 (71%) cases. Furthermore cytogenetic analysis of hyperplastic polyps showed evidence of cytogenetic changes in only 37.5%. These data suggest that for cancers and adenomas these otherwise macroscopically distinct lesions either arose as part of the same neoplastic process or that the same oncogenic environmental factor induced identical chromosomal rearrangements in more than one cell (Bardi 1997).

In summary I detected no evidence of mutational mosaicism with respect to *APC* in the region analysed. When compared to another large series of sporadic colorectal cancer there was an increased incidence of RER+ cancers and MIN cancers in synchronous cases. The majority of cancers were diploid in character. These observations could indicate an underlying MMR deficiency. In conclusion synchronous cancers appear to arise in unstable epithelia in response to a common environmental insult but then progress down different genetic mutational profiles.

9. Final Discussion

The principal aim of my research was to capitalize on other preliminary work to devise a genetic method specific for detecting colorectal cancer from exfoliated lumenal cells obtained from whole gut lavage fluid. If successful, it was envisaged such a test may have practical applications in the field of colorectal cancer screening. Secondly, it was hoped that the technique would provide a useful research tool for studying colorectal carcinogenesis. One specific question related to colorectal cancer tumorigenesis that was identified was the possibility of *APC* mutational mosaicism in patients with an underlying mismatch repair defect.

I have successfully demonstrated that it is possible to use the cells obtained in whole gut lavage fluid for DNA analysis by PCR based techniques. However, confounding factors that prohibited further development of PCR based assays were identified. Firstly, the vast majority of cells obtained by this method are inflammatory or squamous cells. Secondly, in half the whole gut lavage fluid samples obtained PCR amplification was not possible either due to reaction inhibition or insufficient DNA. Repeated sampling of whole gut lavage fluid (as for FOB) may be required to improve the sensitivity and specificity of stool based genetic tests. Finally, a specific mutation enrichment strategy is required to identify mutant alleles in a vast excess of wild type alleles. The net effect of my methodology was to limit DNA analysis to known point mutations suitable for restriction enzyme enrichment techniques. Analysis of *APC*, a key gene in colorectal carcinogenesis, was therefore severely limited. Development of better DNA extraction techniques which allow the use of the protein truncation test for *APC* mutation detection may provide a useful line of further research.

Whilst undertaking this period of research the link between *TGFß RII* mutations and RER+ cancers was established. My initial attempts to repeat these assays were unsuccessful but later published SSCP analysis was easily reproducible. My small series

confirmed *TGFβ RII* mutations in a smaller percentage of RER+ cancers than earlier work suggested. My figure is, however, in broad agreement with other studies published subsequently. The predominant mutations encountered in *TGFβ RII* are 1-2 bp deletions in the A_{10} segment of exon 3. The nature of this mutation precludes it from restriction enzyme enrichment techniques.

Another significant advance made while my research was being conducted was the identification of telomerase activity in up to 90% of colorectal cancers. This is therefore an obvious target for molecular detection of colorectal cancer eliminating the need to screen for point mutations at many sites. I was fortunate to be able to include some preliminary work on telomerase into my research. Telomerase assays are now available in kit form. Initial validation established the reliability of the commercial TRAP assay. Application of the TRAP assay to whole gut lavage fluid identified PEG inhibition of the PCR amplification at high concentrations. Despite correction for this TRAP assay analysis of whole gut lavage fluid was still not possible. The thermoliability of telomerase and its sensitivity to endogenous RNase activity may have some bearing on this. Different methods of sample collection and processing may remedy these problems and therefore offer an opportunity for continuing research.

I was not able to address the question of *APC* mutational mosaicism directly using whole gut lavage fluid techniques. I therefore turned my attention to the analysis of synchronous colorectal cancers. The identification of identical *APC* mutations in synchronous cancers would be strong evidence in support of such mosaicism. I was unable to identify single case with identical mutations. This suggests synchronous cancers develop as result of independent genetic events. There was however a tendency towards cancers with an RER+ which suggests synchronous cancers may arise in individuals with an underlying genomic instability for example mismatch repair deficiency. Further characterisation of the *APC* mutations in this study population may support this theory. This does not preclude the possibility that synchronous cancers in

susceptible individuals could occur as independent progressions following the same environmental insult.

