An investigation into the genetic pathways of colorectal cancer.

Fradley, Susannah Ruth

AN INVESTIGATION INTO THE GENETIC PATHWAYS OF COLORECTAL CANCER

SUSANNAH RUTH FRADLEY

A thesis submitted for the partial fulfillment of the requirement for the degree of Doctor of Philosophy

Human Molecular Pathology Group
School of Biological Sciences
University of Wales Swansea

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Declaration and Statements

Declaration

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree

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Statement 1

This thesis is the result of my own investigations, except where otherwise stated.

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Summary

The mortality associated with colorectal cancer is extremely high, however if the disease is caught at the earliest stage then there is a 95% 5 year survival. An improved screening technique which is less invasive than current methods may increase uptake by the public. Also, an increase in knowledge of the different genetic pathways of colorectal carcinogenesis may help to tailor treatments to individual patients.

The experiments in this thesis address these aims by searching for possible biomarkers which may be detectable in stool samples and by looking for early genetic alterations at the molecular, gene expression and chromosomal levels. This thesis also explored various different techniques such as single stranded conformation polymorphism, fragment analysis, sequencing, cDNA arrays and fluorescent *in situ* hybridisation (FISH).

The findings of these experiments include mitochondrial DNA mutations occurring frequently in late stage colorectal carcinogenesis. These mutations predominantly occurred in microsatellites within the mitochondrial genome. Nuclear microsatellite instability (MSI) levels within the South Wales population studied are within the levels found in the rest of the Western world. There was no correlation found between mitochondrial and nuclear MSI suggesting the presence of separate mismatch repair systems. Various genes were found down-regulated in the early stages of colorectal cancer, including PTEN, TGF-ßRII and p21. These genes may be key to the early stage development of a significant proportion of colorectal cancers. I developed a FISH probe for p21 which was simple to make and apply, as well as being cost effective. Chromosome 6 aberrations were detected in an increasing frequency in samples with increasingly developed tumours. A large variation was found in chromosome 6 copy number within different regions of an individual tumour. This variation shows how essential it is that research and diagnostic tests are based on either large portions of a tumour or from multiple smaller biopsies.

Abbreviations

μg	Micrograms
μl	Microlitres
A	Adenine
APC	Adenomatous Polyposis Coli
bp	Base pair
C	Cytosine
CDK	Cyclin Dependent Kinase
CIN	Chromosome Instability
D-Loop	Displacement loop
DNA	Deoxyribonucleic acid
etOH	Ethanol
FAP	Familial Adenomatous Polyposis
FISH	Fluorescent *in situ* hybridisation
G	Guanidine
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
hMSH3	Human *Saccharomyces cerevisiae* MutS homolog 3
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
IGFIIR	Insulin-like Growth Factor II Receptor
min	Minute
MMR	Mismatch Repair
MSI	Microsatellite Instability
mtDNA	Mitochondrial DNA
mtMSI	mitochondrial MSI
nDNA	Nuclear DNA
nMSI	nuclear MSI
OXPHOS	Oxidative phosphorylation
p21	Cyclin-dependent kinase inhibitor 2C
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PTEN	Phosphatase and tensin homolog
$RER^{+/-}$	Replication Error positive/negative
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
SDS	Sodium doecyl sulphate
sec	Second
SSC	Saturated Sodium citrate
SSCP	Single Stranded Conformation Polymorphism
ssDNA	Single Stranded DNA
T	Thymidine
TGF-βRII	Transforming Growth Factor-Beta Receptor type II
UV	Ultra Violet

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Chapter 1
General Introduction

1.1 General introduction to cancer

Cancer is a worldwide disease. In the UK alone there are over ¼ million cases diagnosed every year, approximately 1 in every 3 of us will develop a form of cancer and approximately 50% of those with the disease will die as a result. In terms of financial cost, the annual NHS hospital costs for diagnosis, surgery, chemotherapy and other treatments and support for sufferers are greater than £1 billion (Department of Health statistics, 2005).

Cancer is defined as any malignant neoplasm; an uncontrolled growth of cells that exhibits invasiveness and remote growth, these neoplasms seem to evade the normal control from nerves or hormones (Strachan and Read, 1998). Gene expression patterns are different to those of normal tissue, for example, tumour suppressor genes (which are discussed further in section 1.1.1.5) are frequently down regulated in tumour cells (Kitahara *et al.*, 2001). There are many forms of the disease as virtually any cell type can become cancerous and grow in an uncontrolled manner. For example, a breast cell that eludes normal regulation and divides out of control may form a breast cancer tumour. If cells from this then grow and spread into the bloodstream or lymphatic system then the cancer can spread to neighbouring tissues and via the circulatory system throughout the body causing secondary cancerous growths. The spread of cancer cells beyond the original site is known as metastasis. Cancers can also form in the blood; leukaemia is a form of cancer in which the white blood cells proliferate. Because of the nature of the cell type solid tumours are not formed however the cancerous cells circulate through the body where they may grow in other tissues. The chance of cancer detection depends on the location of the neoplastic growth. For example, skin cancers are often detected at early stages of cancer progression as the skin is easily monitored for any signs of cell change. The success rate of treatment depends upon the tissue type affected and the stage of

progression at which the cancer has been detected at as well as the form of treatment selected. The incidence of the most common forms of cancer in the UK can be seen in Figure 1.1.

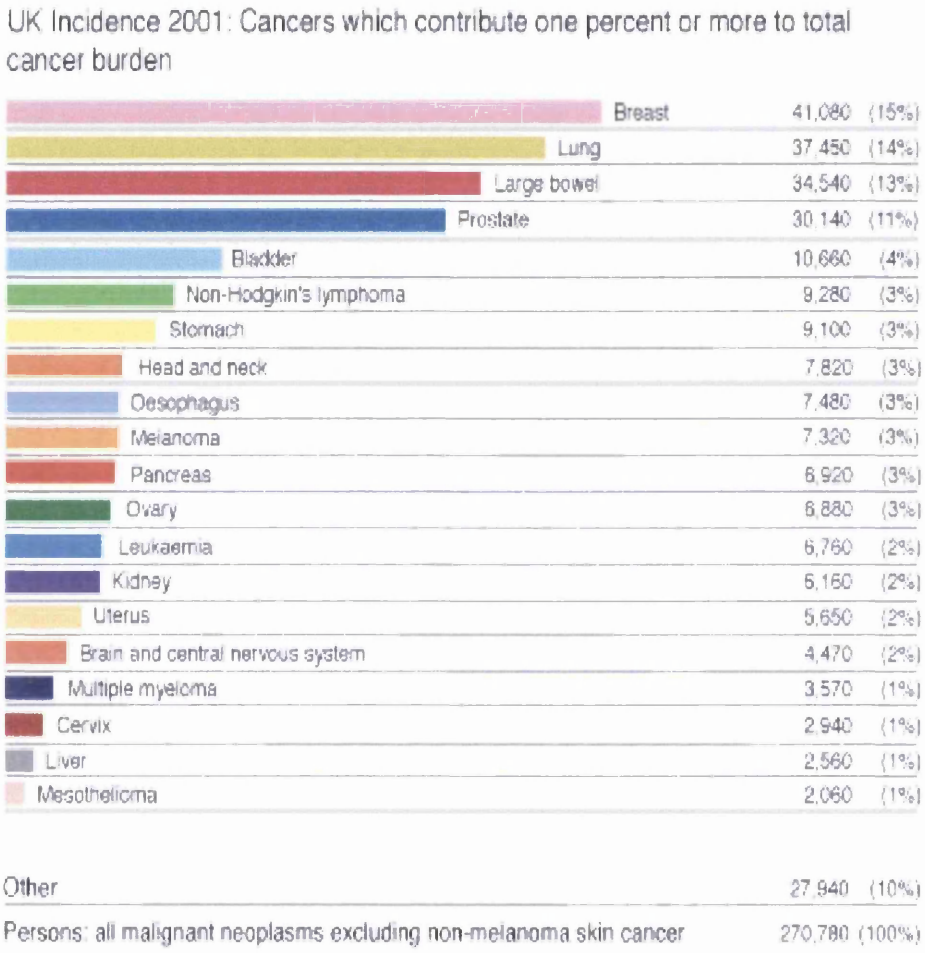


Figure 1.1

This graph illustrates the frequency of various cancer types in the UK in 2001. From www.cancerresearchuk.org

There are many factors that influence an individual's risk of developing cancer such as age and, for various cancer types, sex. Lifestyle also is highly important; whilst smoking increases the risk of many different forms of cancer, eating a diet high in fruits and vegetables can lower the risk. The risk factors for colorectal cancer are discussed in Section 1.2.1.

1.1.1 Basic genetics of cancer

All cancer cells share a common cause, mutations or disruption to the gene expression of the genes which regulate cell growth, apoptosis or DNA repair (Bertram, 2001). In a normal cell there are highly developed systems to repair damage to the DNA of the cell, regulate cell growth and cell death through either necrosis or apoptosis. Tumours develop by the accumulation of damage to these genes and a multistep hypothesis suggests that for a tissue to become cancerous between 3-6 mutations are required to these growth control and/or repair genes (Vogelstein and Kinzler, 1993). Genomic instability is required to allow a sufficient amount of genetic change to accumulate to permit the neoplastic phenotype to develop.

1.1.1.1 Cell cycle

Cells are required to replicate in order for organisms to grow and also to replace cells which die either from age or as a result of cellular damage. This occurs through a process known as the cell cycle. The cell cycle is divided into four phases, first growth or gap phase (G_1), synthesis (S), second growth or gap phase (G_2) and mitotic phase (M). The mitotic phase consists of prophase, metaphase, anaphase and telophase. These can be seen in Figure 1.2. The length of time taken for the cell to complete a cycle varies from type to type with skin cells dividing approximately every 12 hours and liver cells dividing every 2 years. This variation occurs when cells are not dividing and are said to be in G_0 phase (Lodish *et al.*, 1995). Throughout the cell cycle there are checkpoints which control the progression through the cycle, these checkpoints are detailed in Table 1.1. The cell cycle is carefully regulated by cyclin dependant kinases (CDKs) which are activated by

cyclins and inhibited by CDK inhibitors (Grana and Reddy, 1995). The genes which code for these proteins are frequently mutated in cancer cells which results in uncontrolled cell division. The regulation of cell cycle is further discussed in Chapter 6, Section 6.1.6.1.1., when relevant to specific parts of my thesis.

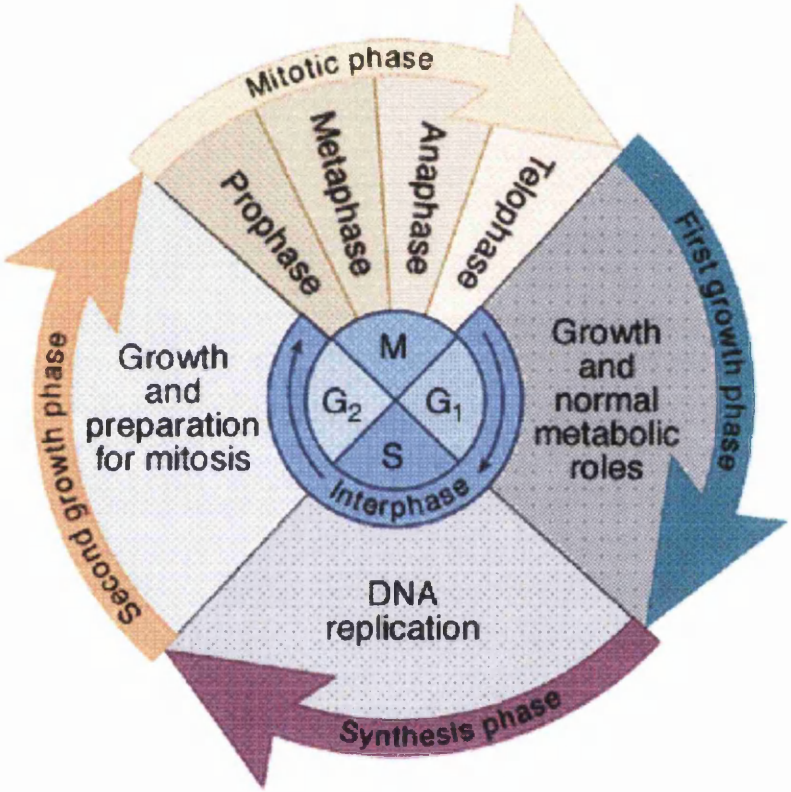


Figure 1.2

This diagram shows the phases of the cell cycle. The cycle is not shown to time scale as the size of each section is not representative of the time taken to complete each stage. The average period of time taken to complete the cell cycle is generally considered to be around 16 hours. Mitosis is a short stage lasting around 1 hour with interphase lasting for the remaining 15 hours (Lodish *et al.*, 1995). From www.bmb.psu.edu.

Phase		Events
Interphase	G_1	Cell grows and produces RNA and synthesizes proteins The interval between the completion of mitosis and DNA synthesis
	G_1 Checkpoint checks if the cell is large enough and the environmental factors are favourable	
	S	DNA synthesis. The cell's DNA is duplicated prior to cell division
	G_2	Cell starts to produce proteins required for cell division Mitochondria are duplicated to meet the increased energy demands of the cell In the late G_2 condensation of chromatin starts
	G_2 Checkpoint assesses integrity of DNA and also checks if the cell is large enough and if the environmental factors are favourable	
Mitosis	Prophase	Chromosomes condense Centrioles move to opposite poles of the cell
	Pro-metaphase	Nuclear membrane is broken down Kinetochores begin to form on each chromosome
	Metaphase	Spindle fibres connect centrioles to centromere Chromosomes align along equator of cell
	Metaphase Checkpoint checks the chromosomes are aligned on spindles	
	Anaphase	Centromeres spilt Chromatids separate and move to opposite poles
	Telophase	Spindle fibres disperse Nuclear membranes form around each set of chromosomes
Cytokinesis		A ring of actin filaments form around the equator of the cell and tightens to form a cleavage furrow The cell is divided into two daughter cells

Table 1.1

This table details the stages and checkpoints of the cell cycle

1.1.1.2 Mutagenesis

A mutation is a change to the nucleotide sequence and can be in the form of a single base change or deletions or insertions to the sequence. Depending upon where in the DNA sequence the mutation occurs the affect of the mutation can vary from mutations in non-coding regions which have little or no effect on the cell function to frameshift mutations (Strachan and Read, 1998). These latter mutation types may create a stop codon and lead to the production of a truncated protein with significant loss of function. For example, the adenomatous polyposis coli (APC) gene is frequently mutated in colorectal cancers. This in turn leads to the production of truncated protein and the tumour suppressor function of wild type APC is lost (Fearnhead *et al.*, 2001).

Single base substitutions are the most common form of mutation. These can be either transition or transversion mutations. Transition mutations are changes from one purine to another e.g., A → G or pyrimidine to pyrimidine such as T → C. Transversion mutations are changes from purine to pyrimidine and vice versa such as A → T mutations. Transition mutations often form due to transient tautomers forming non-standard base pairs within the double helix. These tautomers are the rare forms of bases where, for example, the amino group (-NH2) on an adenosine base can tautomerize to an imino form (=NH) which allows the pairing of this form of adenosine to pair with cytosine (Stryer, 1995). If not repaired, in further rounds of replication the daughter strands created would consist of one normal sequence of DNA and one mutated strand. Transversion mutations can occur as a result of damage to the bases such as an incorporation of an oxygen atom onto the purine guanine base. This gives rise to 8-hydroxy-2'-deoxyguanosine which can mispair with the purine adenosine (Grollman and Moriya, 1993). This is further discussed in Chapter 3, section 3.1.1.4.

1.1.1.3 Repair

DNA is damaged by environmental factors such as mutagenic chemicals and radiation as well as intrinsic factors such as oxidative damage. During DNA replication the wrong base is sometimes incorporated by the DNA polymerase causing point mutations or

strand slippage can occur giving either deletion or insertion mutations. This mutational load would be too great for cell survival if left unchecked and so systems have evolved to repair the damage. There are three types of DNA repair, base excision (BER), nucleotide excision (NER) and mismatch repair (MMR).

BER removes defective DNA bases such as those damaged by reactive oxygen species (Nilsen and Krokan, 2001). Damaged bases are repaired by BER where the base is removed by DNA glycosylase to leave an abasic site also known as an AP site. This is then recognised by AP endonuclease which removes the AP site and neighbouring nucleotides. The resulting gap is then filled by DNA polymerase I and DNA ligase. Biallelic mutations in the BER DNA glycosylase gene MutYH have been found to lead to adenomatous colorectal polyposis and a greatly increased risk of colorectal cancer (Cheadle and Sampson, 2003).

NER removes bulky DNA lesions such as adducts produced by chemical carcinogens or UV-induced lesions (Benhamou and Sarasin, 2000). These lesions distort the DNA double helix which is detected and repaired by NER. During this process the oligonucleotide containing the lesion is excised from the DNA by endonuclease and the resulting single-stranded gap is then filled in by DNA polymerase I and then DNA ligase (Voet and Voet, 1995). Xeroderma pigmentosa (XP) is a heritable disease where patients have genetically defective NER. Exposure to UV irradiation from sources such as sunlight causes the patient's skin to develop erythematous patches which later develop into skin keratoses (Kornberg and Baker, 1992).

MMR repairs the DNA where errors have occurred during DNA synthesis, recombination or chemical damage (Kolodner, 1996). In order to establish which base is incorrect from a base pair the system has to be able to distinguish between the template strand and the newly synthesised strand. The template strand is methylated whereas the newly synthesised strand is not at this stage and so the strands can be differentiated. The incorrect bases are then excised and the gap filled by DNA polymerase III and DNA ligase (Lodish *et al.*, 1995). Mutations to the genes involved in this pathway such as

hMSH2 and hMLH1 are mutated in hereditary non-polyposis colorectal cancer (HNPCC) which is further discussed in section 1.3.1.

1.1.1.4 Proto-oncogenes

Proto-oncogenes are generally involved in the control of cell proliferation which, if over expressed, can contribute to the transformation of a normal cell into a tumour cell. Once activated these genes are known as oncogenes, this can occur by mutation, hypomethylation or by larger scale genetic damage such as gene amplification or chromosomal translocations or rearrangements (Laird *et al.*, 1995; Fearon, 1996). Examples of these genes are K-ras which is mutated in 60% of late colorectal adenomas (Weinberg, 1991) and bcl-2 the over expression of which makes cells resistant to apoptosis (Vaux *et al*, 1988).

1.1.1.5 Tumour suppressor genes

Tumour suppressors are genes which in normal circumstances negatively regulate cell proliferation, but when mutated or down-regulated the cell can over proliferate and become cancerous (Collins *et al.*, 1997). These genes are often involved in the checkpoint controls of cell cycle such as p53 which when the DNA is damaged promotes either apoptosis or halts the cell cycle at G_1 checkpoint in order for repair to occur prior to cell division (Lane, 1993). Hypermethylation is responsible for the inactivation of some tumour suppressor genes such as the mismatch repair gene hMLH1 (Herman *et al.*, 1998; Cunningham *et al.*, 1998).

1.2 Colorectal cancer

As the second most common cause of cancer related death (see Figure 1.3), colorectal cancer has received attention from many research groups around the world. In the UK over 31,000 people develop colorectal cancer annually, 13% of all new cancers diagnosed. Despite advances in medicine, with surgery as the main form of treatment, there is 70% mortality, largely due to patients initially presenting with later stages of colorectal cancers (Mella *et al.*, 1997). Over 50% of Westerners develop a colorectal

tumour by the age of 70. 1 in 10 of these will progress to malignancy (Navaratnam *et al*, 1999). In Wales, figures from 1994 show that there are around 2000 cases of colon cancer per year. Worldwide, in 1990 there were approaching 800,000 new cases diagnosed and 400,000 deaths with the number having risen rapidly over the last 25yrs. Colorectal cancer rates are lower in Africa and Asia. This is likely to be due to environmental and lifestyle factors such as diet, as opposed to genetic background because migrating populations develop similar relative risks as to the area to which they have migrated (Haenszel and Kurihara, 1968).

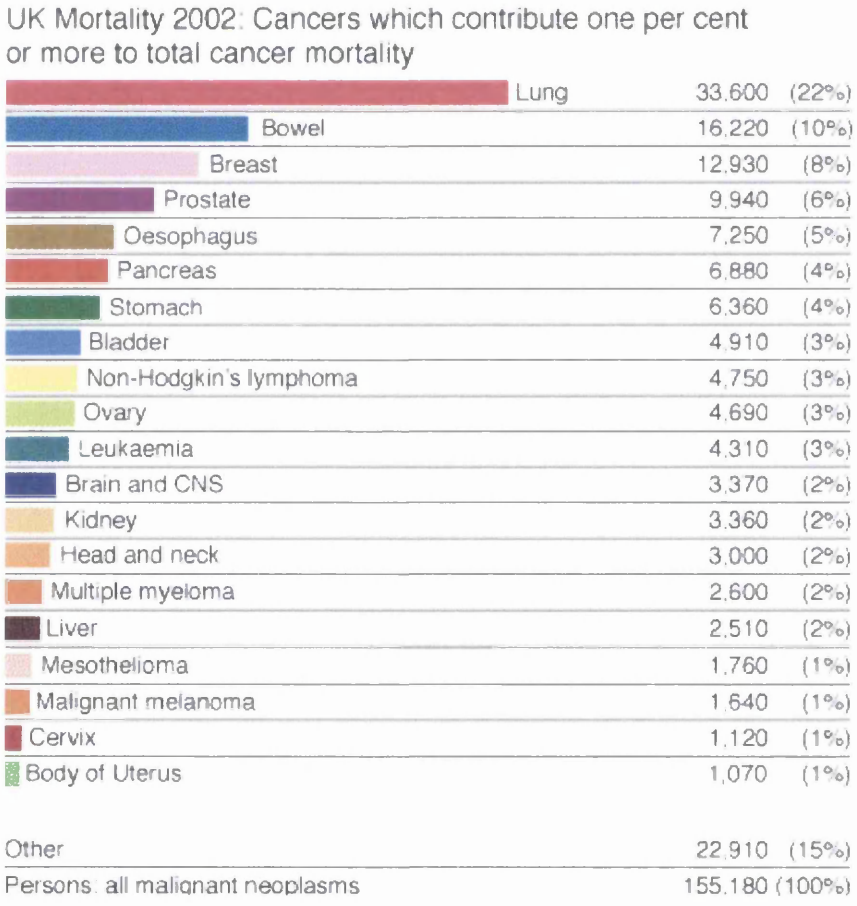


Figure 1.3

This graph illustrates the mortality of cancers in the UK as surveyed in 2002. It can be seen that colorectal cancer is the second most common cause of cancer related death with 10% of cancer deaths resulting from this type. From www.cancerresearchuk.org

In Western countries approximately 60% of primary colorectal cancers arise within either the rectum or sigmoid and around 20% are located in the caecum (Houlston, 2001); more details of the proportion of colorectal cancers within each region of the colorectum can be seen in Figure 1.4. Cancers of the colorectum are known, as with other cancers of epithelial tissue, as carcinomas.

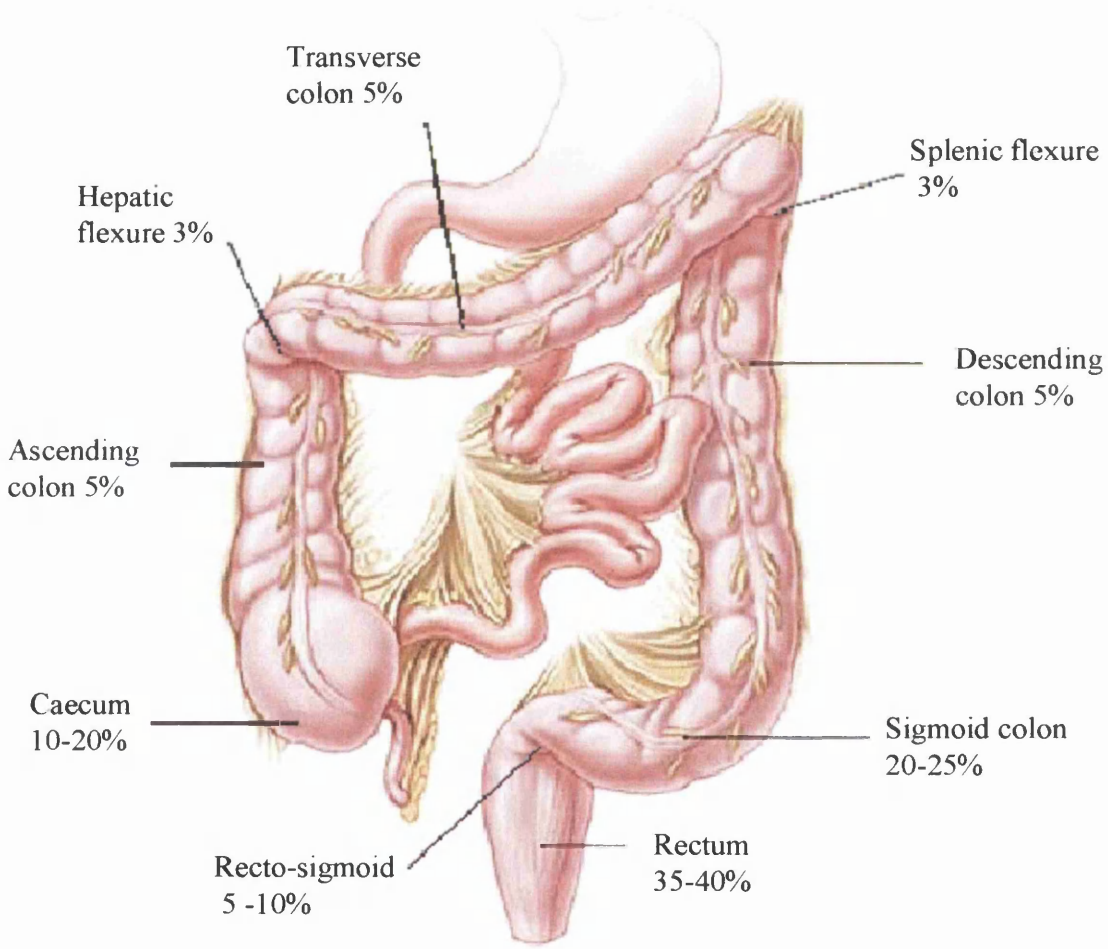


Figure 1.4

This diagram shows the regions of the colorectum and also the proportion of colorectal cancers which occur in each region. Adapted from www.hokins-gi.org and Mella *et al.*, 1997.

Although there are some similarities in the gastrointestinal epithelium of both the small intestine and the colon the rates of cancer incidence are very different with less than 1% of cancers occurring in the small intestine (Cancer Research UK, www.cancerresearchuk.org). There are several possible explanations for this including a lower rate of spontaneous apoptosis in the proliferative region of the large intestine in comparison to the small intestine. The rate is approximately 10 times lower in the colon, with an apoptotic cell occurring in around 0.1% of colonic crypt cells and proportionally fewer of these are in the stem cell region of the crypt (Hall *et al.*, 1994). This reduced ability for the colon to remove excess stem cells may result in hyperplastic colonic crypts, which may become targets for carcinogenesis (Potten *et al.*, 1997).

1.2.1 Clinical aspects of colorectal cancer

Colonic tumours may be asymptomatic or the patient may present with changes in bowel habit, constipation, diarrhoea, narrow stools, passage of blood or mucous par rectum, obstruction or rectal pain. The symptoms may also be more general such as abdominal discomfort including frequent gas pains, bloating, fullness and cramps. The symptoms also vary on the location of the lesion with the right-sided lesions resulting in weight loss and iron deficiency anaemia secondary to chronic occult blood loss (Petersen and Shepherd, 2000). The symptoms however are frequently ignored until the cancer has progressed to later stages. The delay in patients presenting to their doctor or delays in treatment are likely to be responsible for the lower survival rates in England and Wales compared to the rest of Western Europe.

Colorectal cancers in the UK are classified by Dukes' staging according to how far the cancer has progressed (Dukes and Bussey, 1958). This classification system uses the letters A-D where Dukes' A is cancer of only superficial layers of cells through to Dukes' D which is metastatic disease where the cancer has spread to distant organs. These stages are represented and explained in Figure 1.5. Dukes' A and B stage colon cancer have

very high cure rates, 95% and 80% estimated 5-year survival rate respectively (Rhodes, 2000). However only around a third of new cases are diagnosed at these stages, the remainder are diagnosed with stage C and D colon cancer. For these later stages the survival rates drop to 50% for stage C and only 3-5% for stage D (Rhodes, 2000). For the earlier stage the usual treatment is surgery, whereas the later stages C and D are usually treated with a combination of surgery and chemotherapy and for rectal tumours sometimes radiation is used, see Figure 1.5.

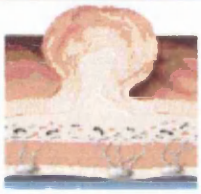
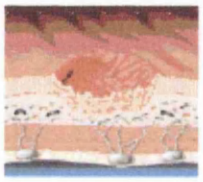
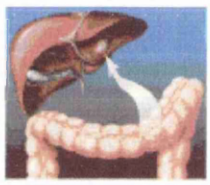
Classification	Dukes' A	Dukes' B	Dukes' C	Dukes' D
Development of Disease				
Explanation of Cancer Progression	Cancer confined to most superficial cell layers of colon or rectum. (e.g. the top of this polyp).	Cancer may extend completely through wall of colon or rectum, but there is no lymph node involvement.	Cancer may extend completely through wall of colon or rectum and has spread to lymph nodes.	Metastatic disease. The cancer has spread to distant organs, such as the liver.
Usual Treatment*	Surgery	Surgery	Surgery and Chemotherapy (possibly radiation for rectal cancer).	Surgery and Chemotherapy (possibly radiation for rectal cancer).
Estimated 5-Year Survival Rate	95%	80%	50%	5%
Percent Diagnosed at Stage	37%		63%	

Figure 1.5

This figure shows the progression of colorectal cancer as well as the likely treatments, patient survival and the percent of patients that present at these stages. From http://www.exactsciences.com/pregen26/professionals/about_hnpcc/.