Acknowledgments

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I would like to thank my advisor Mr. Malcolm Dunlop, Reader in Surgery for introducing me to Professor Wyllie which ultimately enabled me to undertake this research. I also thank him for his helpful advice in drafting abstracts and presentations. My thanks also to the late Professor Anne Ferguson, Professor of Gastroenterology, for making the facilities at her laboratories so freely available, her support, and for her constructive comments during the drafting of this manuscript.

Thanks also to Pearl Culbert and the nurses in the Gastrointestinal laboratories for their help in obtaining whole gut lavage fluid samples and to Mr. Norman Anderson for his instruction on ELISA technique. Special thanks go to Mr. Robert Morris Chief MLSO for his tireless patience in instructing me in the finer points of laboratory technique. I would also thank Dr. Susan Farrington for her help and instruction on the protein truncation test.

Finally I would like to thank Professor Wyllie for allowing me the privilege of working in his laboratory and for his continued support and discussion.

I would also like to express my gratitude to my wife Elizabeth for her understanding following long nights at the computer and to my children Emily, Eilidh and Daniel for their continual source of amusement and questions.

Publications and Presentations

1. *K-ras* mutations in Stool and Whole Gut Lavage Fluid. M.A.Potter, R.G.Morris, A. Ferguson, A.H.Wyllie *Gastroenterology* 1997;**112**:1427-1428

2. Detection of Mutations associated with colorectal cancer in DNA from whole gut lavage fluid. M.A.Potter, R.G.Morris, A. Ferguson, A.H.Wyllie. J. *Natl. Cancer Institute* 1998;**90**:623-626

3. Whole gut lavage a possible method for screening for Colorectal cancer. M.A.Potter, R.G.Morris, A. Ferguson, A.H.Wyllie. The School of Surgery Day (Chiene Medal Session), Royal College of Surgeons of Edinburgh, 30 November 1996.

4. The detection of Mutations associated with colorectal cancer in DNA extracted from whole gut lavage fluid. M.A.Potter, R.G.Morris, A. Ferguson, A.H.Wyllie. Plenary session for the Patey Prize. Tripartite meeting of the Surgical Research Society, Nottingham 9-11 July 1997.

5. The detection of Mutations associated with colorectal cancer in DNA extracted from whole gut lavage fluid. M.A.Potter, R.G.Morris, A. Ferguson, A.H.Wyllie. The School of Surgery Day (Chiene Medal Session), Royal College of Surgeons of Edinburgh, 30 October 1997.

Appendix: PCR conditions

Ki-ras

Primer sequences

Forward: 5'- TCA AAG AAT GGT CCT GGA CC

Reverse: 5'- GAC TGA ATA TAA ACT TGT GG

Reaction volume 50µl, primer concentration 0.4µM, Mg^{2+} concentration 1.5mM

Tm 55°C, annealing time 30 s. 35 cycles.

Cytochrome b

Primer sequences

Forward: 5'- AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA

Reverse: 5'- AAA CTG CAG CCC CTC AGA ATG ATA TTG TCC TCA

Reaction volume 50µl, primer concentration 1µM, Mg^{2+} concentration 1.5mM

Tm 55°C, Annealing time 1 min. 35 cycles.

Cytochrome oxidase

Primer sequences

Forward: 5'- ACG CCT AAT TCT ACT CCA CCT CAA TC

Reverse: 5'- ACG ATG TCT AGT GAT GAG TTT GCT A

Reaction volume 50µl, primer concentration 1µM, Mg^{2+} concentration 1.5mM

Tm 55°C, annealing time 1 min. 35 cycles.

APC SSCP section 1

Primer sequences

Forward: 5'- TGG AAC TTC GCT CAC AGG AT

Reverse: 5'- AAG TGG CAG CCT CAA AAG G

Reaction volume 50µl, primer concentration 1µM, Mg^{2+} concentration 3 mM. Product size 256 bp. Hot Start touch down PCR.

Annealing temp (°C)	63	60	57	55
Annealing time (s.)	30	30	30	30
Cycles	1	1	1	33

APC SSCP section 2

Primer sequences

Forward: 5'- TCA GAC GAC ACA GGA AGC CAG

Reverse: 5'- GTA CAT CTG CTA AAC ATG AGT GGG

Reaction volume 50µl, primer concentration 0.5µM, Mg^{2+} concentration 1.5mM. Product size 294 bp. Hot start, Tm 55°C, annealing time 30 s., 35 cycles.