Current recommendations suggest that to detect colon cancer in these early stages, when the chance of survival is highest, everyone over the age of 55 should have bi-annual

endoscopic examinations of the colon (Winawer *et al.*, 1997; Rex *et al.*, 2000). This comes with risks connected to the procedure itself with 1 in every 2000 colonoscopic examinations having complications including problems associated with sedation and perforation of the bowel, which can, in extreme circumstances, result in death. Screening using faecal occult blood tests (FOBTs) is currently being used across the UK as studies in other European countries and the US have shown these tests could reduce mortality by 50% (Hobbs, 2000). A 5 year survival rate for patients with colorectal cancer should be achievable as this relatively recent screening programme becomes established. Although FOBTs are inexpensive they also have a high number of false positives which may cause unnecessary alarm. Only between 2-18% of those with a positive result will actually have cancer or large polyps (Ransohoff and Lang, 1997). As a result, FOBTs do not prevent unnecessary colonoscopies which are both expensive and come with the aforementioned risks. Barium enemas can be used to detect growths and abnormal masses and although uncomfortable, no sedation is required and there is no risk of bowel perforation. However, further examination is required in the form of a colonoscopy if anything abnormal is discovered and also smaller polyps or flat adenomas may be missed using this procedure (Winawer *et al.*, 2000). The proportion of cancers within reach of sigmoidoscope has fallen to <50% within the last 20 years with tumours now tending to have a more proximal location (Spence and Johnston, 2001). This trend could have serious implications as a sigmoidoscope is often the first diagnostic test carried out and if cancers are being missed at this stage there is the possibility of patients being misdiagnosed. This shows the urgent need for a non-intrusive test such as the FOBT but with lower levels of both false positive and negative results.

Early colorectal lesions may be evident as simple polypoid lesions, flat adenomas or depressed lesions. Tumour progression is not inevitable but the factors that govern this are not fully understood. Cancer of the colon is mainly sporadic or non-familial with around 5% due to inherited syndromes such as familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC), which are both discussed later. The adenoma-carcinoma pathway is not the only route to colorectal cancer; in ulcerative colitis associated neoplasm (UCAN), carcinomas arise in the absence of adenomas.

1.2.2 Risk Factors for colorectal cancer

Over 90% of colorectal cancers occur in patients over 50 years of age with the peak age of incidence occurring with people in their 70's, as can be seen in Figure 1.6 (Ionov *et al.*, 1993; Thibodeau *et al.*, 1993). Only 1-2% of sporadic colorectal cancer patients are under 36 but these patients may require more drastic surgical measures, such as total colectomy, than older patients as the chance of a later second colorectal cancer is much greater. Genetic testing for MMR gene mutations and genetic counselling for the patient and family are also particularly important in this subset of patients (Liu *et al.*, 1995a). Both males and females are almost equally at risk from this disease however rectal cancer is marginally more common in men (Hayne *et al*, 2001).

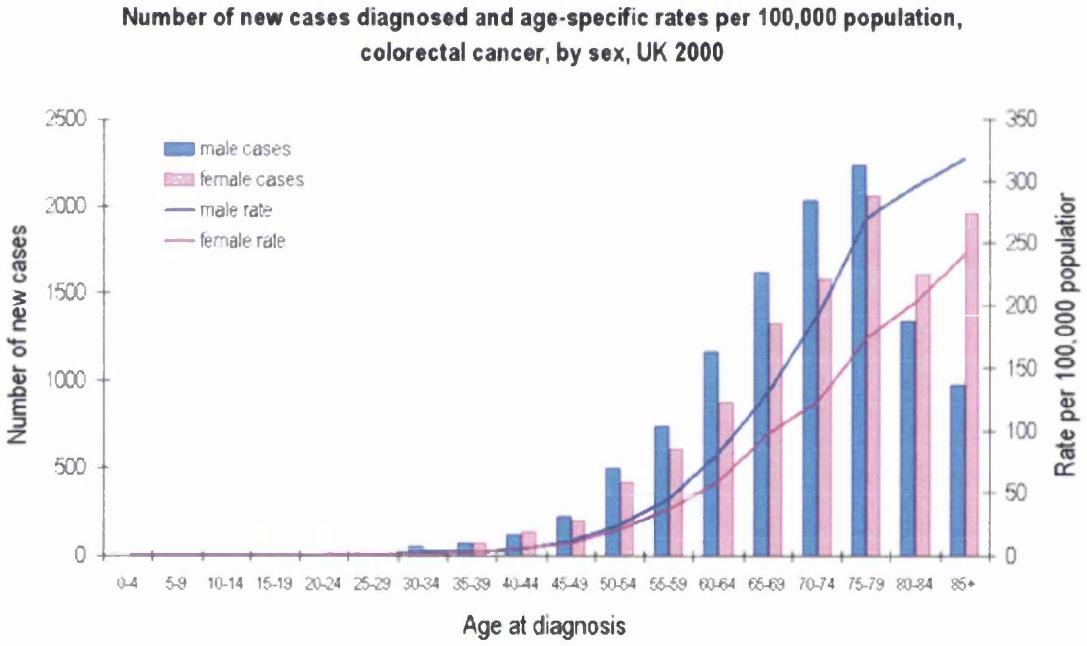


Figure 1.6

Graph showing the number of new cases and cancer rates of colorectal cancer in the UK. From www.cacnerresearchuk.org.

Studies have shown a positive correlation between mortality and deprivation (Boyle and Langman, 2000). In the United Kingdom the rates are highest in Scotland where the diet is generally higher in animal fats and red meats, which have been shown to increase the risk of colorectal cancer (Giovannucci and Willett, 1994; Sandhu, 2001). Lifestyle risk factors such as smoking and alcohol consumption have also been linked to a higher risk of developing carcinomas of the colon, whereas diets high in fibre, fruit, vegetables, calcium and folate help to lower that risk (Michels *et al.*, 2000; Lipkin *et al.*, 1999; Giovannucci *et al.*, 1998). Hormone replacement therapy (HRT) in postmenopausal women can also reduce colorectal cancer risk (Grodstein *et al.*, 1998). Fibre is thought to reduce colorectal cancer risk by diluting any carcinogens within the stools and by reducing the transit time in which they travel through the colon (Burkitt, 1971). Diet is thought to responsible for up to 80% of colorectal cancer (Bingham, 2000).

A study feeding rats and mice a Western-style diet for 12 weeks showed that a diet of low calcium and vitamin D as well as an increase in fat and phosphate cause colonic hyperplasia and hyperproliferation. These diets were devised by nutrient density equivalent to the dietary intakes of the U.S. population and were administered to the rodents without any additional carcinogen. As the study saw these changes in the colonic mucosa after such a short duration it is reasonable to consider that hyperplasia and hyperproliferation may be factors in tumour promotion and, based on epidemiological data, may contribute to eventual colonic neoplasia (Newmark *et al.*, 1990). Further experiments showed that with high calcium intake, hyperplasia and hyperproliferation in mice and hyperproliferation in rats was reduced to the level of control amounts despite the levels of fat, phosphate and vitamin D remaining unchanged from the first experiments (Newmark *et al.*, 1991). These experiments provide promising evidence that perhaps food supplements such as calcium and vitamin D can be used to lower the risk of colon cancer without reducing fat and phosphate intake which is an intrinsic part of the meat and processed foods rich Western-style diet. The gene for the vitamin D receptor is down-regulated in colorectal cancers and may explain the need for increased intake in these patients (Kitahara *et al.*, 2001).

Non-steroidal anti-inflammatory drugs, (NSAIDs), reduce the risk of colorectal cancer as proved by epidemiological studies and data from murine models. There is a decreased death rate for colorectal cancer associated with aspirin use; the size and number of tumours are reduced by the NSAID sulindac in patients with the hereditary disease familial adenomatous polyposis (FAP). These drugs inhibit cyclo-oxgenase (COX) genes, which results in a decrease in prostaglandin production (reviewed in Prescott and White, 1996). NSAID derivatives that lack ability to inhibit COX also inhibit tumour growth, which demonstrates that there are other additional cellular targets of NSAIDs (He *et al.*, 1999).

Chemical carcinogens such as those in the diet often cause transversion mutations and have signature mutations, but these have not been found in colorectal cancers implying that the mutations arise spontaneously and that diet affects tumour development by another mechanism. It is though more likely that the signature mutations are not seen as there are many different chemicals acting and a distinct pattern cannot be traced to any specific mutagen (Ilyas *et al*, 1999; Greenblatt, 1994).

Another lifestyle factor that affects the relative risk of developing colorectal cancer is the level of physical activity. People with sedentary occupations are more at risk of developing colorectal cancer than those whose jobs are more physically demanding (Mehigan and Monson, 2000).

Colonic flora have also been associated with colorectal cancer. The colon is host to a large variety of anaerobic bacteria which live within the colon, many of which are essential for fermentation of carbohydrates and also help to lower colorectal cancer risk by increasing stool mass by the increase in microbial biomass (Bingham, 1996). Conversely, *Escherichia coli* and a virulent variant of *Helicobacter pylori* have both been linked with an increase in colorectal cancer risk (Swidsinski *et al.*, 1998; Shmuely *et al.*, 2001). All the factors discussed here that influence colorectal cancer risk are summarised in Figure 1.7.

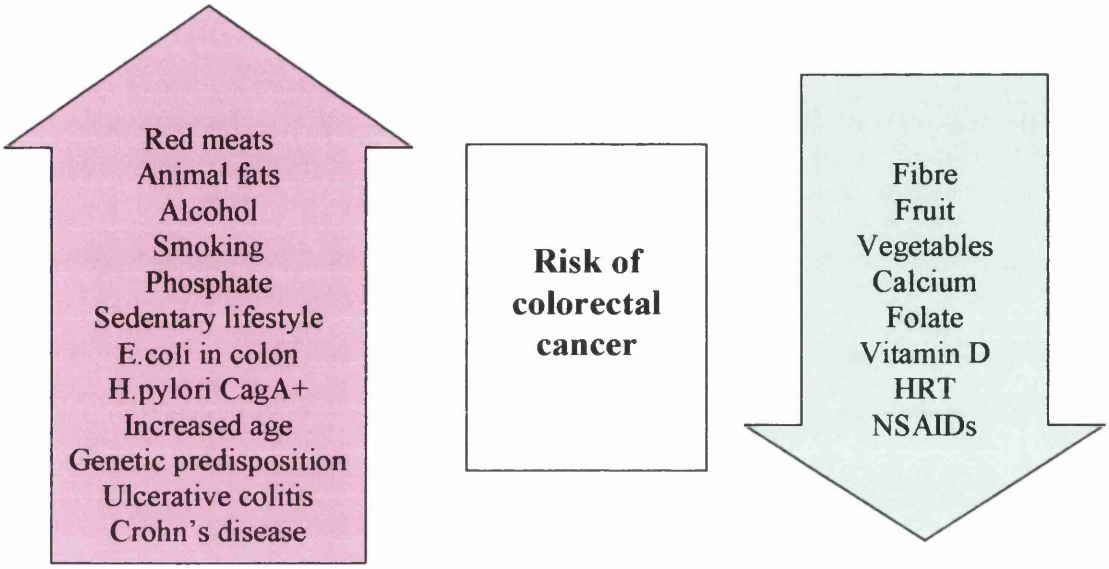


Figure 1.7

This diagram summaries the factors which increase (in the red arrow) and decrease (in the green arrow) the risk of colorectal cancer.

1.2.3 Genetic pathways of colorectal carcinogenesis

The study of colorectal cancer has been facilitated as all the stages of colorectal cancer from normal mucosa to metastatic disease can often be studied from a single patient and as a result many of the details of the genetic pathways of colorectal cancer have become established. Vogelstein and Fearon published the first proposed pathway of colorectal carcinogenesis in 1990 outlining the genetic changes which occurred as tissue changed from normal colonic epithelium through the adenoma stage through to carcinoma, see Figure 1.8. Since this paper there have been many additions and alterations to this pathway proposed with, for example, colorectal carcinomas arising from mismatch repair deficient cells following an alternative pathway than the majority of colorectal cancers.

The mismatch repair pathway is further discussed in Chapter 4, section 4.1.5. These pathway diagrams are often termed Vogelgrams after the author of the first proposed pathway.

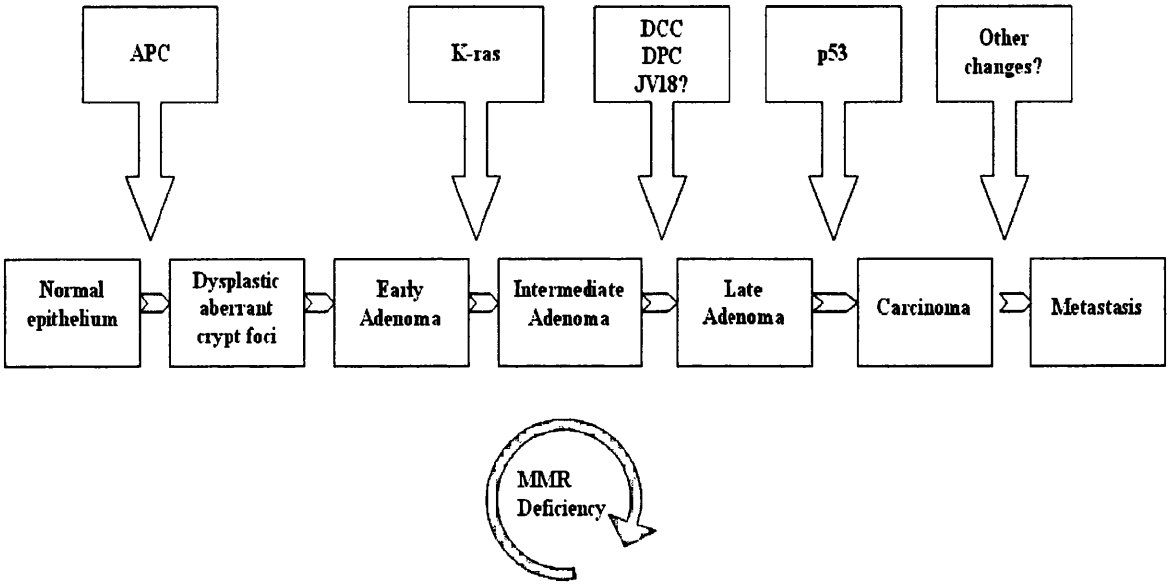


Figure 1.8

This diagram is a copy of the first proposed pathway of colorectal carcinogenesis published in 1990 by Vogelstein and Fearon. At the time of publication chromosome 18q21 was identified as key in the progression from intermediate adenoma to late adenoma but it was not known which of the genes on this chromosome region, deleted in colorectal cancer (DCC), deleted in pancreatic cancer (DPC) or JV18-1 were involved.

As more research is carried out these pathways can become more detailed and alternative pathways become substantiated by evidence. Detailed clinical studies have also enabled the classification of tumours into these different pathways by various features. For example, colorectal cancers can be divided into two different groups, mucinous and non-

mucinous, according to the presence or absence of secretory mucins in the carcinoma tissue. These different phenotypic groups of colorectal carcinoma are thought to arise via different genetic pathways as mucinous tumours have a higher frequency of K-ras mutations and a low level of p53 mutations as well as other differences such as overexpression of the tumour suppressor p21 and the intestinal mucin coding gene MUC2 (Hanski, 1995; Backert *et al.*, 1999).

The study of hereditary colorectal cancer has also provided information not only of these diseases but also of the sporadic cases which account for the majority of colorectal cancer cases.

1.3 Hereditary Colorectal Cancer Diseases

1.3.1 Hereditary non-polyposis colorectal cancer (HNPCC)

Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant disease responsible for approximately 5% of colorectal carcinomas in Western society. HNPCC is the most common form of heritable cancer. Patients have a germline mutation in one of several mismatch repair genes. 98% of the mutations are in the hMSH2 or hMLH1 genes with hPMS2 mutated in most of the remaining cases (Liu *et al*, 1996). The germline mutation gives a heterozygous phenotype, a later somatic mutation in the other copy of the gene results in a loss of heterozygosity. The result is a defective mismatch repair system that gives rise to microsatellite instability. Tumours formed due to this are termed replication error positive as they show major microsatellite instability that reflects the deficiency of mismatch repair. These tumours are predominantly found on the right side of the colon. The patient may also develop cancers in the small intestine, stomach, ovary, bladder and endometrium.

Around 60% of HNPCC adenomas have high levels of MSI (MSI-H) (Hamilton and Aaltonen, 2000). In adenomas with microsatellite instability, 50% were found to have mutated copies of the APC gene. This is a similar spectrum to that found in FAP and

sporadic colorectal cancers. The β-catenin gene was mutated in 14% of the adenomas and has been shown to be associated with microsatellite instability (Akiyama *et al*, 2000). TGF-β RII also has a crucial role in HNPCC tumourogenesis.

The majority of cases are diagnosed when the patients are in their 40's. Unlike FAP and sporadic colorectal cancers which affects males and females equally, in HNPCC the sex ratio of patients is 1.5:1 males: females. Despite the name of the disease, HNPCC patients do have polyps, up to 10-20, but these are present at far lower levels than patients with the heritable condition FAP who have hundreds to thousands of polyps.

The Amsterdam criteria were established for the clinical identification of HNPCC. The revised diagnostic criteria known as Amsterdam criteria II state that there should be at least three relatives with an HNPCC-associated cancer: colorectal cancer, or cancer of the endometrium, small bowel, ureter or renal pelvis. One patient should be a first degree relative of the other two, at least two successive generations should be affected, at least one tumour should be diagnosed before age 50, FAP should be excluded in the colorectal cancer cases and tumours should be verified by histopathological examination (Hamilton and Aaltonen, 2000).

1.3.2 Familial adenomatous polyposis (FAP)

Familial adenomatous polyposis (FAP) is an autosomal dominant genetic disease with an incidence of 1/7000. It accounts for less than 1% of colorectal cancers in the United States (Navaratnam *et al*, 1999). Patients with FAP have a germline mutation in the APC gene that leads, after further mutations of other genes, to profuse polyposis of the colon with, by definition, >100 polyps. The polyps themselves are benign but as they often number into their 1000s it is an almost certainty that some will progress into carcinomas. Mutations at the 3' region of the APC gene gives attenuated phenotype with fewer polyps and a later onset of cancer (Brensinger *et al*, 1998; Spiro *et al*, 1993). For FAP patients there is an almost 100% chance of developing colorectal cancer by the age of 40yrs although the onset of cancer occurs in most patients between 10-30yrs (Mehigan and

Monson, 2000). The rate limiting step in the initiation of tumours in FAP patients is the somatic mutation of the wild type APC allele inherited from the non-infected parent (Levy *et al*, 1994). Unlike HNPCC where tumour progression is accelerated, in FAP it is the initiation of tumours that is increased (Lynch *et al*, 1996). The treatment for the disease is often a total colectomy. Other manifestations of the disease include retinal lesions, desmoids of the skin, brain tumours and benign bone tissue tumours called osteomas.

1.3.3 Ulcerative colitis associated neoplasm (UCAN)

Ulcerative colitis associated neoplasm is another route to colorectal cancer. Ulcerative colitis is a condition in which the lining of the colon becomes inflamed and develops ulcers. Patients with ulcerative colitis are offered regular screening colonoscopies as they have a higher risk of developing colorectal cancer which increases according to the length of time the disease has been present up to a 18% increased risk after 30 years (Eaden *et al.*, 2001). UCAN is much less common and follows a different pathway than the previously mentioned diseases. K-ras mutations in UCAN tumours are half as frequent as they are in sporadic cases (Redston *et al.*, 1995; Benhattar and Saraga, 1995). Also, p53 allele loss occurs in most dysplasias preceding the appearance of cancer (Brentnall *et al.*, 1994). Crohn's disease is another condition in which the lining of the colon becomes inflamed and again carries an increased risk of colorectal cancer (Gillen *et al.*, 1994).

1.4 General aims of this thesis

The mortality associated with colorectal cancer is extremely high, however if the disease is caught earlier at Dukes' stage A then there is a 95% 5 year survival. As well as increased awareness of colorectal cancer, it's symptoms and risk factors, an improved screening technique which is less invasive than current methods such as colonoscopy may increase uptake by the public. Also, an increase in knowledge of the different genetic pathways of colorectal carcinogenesis may help to tailor treatments to individual patients.

The experiments described in this thesis address these aims by searching for possible biomarkers which may be detectable in stool samples and by looking for early genetic alterations at the molecular, gene expression and chromosomal levels.

1.5 Hypothesis

By researching the pathways of colorectal cancer, potential molecular markers for the detection of early stage cancers may be identified.

Chapter 2
General materials and methods

2.1 General technical information

All research was performed wearing suitable safety clothing including sterile powder-free latex gloves (Diamond grip plus, Microflex, Vienna, Austria). All surfaces were cleaned using 70% ethanol prior to each experiment. Filter tips and microcentrifuge tubes were obtained from Starlab (UK) Ltd (Milton Keynes, UK). All glassware, plastics and solutions were sterilized either by autoclave or filter sterilization and all water used was deionised, purified water (Milli-Q plus PF, Millipore). This chapter contains general materials and methods used, for the specific use of each technique there is more detail in the relevant chapter.

2.2 Sample collection

Ethical consent was gained for the samples to be taken for research. Informed consent was then obtained from each patient wishing to participate prior to either endoscopy or surgery. Samples were taken either from Mr. J. Beynon's endoscopy clinic or from tissue removed from surgery, both at Singleton Hospital, Swansea. Biopsies from endoscopy clinic were divided into two parts with a sterile scalpel with one part taken to the pathology department for diagnostics and the remaining tissue available for this research. For the tissue taken from surgery these samples were taken to the pathology department where the consultant pathologist opened up the specimen, washed the surface of the colorectum with water, dried the specimen by blotting with disposable towels and provided advice from where to sample the tissue. Samples in all cases were taken from the colorectal tumour as well as normal mucosa adjacent to the tumour and distant to it. If the carcinoma was large enough, samples of the tumour from a central location and the periphery of the tumour were collected. Where possible, samples were also taken of polyps and of lymph nodes where these were suspected to be metastatic. At a later date the pathology reports on the samples were

received after Dr. Varsha Shah of the histopathology department, Singleton Hospital, Swansea, had carried out the histology on the remaining tissue.

The samples were taken differently depending on the further experiments to be carried out. For DNA extractions the samples were placed inside sterile bijoux tubes; for RNA extractions the samples were thinly sliced and placed immediately into RNALater completely immersing the tissue and gently agitated to ensure the slices were broken up to allow penetration of the RNA preservative. For FISH, brushings were taken using cytology brushes which then had the brush end cut off and immersed in 10ml ETN buffer (0.1M EDTA, 0.01M Tris-HCl, 0.02M $NaCl_2$). All samples were placed on ice immediately. On return to the laboratory the tissues were kept at -20°C and samples in RNA later kept at 4°C until use. The brushings were processed immediately as described below.

2.2.1 Brushings

The cytology brushes were immersed in 10 ml ETN buffer and the cells then suspended into the buffer by vortexing. The brush was then removed and the cells spun down at 9000 x g for 10 minutes. The supernatant was discarded and cells re-suspended in a further 10ml ETN prior to centrifugation at 9000 x g for 10 minutes. To ensure the cells were properly washed, this step was repeated once more. The cells were then re-suspended in between 0.5ml and 1ml ETN depending on pellet size and 100µl applied to a cytospin and spun onto coded slides at 9000 x g for 8 minutes. After air-drying the cells were fixed in 90% methanol at -20°C for 10 minutes. The slides were then air dried and stored at -20°C until use.

2.3 DNA extraction

2.3.1 Fresh tissue sample disruption for isolation of genomic DNA by phenol/chloroform extraction

Between approximately 6mg and 200mg of colonic tissue was used for each DNA extraction depending on sample size available. The tissue was suspended in 10µl of

STE buffer (0.1M NaCl, 0.05M trizma base, 0.01M EDTA) and dissected into small pieces using a scalpel on a glass slide prior to homogenisation. This aided the break down of the cells and was especially important for the mucosa samples, which were more difficult to homogenise given the elastic property of this tissue type. The sample was then transferred to a glass (Dounce) homogeniser with 300µl STE and was thoroughly homogenised. The homogenate was then added to 200µl STE, 100µl SDS (10g/100ml lauryl sulfate) and 40µl proteinase K (100µg/ml) prior to incubation at 55°C for 1h under continuous agitation. Total genomic DNA was isolated from the tissue samples by either phenol-chloroform extraction or using a high salt DNA extraction kit (Stratagene).

2.3.2 DNA extraction - Phenol/chloroform

For each sample, 600µl phenol was added and shaken to form an emulsion. The organic and aqueous fractions were separated by centrifugation for 5 minutes at 10,000 x g and the latter phase transferred to a fresh tube taking precaution not to draw any cellular debris with the fluid. To this was added 300µl phenol and 300µl chloroform and the process repeated. Following this the procedure was repeated using 600µl of chloroform. The nucleic acid was recovered by precipitating with 600µl iso-propanol. The nucleic acid was centrifuged and supernatant discarded prior to washing with ice cold 70% (v/v) ethanol. The washed nucleic acid was then centrifuged and air-dried in an incubator at 37°C for 1h. The DNA was resuspended in 100µl sterile H_2O and stored until use at 4°C.

2.3.3 DNA extraction from tissue samples - high salt extraction

DNA was extracted using the Stratagene DNA extraction kit. Between approximately 6mg and 200mg of colonic tissue was used for each DNA extraction. The tissue was dissected into small pieces using a scalpel on a glass slide prior to homogenisation. The sample was then transferred to a 15ml glass homogeniser (Wheaton, USA) and was homogenised in 7ml lysis buffer (10mM Tris-HCl, pH 8.2, 400mM NaCl, 2mM EDTA). The resulting cell suspension was transferred to a 15ml centrifuge tube and 100µg/ml pronase (Stratagene) added before incubation, with shaking, at 37°C

overnight to fully lyse the cells. The samples were then chilled on ice for 10 minutes. 2.5ml of 6M NaCl was added and incubated on ice for a further 5 min to precipitate the proteins. This precipitate was pelleted by centrifugation for 15min at 8000 x g after which the supernatant containing the nucleic acids was transferred to a sterile 0.5ml centrifuge tube and incubated with 20µg/ml RNase (Stratagene) for 15 min at 37°C. DNA was precipitated an equal volume of iso-propanol, DNA was centrifuged and supernatant discarded prior to washing twice with ice cold 70% (v/v) ethanol. The washed nucleic acid was then centrifuged and air-dried in an incubator at 37°C for 1h. The DNA was resuspended in 100µl sterile H_2O and stored until use at 4°C.

2.3.4 DNA quantification

DNA concentration was determined using a Beckman DU-65 spectrophotometer. DNA was diluted with H_2O before adding to a 100µl 1cm quartz cuvette. Readings of optical density were taken at 260nm to calculate DNA concentration (µg/ml) using the equation:

DNA concentration (µg/ml) = optical $density_{260nm}$ x spectrophotometric conversion x dilution factor x pathlength

DNA concentration (µg/ml) = A_{260} x 50µg/ml x dilution factor x 1

DNA solutions were diluted to a working concentration of 0.1µg/µl and stored at 4°C. The remaining stock DNA solutions were stored at –20°C.

2.4 Polymerase Chain Reaction

The polymerase chain reaction (PCR) reaction mixtures were set up in thoroughly cleaned laminar flow hoods (MDH) in a dedicated PCR laboratory. Pipettes (Finn pipette, Thermo Life Sciences) solely for PCR were used with filter tips to prevent contamination. 200µl thin walled tubes were used for all reactions to ensure a rapid temperature change within the vessel. All tips and tubes used were autoclaved and opened only within the flow hoods. Racks and pipettes were cleaned with 70% ethanol prior to use.

The PCR reaction mixture consisted of 5µl reaction Buffer A (50mM KCl, 10mM

Tris-HCl pH 9, 0.1% (v/v) Triton®X-100, Promega), 3µl $MgCl_2$ (25mM, Promega), 1µl of each dNTP (200µM, Promega), 20pmol of each primer, 1µl DNA, 1 unit *Taq* DNA polymerase (Promega) and filtered sterilized deionised H_2O to a final volume of 50µl. Reaction mixtures were briefly vortexed to ensure uniform distribution within each tube and centrifuged for 30 seconds prior to thermal cycling (PTC-225 Peltier thermal cycler, MJ Research).

The PCR parameters used were denaturation at 94°C for 2 min to ensure complete denaturation of the template DNA followed by cycles of denaturation at 94°C for 2 min, primer annealing at 59°C for 30 seconds and primer extension at 72°C for 1 min. For the last cycle, a further 5 min stage at 72°C occurred to ensure full double-stranded molecules had been produced. Cycle numbers were optimised for each set of primers to prevent the production of ambiguous PCR products that suggests the plateau phase of PCR has been reached. Optimisation of this PCR protocol in terms of $MgCl_2$ volume, primer annealing temperature, annealing and extension time and cycle numbers for each primer set, as well as primer sequence can be found in the relevant chapters.

2.5 Electrophoresis

2.5.1 Polyacrylamide gel electrophoresis

The PCR products were primarily checked by running on a 6% polyacrylamide gel. Each gel was prepared using 3.75ml 10 x TBE buffer (0.09M trizma base, 0.09M boric acid, 0.002M EDTA), 7.5ml liquid polyacrylamide (acrylamide: bisacrylamide [29:1], NBL Gene Sciences) diluted in 27.25ml deionised H_2O. The solution was vacuum degassed for 5min prior to polymerisation with 110µl APS (10g/100ml ammonium persulfate) and 22.5µl TEMED (Life Technologies). The solution was poured into a glass gel cast (Protean III gel system BIO-RAD), a 15 tooth gel comb inserted and allowed to set at room temperature for a minimum of 30 minutes. The gels were loaded in designated wells with 7µl PCR products mixed with 2µl loading buffer (1% v/v bromophenol blue, 0.1M EDTA, 1% v/v SDS, 50% v/v glycerol). The samples were run alongside a marker consisting of 1µl 100bp DNA ladder (Promega), 2µl loading buffer and 5µl water. Gels were placed in vertical electrophoresis tanks

(BIO-RAD) filled with 1 x TBE buffer. Each gel was run at a consistent 140V supplied by a power pack (BIO-RAD, Power Pac 300) and electrophoresis was stopped after 35min when the loading dye was close to the end of the gel.

2.5.2 Agarose gel electrophoresis

3% agarose gels were made by melting 3g of agarose into 100ml TBE in a microwave set at medium power until the agarose had just melted and was mixed by gentle swirling taking care not to incorporate air bubbles. This solution was then poured into gel moulds, a ten tooth well comb inserted, covered to prevent contamination and left to set for 40 min. These gels were run on horizontal gel electrophoresis tanks at 100V for 2 hr or until the loading dye had run a sufficient length.

2.5.3 Silver staining

For non-quantative staining the gels were silver stained. Each gel was placed in 0.1% silver nitrate (Sigma) for 5 min, washed twice with H_2O and then incubated for 5 min in 1.5% sodium hydroxide (Fisher) and 0.15% formaldehyde (Sigma) to develop the gel and visualise the PCR products and marker lane.