APC SSCP section 3

Primer sequences

Forward: 5'- CAG GAG ACC CCA CTC ATG TT

Reverse: 5'- CAG CAT TTA CTG CAG CTT GC

Reaction volume 50µl, primer concentration 0.5µM, Mg^{2+} concentration 1.5mM. Product size 291 bp. Hot start, Tm 56°C, annealing time 30 s., 35 cycles.

APC SSCP section 4

Primer sequences

Forward: 5'- AGA GTG GAC CTA AGC AAG CT

Reverse: 5'- CAT TTT CCT GAA CTG GAG GC

Reaction volume 50µl, primer concentration 0.5µM, Mg^{2+} concentration 1.5mM. Product size 203 bp. Hot start, Tm 55°C, annealing time 30 s., 35 cycles.

APC PTT segment 2/G2

Primer sequences

Forward: 5'- GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGA GAA CAA CTG TCT ACA AAC T

Reverse: 5'- ATG AGT GGG GTC TCC TG

Reaction volume 50µl, primer concentration 0.5µM, Mg^{2+} concentration 1.5mM. Product size 2217 bp. Hot start, Tm 60°C, 94°C 1 min., 60°C 2 min., 72°C 2 min., 37 cycles.

APC PTT segment 3/J2

Primer sequences

Forward: 5'- GGA TCC TAA TAC GAC TCA CTA TAG GGA GAC CAC CAT GGT TTC TCC ATA CAG GTC ACG G

Reverse: 5'- GAG CCT CAT CTG TAC TTC TGC

Reaction volume 50µl, primer concentration 0.5µM, Mg^{2+} concentration 1.5mM. Product size 1806 bp. Hot start, Tm 63°C, 94°C 1 min., 63°C 2 min., 72°C 2 min., 37 cycles.

Microsatellites

BAT 26

Primer sequences

Forward: 5'- TGA CTA CTT TTG ACT TCA GCC

Reverse: 5'- AAC CAT TCA ACA TTT TTA ACC C

Reaction volume 50µl, primer concentration 1µM, Mg^{2+} concentration 1.5mM. Product size 120 bp. Tm 50°C, annealing time 30 s. 35 cycles.

BAT 40

Primer sequences

Forward: 5'- ACA ACC CTG CTT TTG TTC CT

Reverse: 5'- GTA GAG CAA GAC CAC CTT G

Reaction volume 50µl, primer concentration 1µM, Mg^{2+} concentration 1 mM. Product size 109 bp. Tm 50°C, annealing time 30 s. 35 cycles.

D2S123, D5S346, D13S160 CA repeats

Primer sequences

D2S123 Size

Forward: 5'- AAA CAG GAT GCC TGC CTT TA

Reverse: 5'- GGA CTT TCC ACC TAT GGG AC

D5S346 Size

Forward: 5'- ACT CAC TCT AGT GAT AAA TCG GG

Reverse: 5'- AGC AGA TAA GAC AAG TAT TAC TAG TT

D13S160 Size

Forward: 5'- CGG GTG ATC TAA GGC TTC TA

Reverse: 5'- GGC AGA GAT ATG AGG CAA AA

Reaction volume 50µl, primer concentration 0.5µM, Mg^{2+} concentration 1 mM. Product size 120 bp. Hot start, Tm 55°C, annealing time 30 s. 35 cycles.

***TGF Beta Receptor II Exon* 3**

Primer sequences

Forward: 5'- CCT CGC TTC CAA TGA ATC TC

Reverse: 5'- TTG GCA CAG ATC TCA GGT CC

Reaction volume 50µl, primer concentration 0.5µM, Mg^{2+} concentration 2 mM. Product size 267 bp. Tm 55°C, annealing time 30 s. 35 cycles.