2.5.4 SYBR gold staining

The fluorescent stain SYBR gold (Molecular Probes) was used to visualise the DNA bands. The polyacrylamide gels were incubated in the stain (10µl SYBR gold, 100ml 1 x TBE buffer) in the dark at room temperature for 1hr.

2.5.5 Image capture of gels

Images of gels once stained were obtained using the GelDoc 2000 (BIO-RAD) system. Silver stained gels were illuminated using white light and focused manually for clarity. Images of SYBR gold stain gels were captured under UV light to excite the stain bound to the DNA. Images were saved to computer and hard copies produced using heat sensitive printer paper by a thermal printer (P91, Mitsubishi, Japan).

2.6 PCR purification

PCR products were purified using a QIAgen PCR purification kit (QIAgen) to remove excess primer, free nucleotides and short DNA fragments less than 40bp from the PCR products. The kit was used as instructed by the manufacturer using a microcentrifuge. 200µl buffer PB was added to 40µl of PCR product, the sample mixed and applied to the QIAquick column to bind the DNA to the silica-gel membrane. The column was centrifuged for 1 min at 11,000 x g and the flow through discarded. The membrane was washed with 0.75ml buffer PE and centrifuged for 1 min at 11,000 x g, the flow through was discarded and the column centrifuged again for a further 1 min at 11,000 x g to remove any traces of the wash buffer. The column was transferred to a new 1.5ml microcentrifuge tube and the DNA eluted by adding 30µl H_2O directly to the membrane, incubated at room temperature for 1 min prior to centrifugation for 1 min at 11,000 x g. The resulting purified PCR product was stored at 4°C until use.

2.7 Single Stranded Conformational Polymorphism (SSCP)

2ml of deionised formamide (Sigma) and 2ml of loading buffer was added to 2ml of each purified PCR product, these were well mixed and heated to 95°C on a thermocycler for 10 minutes. After this they were immediately taken from the thermocycler and pushed into ice and put into a freezer at -20°C for 10 minutes. The samples were then loaded into wells of a 10% polyacrylamide gel; loaded gels were then placed in vertical electrophoresis tanks (Hoeffer SE 660) filled with 1 X TBE. Each gel was run at 140V supplied by a power pack (Pharmacia, EPS 3500XL) for 16 hours at 4°C. The gels were then stained with silver staining and the image captured on the gel doc system as described in sections 2.5.3 and 2.5.5 respectively.

2.8 DNA Precipitation

Purified PCR products were precipitated using 3µl 3M sodium acetate and 75µl ice-cold ethanol per 30µl sample. After incubation for 30 min at –20°C the DNA was pelleted by centrifugation for 15min at 10000 x g at 4°C using a desktop centrifuge

(Micromax, International Equipment Company, USA). The supernatant was carefully discarded and the remaining DNA pellet washed for 15 min in 70% ethanol on a cell washer (CM4400, Voss). The washed DNA was centrifuged for 15min at 10000 x g at room temperature, the supernatant discarded and the pellet dried in an incubator (Forma Scientific) at 37°C for 1 hour. The DNA was then resuspended in 10µl H_2O.

2.9 Sequencing

DNA concentration of the purified precipitated PCR products was determined as described in section 2.3.5. The sequencing reaction was prepared on ice with all reagents stored in a cold block until use. Each sequencing reaction contained deionised H_2O to a total volume of 20µl, 0.5µl-10µl DNA template, 2µl of oligonucleotide primer at 1.5pmol/µl and 8µl quick start master mix (Beckman Coulter). The sequencing reactions in each 200µl thin-walled tube (ABgene) were mixed thoroughly by pipetting and briefly centrifuged prior to thermal cycling (PTC-225 Peltier thermal cycler, MJ Research). 30 cycles of thermal cycling occurred each consisting of 20 seconds at 96°C, 20 seconds at 50°C and 4 min at 60°C. The resulting product was ethanol precipitated, purified and sequenced on a Beckman CEQ 8000 sequencer. Several samples were sequenced in the Beckman CEQ 8000 sequencer in the laboratory; however, due to technical problems the majority were sequenced by Oswel Research Products Ltd (UK) using ABI 377 96 lane fluorescent DNA sequencing technology.

2.10 Fragment analysis

Labelled fragments were generated by carrying out PCR with primers labelled with WellRed dye (Invitrogen). During preparation all PCR reagents were kept on ice. The resulting labelled fragments were diluted 1:10 with SLS (sample loading solution) and, depending on the efficiency of the PCR reaction, 1µl – 5µl of this was added to 40µl SLS and 0.125µl of WellRed dye1PA (red) labelled CEQ DNA size standard - 400 (Beckman). These were well mixed and applied to a 96 well plate and a drop of sterile mineral oil (Beckman) added to prevent evaporation. The prepared samples were loaded into and run on the CEQ 8000 using separation method "frag 3" for fragments of size range 60-400bp. As the primers for the different MSI markers had

been labelled with different coloured WellRed dyes it was possible to multiplex these samples in a single well. For these samples an equal volume of each labeled PCR product was added to the SLS and size standard with the exception of the products labelled with the blue dye as this has a stronger signal. For these products around a third less PCR product was added to the mix to give a balanced signal intensity.

2.11 RNA extraction and quantification

2.11.1 RNA extraction

The fume hood, used solely for the extraction of RNA, and all equipment to be used was thoroughly cleaned using RNaway to remove RNase. RNA extraction was carried out using a QIAgen RNeasy extraction kit with the supplied protocol slightly modified for optimal RNA yields from fresh tissue samples. The samples were dissected into small pieces using a sterile scalpel on a clean slide. These tissue pieces were then transferred into 2ml microcentrifuge tubes and 1.5ml Trizol added prior to homogenisation with rotor-stator homogeniser to disrupt and homogenise the tissue. The resulting cell suspension was then incubated at room temperature for 5 minutes. 300ml chloroform was added and shaken vigorously for 15 seconds prior to incubation at room temperature for 3 minutes. This was then centrifuged at 9000 x g for 15 min at 4°C. The aqueous phase was transferred to a clean 2 ml microcentrifuge tube. 1 volume 70% etOH was added and mixed immediately. The sample was then applied to RNeasy midi column and centrifuged for 5 minutes at 9000 x g. The flow through was discarded, 700µl RW1 reagent was applied and left to incubate at room temperature to stand for 5 minutes prior to centrifugation for 15 seconds at 9000 x g. The column was then placed in a new tube and the column washed twice with 500µl RPE reagent followed by centrifugation for 2 minutes at 9000 x g. The column was transferred to a new 1.5ml lockable tube and the RNA was eluted by applying 30µl H_2O to the column and leaving to stand for 10 minutes prior to 1 min centrifugation at 9000 x g.

2.11.2 Quantification of RNA

RNA concentration was determined using a Beckman DU-65 spectrophotometer. RNA was diluted with H_2O before adding to a 100µl 1cm quartz cuvette. Readings of optical density were taken at 260nm and the concentration calculated using the equation:

RNA concentration (µg/ml) = optical $density_{260nm}$ x spectrophotometric conversion x dilution factor x pathlength

Concentration of RNA sample (µg/ml) = A_{260} x 40 x dilution factor x 1

To establish the purity of the RNA the samples were diluted in 10mM Tris-Cl, pH7.5 buffer, which is more sensitive than water in detecting protein contamination. Readings of optical density were taken at 260nm and 240nm and a A_{260}/A_{240} ratio calculated to determine the quality of the extracted RNA in terms of purity with respect to contaminants such as protein, which absorb UV.

2.12 Arrays

2.12.1 Array hybridization

GEArray Original Series Human Cancer/Tumour Suppressors Gene Array nylon membrane arrays were obtained from SuperArray and were used as directed by the manufacturer's instructions. The arrays were composed of 23 tumour-suppressors, two housekeeping genes β-actin and GADPH as well as bacterial plasmid pUC18 as a negative control. The gene list can be found in table 5.1. The images were captured using the ChemiDoc system from Bio-rad and the array spot intensities measured using the GEArray software package.

2.12 Array analysis

The first stage of the analysis is a consolidation stage. As each gene had at least 2 spots per array, a mean value for these intensities was produced using GEArray

software. Analysis was then undertaken to allow meaningful comparisons between array experiments to be made. The first stage of this analysis is the removal of background noise levels. pUC18 is a bacterial plasmid included on the array as a negative control as there should be none of this RNA present in human tissue samples. The areas on the array for pUC18 confirm there has been no contamination of the array or sample and the intensity level at these spots are used in the array analysis to subtract background noise from the intensities of the cDNA spots. Following the subtraction of background noise, the arrays are normalised so that arrays can be compared. This is carried out by dividing the spot intensities by the average intensity of the constitutively expressed housekeeping gene β-actin. This allows analysis to be undertaken between arrays of, for example, normal mucosa and polyp samples. Ratios were calculated of the expression level of, for example, polyp tissue, divided by the expression level of the normal mucosa. The data were then $\log_2$ transformed to correct for gene expression ratios which give down-regulated genes lower ratios than up-regulated genes which have altered by the same fold difference (Quackenbush, 2002). This treatment of the data gives genes which are up-regulated by a factor of 2 a $\log_2$ ratio of 1 and genes down-regulated by a factor of 2 have a $\log_2$ ratio of -1. Genes which do not alter in expression have a $\log_2$ ratio of 0. Fold changes in gene expression are calculated by taking the anti-$\log_2$ of the absolute value of the $\log_2$ ratio. This is either an increase or decrease in expression change according to the positive or negative value of the $\log_2$ ratio respectively.

2.14 FISH

2.14.1 Pepsin treatment of slides

Slides were treated with pepsin to digest the cellular proteins in the cells to allow the FISH probes and DAPI II to penetrate the nuclei of each cell. The pepsin (300µl/ml 0.01M HCl pH 2.8) (Sigma) and slides were preheated to 37°C prior to application of pepsin for between 0.5 and 7 minutes at 37°C depending upon the cell density on each slide. The pepsin was deactivated by washing the slides for 5 min in phosphate buffered solution (PBS) followed by 5 min in PBS/50mM $MgCl_2$ at room temperature. A wash for 2 minutes in 2 x SSC followed. The slides were then

dehydrated by washing in an ethanol series consisting of 70%, 80% and 95% etOH for 2 minutes in each. The slides were air dried prior to storage until use at -20°C.

2.14.2 FISH with Abbott probes

Commercially available spectrum green labelled centromeric probes for chromosome 6 were purchased from Abbott. Slides were prepared as described above and warmed to 37°C on a slide warmer (Cytocell EC3-133, JLS Ovens Ltd, Birmingham, UK). A probe mixture was made in a black microcentrifuge tube with 3.5µl hybridisation buffer, 1µl H_2O and 0.5µl probe per slide. 5µl of this mixture was then added to the cytodot on each slide and covered with a 1.5cm x 1.5cm plastic coverslip (Oncor). To hybridise the probe with the DNA of the cells, the slides were placed on a covered hotplate at 73°C for 2 minutes and then hybridized in a lightproof humidity chamber in an incubator at 37°C for 30 minutes. The slides were then washed in 0.1% SSC at 73°C for 2 minutes followed by a wash in 2xSSC with 0.3% Nonident P40 for 30 seconds. Slides were air dried and counterstained with 10µl DAPI II (125ng/ml) and the anti-fade p-phenylenediamine in PBS and glycerol (Vysis, UK) for 10min prior to viewing.

2.14.3 Scoring

FISH slides were viewed using an Olympus BX 50 epifluorescence microscope and images captured using a charge-coupled device (CCD) camera operated using Mac-Probe 4.1 software (Applied Imaging, UK). Slides were numerically coded without reference to the histology of the sample to eliminate any bias on scoring. For each sample, 400 complete nuclei were scored, depending upon slide quality this was between 1 and 5 slides per sample. This was then repeated to give a minimum total of 800 nuclei scored per sample. Only clearly defined complete, evenly stained nuclei were scored to avoid including any partial or overlapping nuclei. Nuclei with no clear probe signal were not scored to ensure only sufficiently hybridized nuclei were scored.

2.14.4 The selection and amplification of clones

Clones were obtained from Children's Hospital Oakland Research Institute (CHORI). The clones acquired were RP1-108K11, RP3-431A14 and RP11-52A1. The clones were shipped as bacterial LB agar stab cultures. The plasmids were harboured in DH10 *E.coli*. These host cells were grown in the presence of 20µg/ml chloramphenicol for bacterial artificial chromosome (BAC) clones and 25µg/ml kanamycin for P1 artificial chromosome (PAC) clones to select for *E.coli* that had taken up the plasmids. Upon receipt, the bacteria were inoculated into universal tubes containing 15ml of LB medium containing the relevant antibiotic at the above concentrations and grown overnight in a shaking incubator at 37°C. Stocks of the transformed bacteria were frozen at -85°C after the addition of 25% glycerol. Remaining cultures were then used to harvest plasmids containing the clones.

2.14.5 Plasmid extraction

To isolate the plasmids, two overnight 15ml cultures for each clone were spun down at 9000 x g for 15 minutes and the pellets pooled prior to plasmid extraction using the QIAgen spin miniprep kit. The kit was used as per the manufacturer's instructions using the recommended amendments for larger plasmids. The pellets were resuspended in 500µl Buffer P1 containing RNase and transferred to a 2ml microcentrifuge tube. 500µl Buffer P2 was added and the solution gently mixed by inverting the tube until the solution became viscous and slightly clear. In all cases this was within 5 minutes. 700µl Buffer N3 was added and the solution thoroughly mixed by inverted the tube repeatedly. The cellular debris was separated from the solution by centrifuging for 10min at 11,000 x g and the supernatant removed and applied to the QIAprep column. This was the centrifuged for 1 min at 10,000 x g and the flow through discarded. The column was then washed with 750µl Buffer PB and centrifuged for 1 min at 10,000 x g. This stage was necessary to remove the nuclease activity of the DH10 *E.coli*. The flow-through was discarded and the column centrifuged for a further 1 min at 10,000 x g to remove any remaining traces of wash buffer. The column was placed in a fresh sterile microcentrifuge tube. To elute the vector 30µl H_2O at 70°C was added to the column and left to stand at room temperature for 1 min before centrifuging at 10,000 x g for 1 min. A further 30µl of

H_2O at 70°C was added and left to incubate at room temperature for 5min prior to further centrifugation at 10,000 x g for 1 min. The extracted vector was then quantified by spectrophotometry as described in section 2.3.5.

2.14.6 Nick translation

To create the FISH probe for the region around p21, nick translation was carried out to incorporate fluorescent labelled nucleotides into the DNA from the extracted vector. This was done using the nick translation kit from Invitrogen. For each reaction 5µl dNTP (-dTTP), 0.5µl ChromaTide^{TM} (red) labelled dUTP (1mM, Molecular Probes), 1µg DNA from plasmid, H_2O to a volume of 50µl, 5µl DNase/ DNA polymerase mix were added in that order to thin walled tubes on ice. The reagents were mixed by pipetting and briefly centrifuged. The reactions were then incubated at 15°C for 1-3 hours after which 5µl stop buffer was added to halt the reaction. Products were then run on 3% agarose gels, as described in section 2.5.2, to assess the size of nick translation products which, for optimal results, should be a range of fragments 200-800bp in length. The fragments were then purified using a QIAgen PCR purification kit as described in section 2.6 and quantified as described in section 2.3.5.

2.14.7 Hybridisation Mix

Hybridisation mix was made in a batch for 100 reactions to reduce any pipetting error from the viscous reagents used. This master mix contained 500µl formamide, 100µl 20 x SSC, 200µl dextran sulphate, 100µl 1% salmon sperm DNA and 100µl H_2O. The hybridisation mix was well mixed and divided out into 5 reaction (50µl) aliquots; this was stored at –20°C until use.

2.14.8 Making of the p21 probe

200ng of each of the three nick translation products were added to a 500µl microcentrifuge tube. 15µl human Cot-1 DNA (1mg/ml, Invitrogen), which is placental DNA, enriched for repetitive DNA sequences, was added to the clone DNA to suppress cross-hybridization. To precipitate the DNA, 1/10th of the volume of 3M sodium acetate and 2 volumes 100% ethanol were added, the mixture vortexed and

incubated for 3 min at room temperature. To pellet the DNA the mixture was centrifuged at 11,000 x g for 10 min and the supernatant discarded. The remaining pellet was washed with 70% ethanol and centrifuged at 11,000 x g for 5 min and the supernatant discarded; this washing step was then repeated using 150µl ethanol. The remaining pellet was left to air dry in the dark. The pellet of probe was then resuspended in 10µl hybridisation mix.

2.14.9 FISH with probe made from clones

Slides were prepared as described in section 2.2.1 and treated with pepsin as in section 2.14.1. The p21 probe mixture denatured by heating to 72°C for 10 min in an agitated waterbath and incubated for 20 min at 37°C for the repetitive DNA sequences to pre-anneal. Slides were denatured in 70% formamide / 2 x SSC at 75°C for 5 min in an agitated waterbath. The slides were then quenched in an ice-cold ethanol series of 70%, 80%, 95% and 100% for 2 min in each. The slides were then left to air dry and warm in an incubator at 37°C. The denatured probe was added to the cytodot of nuclei on each slide and covered with a 1.5cm x 1.5cm plastic coverslip (Oncor). The slides were incubated in a lightproof humidity chamber in an incubator at 37°C overnight. The coverslip was then removed and the slides were then washed in 0.1% SSC with at 73°C for 2 minutes followed by a wash in 2xSSC with 0.3% Nonident P40 at room temperature for 30 seconds. Slides were air dried in the dark and counterstained with 12µl DAPI for 10 min prior to viewing.

Chapter 3

Detection of mutations in the mitochondrial genome control region using the single stranded conformational polymorphism technique

3.1 Introduction

3.1.1 Mitochondrion

The mitochondrion is a cytoplasmic organelle and the cellular site of energy production in all eukaryotes. With the exception of erythrocytes, they are present in all human cells. Mitochondria can occupy up to a quarter of the volume of the cytoplasm, where they produce 80% of all the energy required by the cell in the form of adenosine 5'-triphosphate (ATP) by the biochemical process of oxidative phosphorylation (OXPHOS) (Lodish *et al.*, 1995). This process also generates reactive oxygen species, which cause oxidative damage to lipids, proteins and nucleic acid molecules. The role of the mitochondria is not limited to energy production, mitochondria are also responsible for executing the process of programmed cell death, known as apoptosis (Singh 1998; Zamzami *et al.*, 1996).

Mitochondria are generally oval in shape, with their size ranging from 1-2μm in length and 0.5-1μm in width. The organelle is encased by a double membrane structure, the outer of which encloses the mitochondria and the inner membrane consists of many cristae, or folds, which greatly increase the internal surface area. Oxidative phosphorylation occurs on the inner mitochondrial membranes where the enzymes required for this process are located and the number of cristae and therefore the surface area is proportional to the energy production of the cell (Modica-Napolitano *et al.*, 2002). Mitochondria have been observed to be dynamic and have been seen in living cells travelling along a molecular 'highway' created by a network of microtubules and intermediate filaments (Aufderheide, 1980; Summerhayes *et al.*, 1983).

Several features of the mitochondria such as a double membrane structure and circular DNA molecule with mitochondrial specific genetic code indicate the origin of the organelle as a separate oxidative bacterium which formed a symbiotic relationship with a proto-eukaryotic cell around 1.5 billion years ago (Anderson *et al.*, 1981; Gray, 1992; Wallace, 1999).

The mitochondria in all the cells of the human body are derived from those present in the oocyte at fertilization and are therefore inherited only from the maternal lineage. There are two main theories to explain maternal inheritance, either this is simply a result of dosage as the oocyte has a 10^3-fold greater number of mitochondria compared to the sperm (Hiraoka and Hirao, 1988) or a degradation of the paternally inherited mitochondrial genome (Birky 1994). The reality maybe a combination of both of these factors.

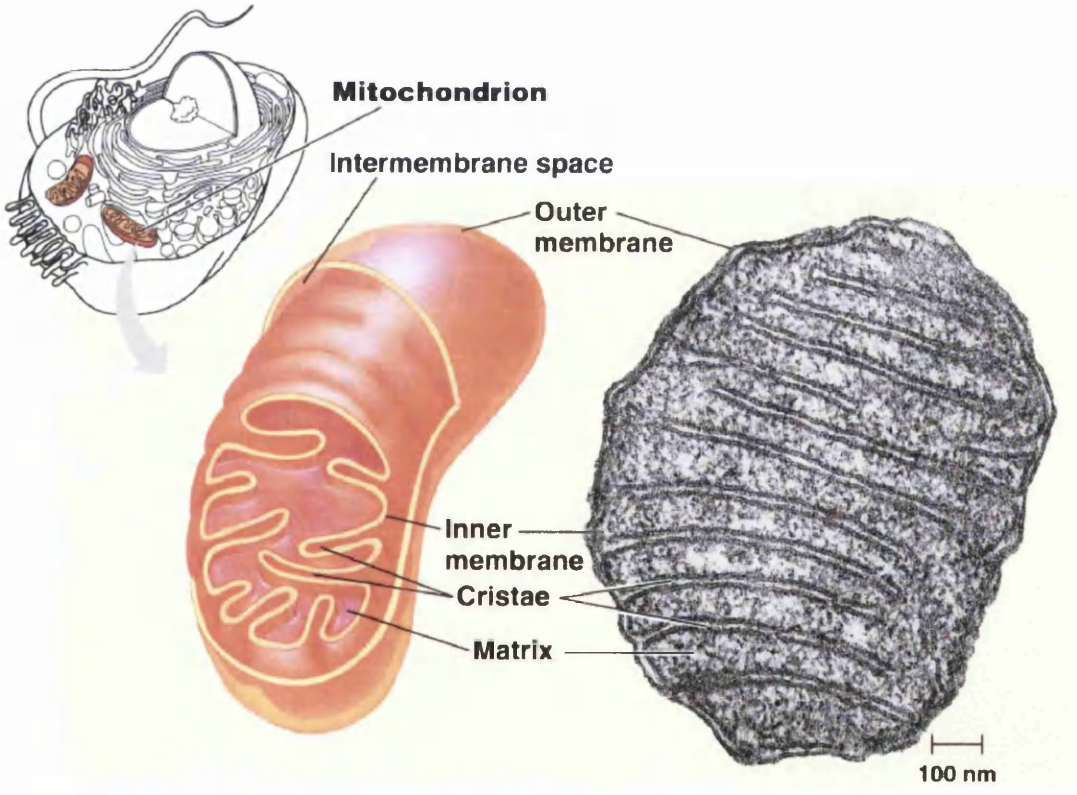


Figure 3.1

This diagram illustrates the position of the mitochondrion within the cytoplasm of the cell as well as the structure of the mitochondrion itself. The electron micrograph shows the scale of the organelle with this particular example having a length of approximately 1μm. Illustration derived from Campbell, 1996.

3.1.1.2 The mitochondrial genome

As well as nuclear DNA, human cells also contain DNA in their mitochondria; this is termed mitochondrial DNA (mtDNA) and was first reported by Schatz *et al.* (1963). Within each cell there are between 1 and 2,000 mitochondria depending upon the energy requirements of the cell type (Campbell, 1996). Within the matrix of each mitochondrion there is an average of 4.5 mtDNA molecules; this figure varies between 2 and 60 copies (Kowald and Kirkwood, 1993), resulting in approximately 1000 to 13,000 copies of the circular DNA molecule per cell (Miller *et al.*, 2003).

The mtDNA genome was sequenced in 1981 (Anderson *et al.*, 1981) and is a 16,569bp double stranded circular molecule consisting of a guanine-rich H-strand (H, to denote heavy) and a cytosine-rich L-strand (L, to indicate the lighter). The mtDNA genome encodes for 13 polypeptides required for oxidative phosphorylation, 22 transfer RNAs and 2 ribosomal RNAs involved in mitochondrial protein synthesis, see Figure 3.2. Of these, the H-strand encodes 12s and 16s rRNAs, 14 tRNAs and 12 proteins involved in oxidative phosphorylation (6 subunits of complex I NADH ubiquinose oxidoreductase, 3 subunits of complex III ubiquinol cytochrome c oxidoreductase, 1 subunit of complex IV cytochrome c oxidase and 2 subunits of complex V ATP synthase), whereas the L-strand encodes only 8 tRNAs and NADH dehydrogenase 6 (ND6) a subunit of complex I NADH ubiquinose oxidoreductase. The remaining metabolic enzymes, polymerases, ribosomal proteins, mtDNA regulatory factors and proteins required for OXPHOS found in the mitochondria are encoded by nuclear genes.

The mitochondrial genome is highly compact with 93% of the mtDNA having a coding function; the only non-coding region is the (D) displacement-loop. This is marked in green on the mitochondrial genome map in Figure 3.2. The D-loop contains a promoter region with origins of replication for both the H and L strand and contains elements for the initiation of leading strand replication. The regulation of replication and transcription is mediated by the interaction of mtDNA with nuclear and ribosomal proteins.

Mitochondria contain around 1000 proteins and, other than the 13 coded for by the mtDNA, they are all synthesised in the cytoplasm of the cell and imported into the

mitochondria. As the mtDNA does not encode all the proteins required for the organelle to function it follows that defective nuclear genes which code for the remaining proteins can affect the function of the mitochondria.

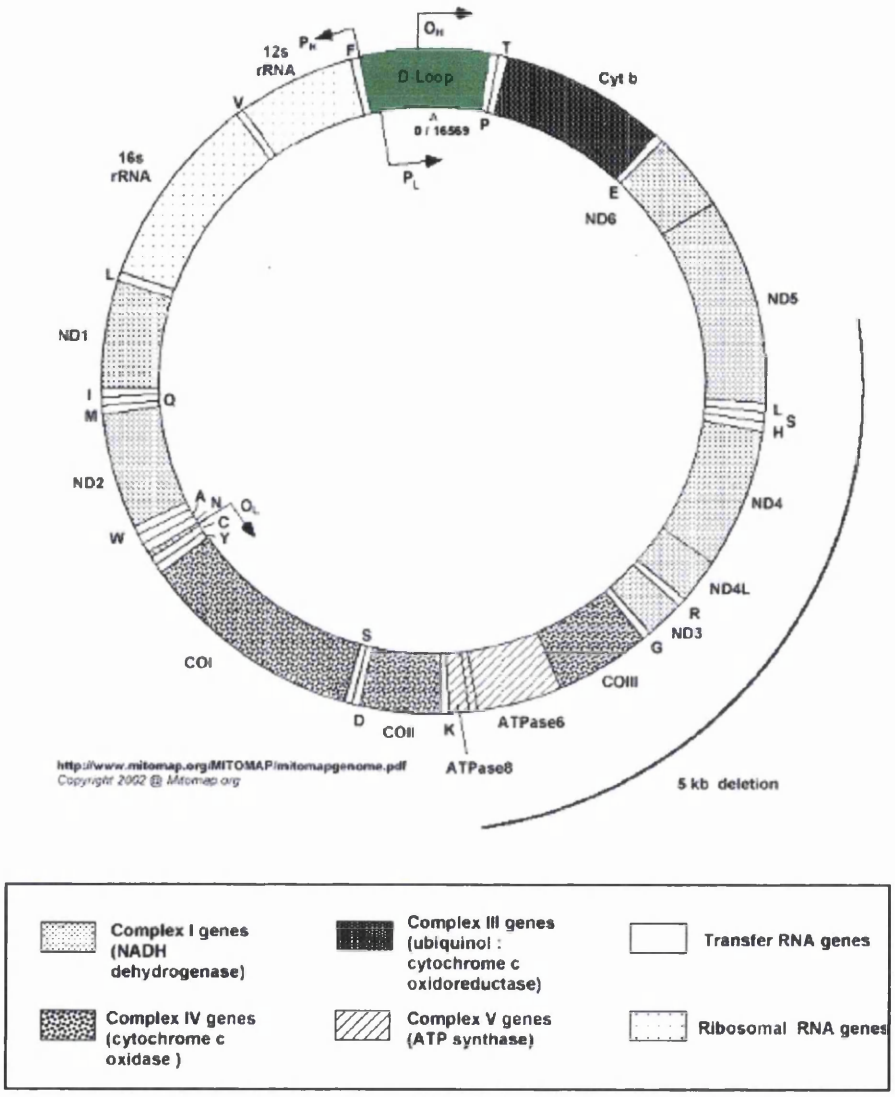


Figure 3.2

The circular human mitochondrial genome with gene locations and the D-loop or control region illustrated. Within the highlighted D-loop the H-strand origin of replication and the H- and L-strand promoters are marked P_H and P_L respectively. Also marked is the portion of the molecule removed in the common 5 kb deletion. This illustration is adapted from Mitomap, 2004.

All the enzymes required for the replication and growth of mitochondria are imported from the cytosol. MtDNA is replicated by the enzyme DNA polymerase γ. DNA polymerase γ subunit 1, the catalytic subunit, is located on chromosome 15q25 of the nuclear genome. Despite an early study of polymerase γ in HeLa cells that demonstrated the enzyme was inaccurate for base substitution errors, DNA polymerase γ has now been shown to have a high fidelity, more accurate than both polymerase α, responsible for nuclear DNA synthesis, and polymerase β, which is responsible for DNA repair synthesis (Kunkel and Loeb, 1981; Kunkel, 1985). DNA polymerase γ has 3'→5' exonucleolytic proofreading activity (Kunkel, 1985; Kunkel and Soni, 1988; Pinz *et al.*, 1995).