Protocol for Polymerase chain reaction optimisation

Volume of each reagent in μL for each optimisation reaction

	Reaction							
Reagent	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
DDW	17	16	15	14	22	21	20	19
dNTP	8	8	8	8	8	8	8	8
Buffer	5	5	5	5	5	5	5	5
Forward Primer	5	5	5	5	3	3	3	3
Reverse Primer	5	5	5	5	3	3	3	3
Mg Cl2	2	3	4	5	2	3	4	5
Detergent	3	3	3	3	3	3	3	3
***Taq* Polymerase**	1	1	1	1	1	1	1	1
DNA	5	5	5	5	5	5	5	5
Total	50	50	50	50	50	50	50	50

DDW	Distilled Deionosed water
dNTP	Nucleotide mixture final reaction concentration of 200 μM
Buffer	10x stock solution diluted to final reaction concentration of 20 mM Tris-HCl, (pH 8.0), 50mM KCl
Primers	10 micromolar
$MgCl_2$	50 mM

Appendix 2 Study population detailing age, sex, site of lesion, stage and mutation detection

			CYTOCHROME	Ki ras		p53		APC	RER			PATHOLOGY		
STUDY NO	SEX	AGE	b	OXIDASE	TUMOUR	LAVAGE	TUMOUR	LAVAGE	TUMOUR	LAVAGE	MIN	TGFβ RII	SITE	DUKES	STAGE
1	F	60	+	+	+	+			SECTION 2				RECTUM	A	pT2 G2 N0
2	M	85	+										DESCENDING	B	
3	M	81	+	+									SIGMOID	C	
4	M	66	+	+			+	+					RECTUM	A	pT2 G2 N0
5	M	73	+	+					SECTION 1		1		SIGMOID	A	pT1 G2
6	M	63	+										RECTUM	C	
7	M	86	+	+					SECTION 3				SIGMOID	B	
8	F	83	+		+	NO PCR			SECTION 1				RIGHT	C	pT3b N0
9	M	62	+	+					SECTION 3				RECTUM	B	pT3b N0
10	M	71	+	+	-	+							TRANSVERSE	C	pT3c G2 N1
11	M	79	+	+					SECTION 1		1		RECTUM	A	pT2 N0
12	M	47											DESCENDING	D	pT3c G3 N0
13	F	74	+		+	+			SECTION 2				RECTUM	B	
14	F	75	+	+	+	-			SECTION 4				SIGMOID	C	pT3b G2 N0
15	F	71	+	+									SIGMOID	C	pT3b G2 N0
16	F	62	+										DESCENDING	C	
17	F	66	+										RECTUM	A	pT1 G2
18	F	39	+	+									DESCENDING	B	
19	F	72	+	+					SECTION 1				RECTUM	B	pT4 N0
20	F	90	+						SECTION 1				RECTUM	C	pT3b G2 N0
21	M	61	+										SIGMOID	D	pT4 G3 N1 M1
22	M	57	+	+									RECTUM	C	pT3 G3 N0
23	F	55	+		+	NO PCR			SECTION 1		1		RECTUM	C	pT3C G3
24	M	73	+	+					SECTION 1				RECTUM	B	pT3a G2 N0
25	M	88	+										SPLENIC FLEXURE	B	pT3b N0
26	F	90	+						SECTION 1				RECTUM	C	
27	M	71	+						SECTION 4		4	+	RIGHT	B	pT3A N0
28	M	72	+				+	+					TRANSVERSE	C	pT3b N1
29	M	64	+										SIGMOID	A	pT2 G2 N0
30	M	78	+								1		RECTUM	B	
31	M	78	+										SPLENIC FLEXURE	B	pT4
32	F	81	+										DESCENDING	B	
33	F	78	+	+	+	NO PCR							SIGMOID	C	pT3a G2 N0
34	F	67	+										RECTUM	C	pT1
35	M	59	+		+	NO PCR							RECTUM	A	pT2 G2 N0
36	M	69	+	+									SIGMOID	B	
37	M	68	+										SIGMOID	C	
38	M	53											RECTUM	C	pT3 G2 N1
39	M	80	+										DESCENDING	B	pT3 G2 N0
40	F	62							SECTION 3		3	+	CAECUM	C	pT3a N1 G3

For Cytochrome data

+	Successful PCR amplification
-	Unsuccessful PCR amplification

For *Ki-ras* and *p53* data

+	Mutation detected
-	Mutation not detected
No PCR	No PCR amplification possible for DNA from whole gut lavage fluid.

For *APC*

Section x	Section of MCR in which *APC* mutation was identified

For RER (Analysis of tumour DNA)

MIN	Number of unstable loci
+	*TGFβ RII* mutation detected in tumour DNA

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