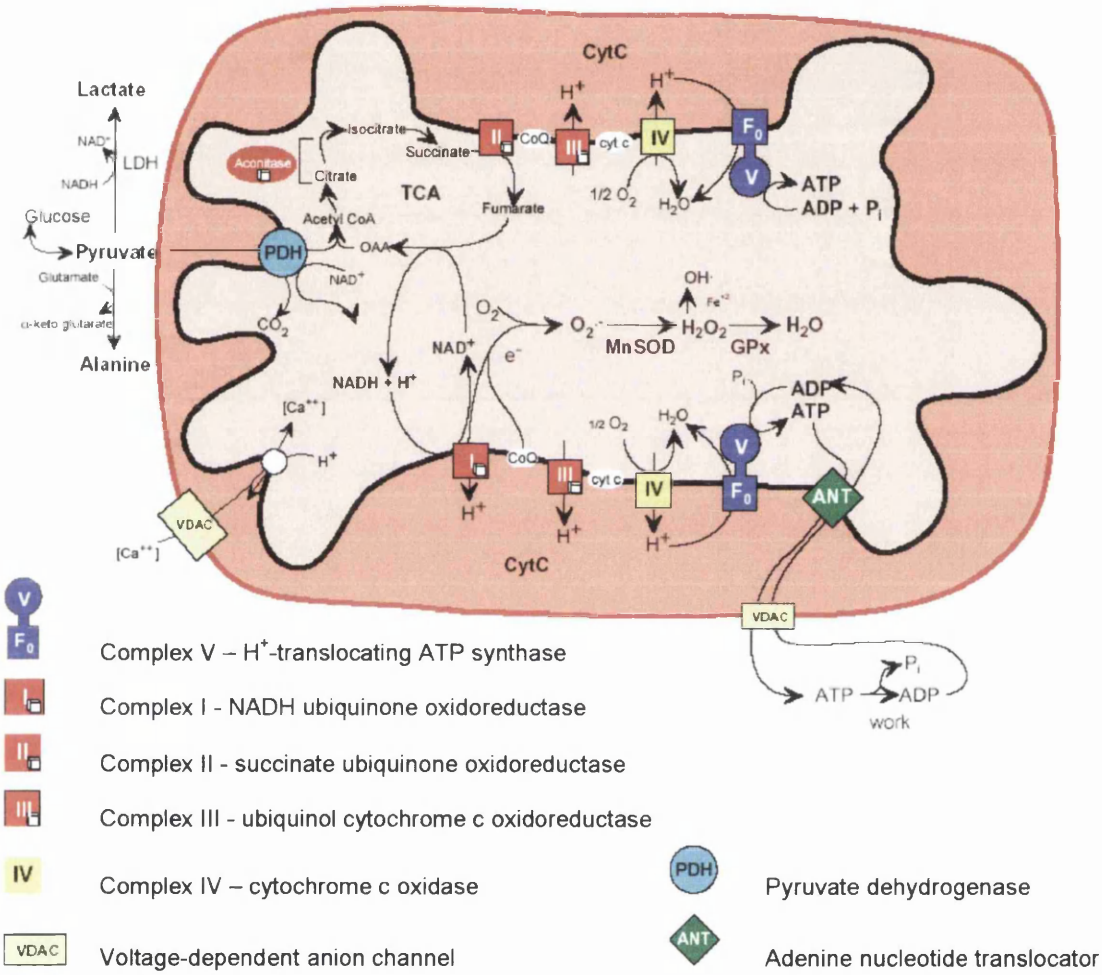
The replication of the mtDNA genome is much slower than that of the nuclear genome and is also highly asymmetric. During replication, the daughter H strand displaces the parental H strand and remains single stranded until the daughter L strand is synthesised. As the spontaneous mutation rate of single stranded DNA is much greater than that of double stranded DNA this is another explanation for the overall higher mutation rate seen in mtDNA (Tanaka and Ozawa, 1994). This higher rate of mutation can also be explained as mtDNA undergoes many more rounds of replication than chromosomal DNA with replication occurring throughout the cell cycle. The lack of protective histones also leaves mtDNA more vulnerable to mutation from causes such as oxidative damage.

Tissues, cells or even single mitochondria may contain a mixture of wild type mtDNA molecules and mutated copies of the mitochondrial genome; this is known as heteroplasmy (Shay and Ishii, 1990). Homoplasmy describes a state in which all the copies of mtDNA have the same sequence. This can occur from a state of heteroplasmy via a process called replicative segregation in which different copies of the mtDNA genome are randomly segregated into daughter cells, which, over many generations, can drift to one version of the DNA molecule (Wallace, 1999).

3.1.1.3 The mitochondria and energy production

Energy, in the form of adenosine triosphosphate (ATP), is produced in the mitochondrion primarily by the process of oxidative phosphorylation. Electrons are

passed along a series of respiratory enzyme complexes situated in the inner mitochondrial membrane. This transfer of electrons releases energy, which is utilised to pump protons across the membrane. This causes an electrochemical gradient that enables ATP synthase to synthesise ATP from ADP and phosphate (Saraste, 1999). The electron transport chain also produces DNA damaging reactive oxygen species (ROS) such as hydroxyl radicals ($OH^{\bullet}$). These processes are illustrated in Figure 3.3.



Complex V – H^+-translocating ATP synthase

Complex I - NADH ubiquinone oxidoreductase

Complex II - succinate ubiquinone oxidoreductase

Complex III - ubiquinol cytochrome c oxidoreductase

Complex IV – cytochrome c oxidase

Pyruvate dehydrogenase

Voltage-dependent anion channel

Adenine nucleotide translocator

Figure 3.3

Diagram showing the energetics occurring in the mitochondria, illustrating oxidative phosphorylation, ATP synthesis and reactive oxygen species generation. Pyruvate enters the mitochondria via pyruvate dehydrogenase (PDH), generating acetyl-CoA which enters the tricarboxylic acid (TCA) cycle by combining with oxaloacetate (OAA) to form citrate. Aconitase converts citrate to isocitrate, this is converted to succinate and succinate ubiquinone oxidoreductase converts this to fumerate which is

then converted into OAA. This cycle produces 10 high energy phosphate bonds for every acetyl-CoA molecule that is oxidized (Stryer, 1995). Lactate dehydrogenase (LDH) converts excess pyruvate along with NADH to lactate (Wallace, 1999). Oxidative phosphorylation occurs as free electrons pass through the electron transfer chain consisting of the complexes I to IV. The resulting proton gradient is used by complex V to make ATP which is passed out of the mitochondria via adenine nucleotide translocator (ANT) and voltage-dependent anion channel (VDAC) (Saraste, 1999). Adapted from Mitomap, 2004.

3.1.1.4 Oxidative damage to mitochondrial DNA

Nuclear and mitochondrial DNA are constantly being exposed to damaging agents, from endogenous and exogenous sources. In particular, reactive oxygen species (ROS) are formed at high levels as by-products of normal metabolism. Upon oxidative attack of DNA many DNA lesions are formed and oxidized bases are generated with high frequency. Radiation from γ-rays also causes oxidative damage to mtDNA whilst nuclear DNA is thought to be protected to a limited extent from this damage by shielding proteins (Richter *et al.*, 1988). MtDNA is not complexed to histones that protect the integrity of the nuclear genome, leaving mtDNA more susceptible to oxidative damage (Richer, 1992). These factors and the lack of efficient nucleotide excision repair system of the nuclear genome lead to the mtDNA having a mutation rate approximately 17-fold higher than that for nuclear DNA (Richter, 1988).

Mitochondrial DNA has been shown to accumulate high levels of the highly mutagenic lesion 8-hydroxy-2'-deoxyguanosine (8-oxo-dG), the product of hydroxylation of guanine at carbon 8 (Figure 3.4a). This form of oxidative damage in syn configuration can mispair with deoxyadenosine and if, left unrepaired, can cause G:C → A:T transversion mutations (Figure 3.4b,c) (Grollman and Moriya, 1993).

The 8-oxo-dG lesion in mtDNA has been reported to occur at a frequency of over 16 times greater than in nuclear DNA at around 1 in 8000 bases (Richter *et al.*, 1988). However, a more recent study using a protocol to isolate mitochondria which is less likely to cause physical mtDNA damage, suggests that the level of this lesion is

actually far lower at 1 in 80,000bp (Higuchi and Linn, 1995) and under physiological conditions the level around 1 in 50,000 bp (Hegler *et al.*, 1993). Another reason for the vast range in oxidative damage levels detected in various studies is that the process of isolating mitochondria itself produces oxidative damage. A comparative study showed that detecting oxidative damage after the isolation of mitochondria gave levels 3 times higher than in studies that did not isolate the mitochondria and thus the higher levels reported are due to an artefact rather than a true representation of the damage levels found within the cellular system (Anson *et al.*, 2000). It has also been found that pure mtDNA is more resistant to hydrogen peroxide (H_2O_2) damage than mtDNA within whole cells implying that intracellular, extra-mitochondrial factors are required to induce mtDNA damage (Higuchi and Linn, 1995).

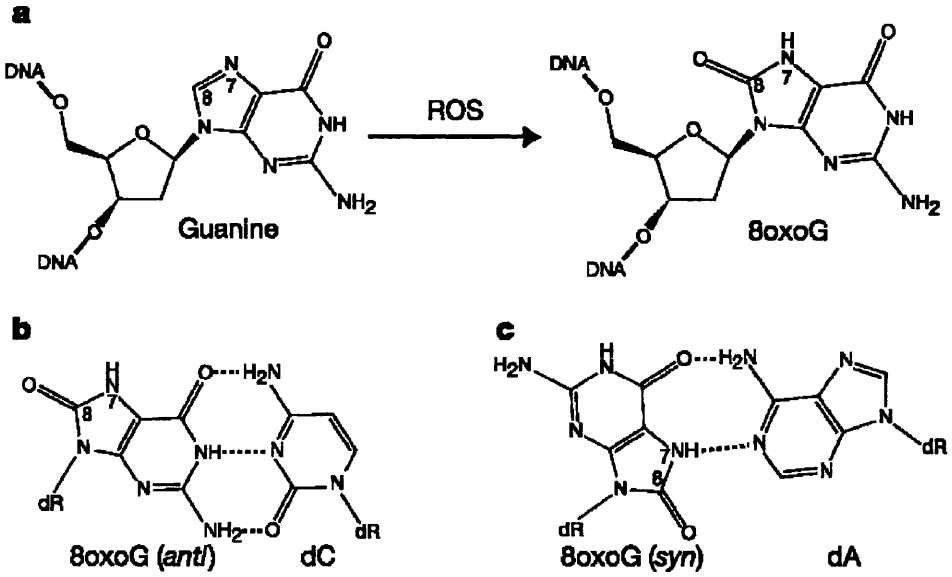


Figure 3.4 Modes of base pairing for 8oxoG. a, Oxidation of guanine at C8 by reactive oxygen species (ROS). b, 8oxoG in base pair with dC. Dashed lines indicate hydrogen bonds. c, 8oxoG (syn) in a base pair with dA (anti). From Hsu *et al.*, 2004.

The ROS superoxide anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) are mainly generated in the mitochondria. Although a variety of enzymes in the cytosol, such as oxygenases, peroxidases and oxidases, generate small amounts of

ROS, it is estimated that more than 95% of $O_2^{\bullet-}$ produced during normal metabolism are generated at the electron transport chain in the inner mitochondrial membrane during oxidative phosphorylation. MtDNA is the main cellular target for ROS induced oxidative damage due to the close proximity of the molecule to the inner mitochondrial membrane. Oxidized bases in mtDNA are found at levels 2–3 times greater than in nuclear DNA (Hudson *et al.*, 1998).

3.1.1.5 The Repair of damage in mtDNA

Less is known about the various repair pathways of mtDNA than that of nuclear DNA. It is recognized that mitochondria do have proficient short patch base excision repair (BER) and also direct reversal DNA repair. There is also evidence that the organelles lack nucleotide excision repair (NER); but there is greater uncertainty over recombination post replication repair and mismatch repair (MMR) (Bohr, 2002).

Mitochondrial DNA polymerase γ misincorporates dA opposite 8-oxo-dG at a high level and this is not removed by the 3'→5' exonuclease activity of the enzyme (Pinz *et al.*, 1995), but most of these small base modifications are repaired by the BER pathway. Despite the initial belief that mitochondria lack DNA repair, experimental evidence, derived from yeast models, now show that mitochondria are very proficient in BER of oxidative DNA damage (O'Rourke *et al.*, 2002). The proteins necessary for this repair pathway, such as apurinic/apyrimidinic (AP) endonucleases and a specific DNA glycosylase, have also been isolated from mammalian mitochondria (Bohr *et al.*, 2002).

Experiments using rats and mice have demonstrated that mammalian mitochondria efficiently remove 8-oxo-dG from their genome and this process becomes more efficient with age (Stevnsner *et al.*, 2002). This removal of 8-oxo-dG has also been shown to occur in human cells, occurring at an equal rate between the light and heavy strands and also between regions of the mitochondrial genome that differ significantly in terms of transcriptional activity (Anson *et al.*, 1998).

3.1.1.6 Mitochondrial damage and aging

Mitochondrial function deteriorates with age and skeletal muscle, liver and brain have all been reported to have reduced OXPHOS enzyme activity with age (Wallace, 1999; Trounce *et al.*, 1989). The reduced respiratory function causes an increase in ROS production through increased electron leak of the electron transport chain. Levels of intracellular antioxidants and free radical scavenging enzymes also decrease with age, which, in turn exacerbates the damage to the enzymes involved in OXPHOS (Wei *et al.*, 1998). These factors give rise to an age associated increase in mutation frequency in the mitochondrial genome.

The mtDNA copy number per cell in humans has been shown to remain constant from neonates to adults over the age of 80 indicating that a cause other than number must be a reason for reduced mitochondrial function in the elderly (Miller *et al.*, 2003). Experiments have shown that mtDNA in skeletal muscle from humans over the age of 50 has a large number and type of rearrangements whereas in skeletal muscle from people aged 40 or under there are virtually no mtDNA mutations present (Melov *et al.*, 1995). The control region for replication of human fibroblast mtDNA from normal elderly subjects contains a high number of point mutations that only appear in a given individual in advanced age (Michikawa *et al.*, 1999). The common 5kb deletion has also been found to accumulate in brain tissue of humans over the age of 75 (Corral-Debrinski *et al.*, 1992). The 5kb deletion region is marked on Figure 3.2. Mutations within the control region of the mitochondrial genome have also been found to accumulate with age in muscle but not in brain tissue (Murdock *et al.*, 2000). The reason for this has not, as yet, been identified.

Inefficient repair of oxidative damage to mtDNA has the potential to lead to the accumulation of mutations and ultimately mitochondrial dysfunction, which is associated with pathological processes such as degenerative diseases and aging. The mitochondrial hypothesis for aging proposes that symptoms of aging and senescence are a result of a reduced bioenergetic capacity of mitochondria due to the accumulation of mtDNA mutations caused by increased levels of ROS associated with age (Harman, 1972). The extent of this effect varies from tissue to tissue

depending upon energy requirements and can explain the damage seen to the brain and heart tissue of the elderly (Wei *et al.*, 1998).

3.1.1.7 The role of mitochondria in apoptosis

The discovery that anucleated cells could be induced to undergo apoptosis sparked research into the role of cytoplasmic structures, including mitochondria, in apoptosis. Early observations noted that, at the start of the apoptosis process, the mitochondrial transmembrane potential drops (Zamzami *et al.*, 1995). To investigate this further, experimental cell free systems were established, these showed that mitochondria with open mitochondrial transition pores were able to induce apoptosis and so had a central role in this process (Zamzami *et al.*, 1996).

Further research has found that the inner mitochondrial membrane space contains several proteins that induce programmed cell death. The mitochondrion initiates apoptosis after increased ROS exposure, reduced ability for energy production or excess uptake of calcium ions. At this point the mitochondrial permeability transition pore (mtPTP) through the inner and outer mitochondrial membranes opens. The mtPTP is created by the interaction of the voltage dependent anion channel, adenine nucleotide translocator, Bax and cyclophilin D at contact sites between the inner and outer mitochondrial membranes. It is through this pore that apoptosis inducing factors are released, including cytochrome c (CytC), caspases and apoptosis-inducing factor (AIF). CytC activates the cytoplasmic protein Apaf-1 which then activates the latent pro-caspase-9 which in turn, degrades the cellular proteins of the cytoplasm. AIF translocates to the nucleus where it induces apoptosis by chromatin condensation and endonuclease-mediated DNA fragmentation (Zamzami *et al.*, 1996; Wallace 1999).

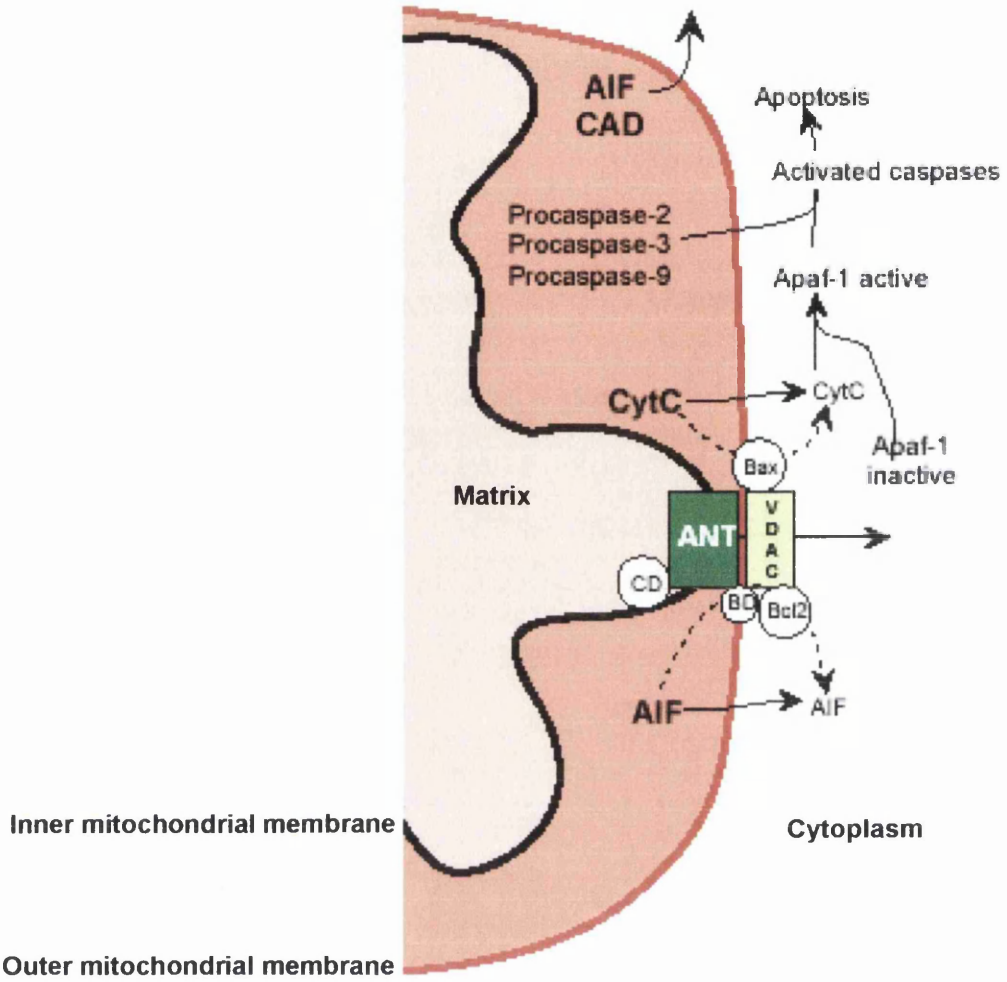


Figure 3.5

This diagram shows a section of a mitochondrion and illustrates the cascade of events leading to apoptosis. The mitochondrial permeability transition pore (mtPTP) is created at points of contact between the inner and outer mitochondrial membranes where the Voltage-dependent anion channel (VDAC), Adenine nucleotide translocator (ANT) cyclophilin D (CD) and Bax come together to form a channel into the cytoplasm. The relative concentrations of the pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 are thought to mediate apoptosis and interact with the benzodiazepine receptor (BD) (Wallace 1999). When the channel is open the apoptosis inducing factor (AIF) and cytochrome C (CytC) are released out of the mitochondrion into the cell cytoplasm where it activates Apaf-1 which in turn activates caspases and leads to apoptosis of the cell. Adapted from Mitomap, 2004.

3.1.1.8 MtDNA and associated disease

In 1988 there were reports of mtDNA missense mutations present in the mitochondrial ATP synthase gene ATP6 in patients suffering from the maternally inherited disease Leber's hereditary optic neuropathy, a degeneration of the optic nerve, (Wallace *et al.*, 1988) and large-scale mtDNA deletion mutations present in patients with Kearns-Sayre syndrome, a degenerative disease of the central nervous system associated with defects of the eye (Zeviani *et al.*, 1988). This prompted mtDNA research into many other diseases and mutations were found in patients suffering from a wide range of symptoms including blindness, deafness, diabetes and heart disease. Tissues most commonly affected by mitochondrial disease are those with high energy requirements such as skeletal and cardiac muscle, nerves and endocrine system. MtDNA mutations are also associated with aging and neurodegenerative diseases such as Alzheimer's disease and Mitochondrial Encephalopathy, Lactic Acidosis and Strokelike episodes (MELAS) (Wallace, 1992; Yoneda *et al.*, 1992). An estimated 0.1% of births in the US are affected by mitochondrial diseases (Singh, 1998).

Patients sharing the same mtDNA mutation may differ greatly in the severity of their symptoms; this can be explained by the degree of heteroplasmy present in the cells. Patients with a high ratio of wild type to mutated mtDNA are likely to have less severe symptoms than those with a low ratio whose mitochondria are less likely to function correctly.

As well as mutations in the mitochondrial genome, mitochondrial associated diseases can arise from mutations in nuclear genes which encode proteins required for mitochondrial function. An example of this is autosomal dominant spastic paraplegia which is associated with the mutation of the gene encoding a mitochondrial protease-like ATPase (Casari *et al.*, 1998).

A delayed onset is a common trait in mitochondrial diseases. This and the progressive nature of these diseases suggests that, in addition to the predisposing mutation, the phenotype of the disease is exacerbated by an age factor that decreases the functional ability of the mitochondrion (Wallace, 1999).

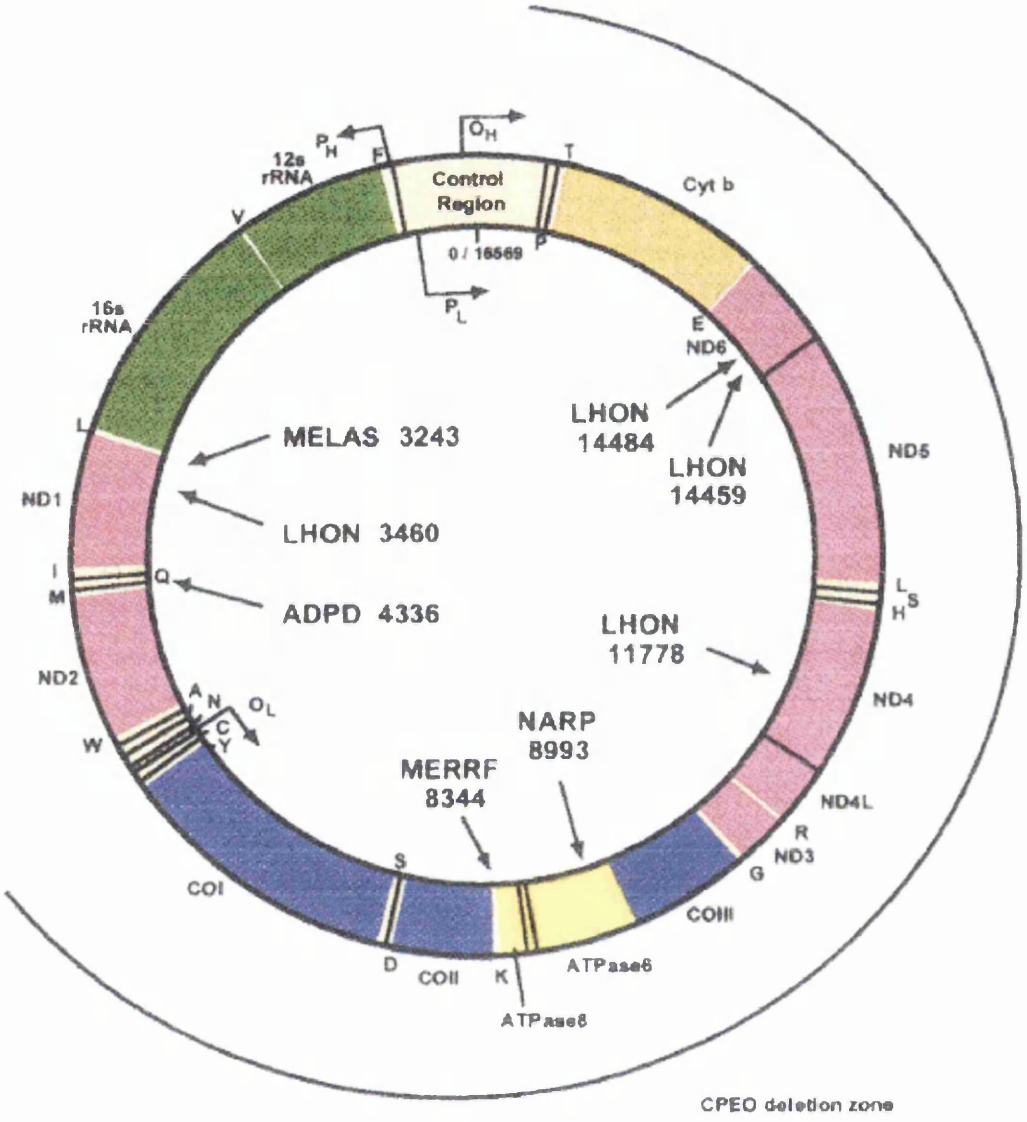


Figure 3.6

Schematic diagram of the mitochondrial genome showing the positions of known mitochondrial disease causing mutations. The acronyms for the diseases marked are Leber's hereditary optic neuropathy (LHON), neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP) also known as Leigh disease, myoclonic epilepsy and ragged-red fiber disease (MERRF), late on-set Alzheimer's disease (ADPD), mitochondrial encephalopathy, lactic acidosis and strokelike episodes (MELAS) which also causes diabetes mellitus, chronic progressive external ophthalmoplegia (CPEO). Deletion of the 4.9kb fragment also causes Kearns-Sayre Syndrome (KSS) (Singh, 1998). Image adapted from Wallace, 1999.

3.1.1.9 Mitochondrial DNA as a biomarker for cancer

It has been known for nearly 50 years that the role of the mitochondrion is different in some tumour cells as studies have shown mitochondrially-mediated oxidative phosphorylation is diminished in cancer cells with some tumours relying on the energy obtained by glycolysis (Warburg, 1956). In addition to this, more recently links with mtDNA mutations and cancer have been established. Mutations in the mtDNA of human solid tumours were first reported in 1989 where mutations within the cytochrome oxidase subunit 1 gene were found in the benign solid tumours known as oncocytomas (Welter *et al.*, 1989). Deletions have been reported in the mtDNA of cirrhotic liver tissue surrounding hepatocellular carcinoma; these were suggested to be an endogenous factor in the induction of nuclear genome somatic mutations and to contribute to carcinogenesis (Yamamoto *et al.*, 1992).

In a gene expression study, 15 nuclear encoded mitochondrial proteins were found to be down-regulated in colorectal cancer. Of these genes, 3 were found to be down-regulated at all Dukes' stages of cancer progression. These were rhodanase (thiosulfate sulfurtransferase), 3-hydroxy-3-methylglutaryl CoA synthase and SCAD (acyl-CoA-dehydrogenase). SCAD was found to have expression decreased by 7-fold in Dukes' A tumours. A deficiency of this protein results in an increase in butyric acid, which at low concentrations stimulates growth, and in higher concentrations stimulates apoptosis. This again provides evidence to indicate a central role for mitochondria in colorectal carcinogenesis (Birkenkamp-Demtroder *et al.*, 2002).

MtDNA may act as transposable elements to modify the nuclear genome. Mitochondrial DNA sequences have been identified within the nuclear genome, depending upon where these sequences are incorporated it is possible for these to disrupt genes which are involved in the maintenance of cell growth and repair and as a result initiate carcinogenesis (Mishmar *et al.*, 2004). Increased oxidative stress is thought to increase the transfer of mtDNA fragments into the nuclear genome (Richter, 1988). These types of mutations have been found in low-grade tumours and in the HeLa cell line (Singh, 1998).

Cell fusion experiments have also shown that extra-nuclear factors also play a role in tumorigenesis. For example, when tumourigenic HeLa cells are fused with other tumourigenic cells the mitochondria are randomly segregated and the tumorigenicity of the cells correlates with the type of mtDNA retained (Shay and Ishii, 1990). In the same series of experiments, the fusion of HeLa cells with non-tumourigenic cells frequently resulted in the loss of the mtDNA originally from the HeLa cells after several weeks of cell culture. The factors which determine this process are still unclear as further cell hybrid experiments have shown this segregation of mitochondrial genome types maybe merely the result of stochastic events (Shay and Ishii, 1990).

Differences between mitochondria in normal and tumour cells provides scope for an alternative cancer treatment to traditional chemotherapy which targets actively replicating cells but with a low specificity for tumourigenic cells (Weissig and Torchilin, 2001). The dosage of mtDNA may also be important in the sensitivity of cells to cancer therapeutic agents. Cells lacking mtDNA have been found to be extremely resistant to cell death induced by the anticancer agents adriamycin and photodynamic therapy (Singh *et al.*, 1999). Pharmacological agents can impede or induce the opening of the mitochondrial permeability transition pores either inhibiting or inducing apoptosis respectively, providing a target for cytoprotective and cytotoxic therapy (Zamzami *et al.*, 1996). Indeed, in a patient trial prostate cancer cells have been selectively killed by adenoviral-mediated delivery of the novel cytokine gene *mda*-7/IL-24 which promotes apoptosis in cancer cells but not in normal cells. The gene is able to do this by promoting mitochondrial dysfunction and ROS production (Lebedeva *et al.*, 2003).

The mitochondrial membrane potential falls in cells undergoing apoptosis (Zamzami *et al.*, 1995), but in cancer cells the membrane potential is increased thus giving the cell a greater resistance to apoptosis (Modica-Napolitano and Singh, 2002). Anti-cancer drugs which contain delocalised lipophilic cations (DLCs) accumulate preferentially in the mitochondria and the membrane potential difference between normal and cancerous cells provides a possible target for these types of drugs. Several of these DLCs have effectively exploited this transmembrane potential difference in

carcinoma cells and providing selective cytotoxicity to these cell types (Modica-Napolitano *et al.*, 1996; Modica-Napolitano and Singh, 2002).

Mutations in the mitochondrial genome have been detected in a wide range of different cancer types including leukaemia, ovarian, thyroid, salivary, renal, liver, lung, colonic, gastric, brain, and breast cancer as well as the benign condition goitre (Modica-Napolitano and Singh, 2002). One study has shown that tumours from the human lung, bladder and head and neck contained a high frequency of mtDNA mutations. These mutations were also detectable in the paired bodily fluids, lung lavage fluid, urine and saliva and were more common than mutated copies of the tumour suppressor gene p53 (Fliss *et al.*, 2000). Mutant mtDNA has been reported to be 220 times more abundant than mutated nuclear molecular markers (Singh, 1999). This may provide a non-invasive method of screening for many types of cancer, which is more sensitive than nuclear DNA molecular markers. A study sequencing the complete mitochondrial genome of 10 colorectal cell lines and primary tumour cells found mitochondrial mutations in 70% of the cell lines (Polyak *et al.*, 1998).

There are several ways in which mtDNA mutations may contribute to the progression of cells through to carcinogenesis such as alterations in cellular energy capacities, increases in mitochondrial oxidative stress and by the decreased initiation of apoptosis (Modica-Napolitano and Singh, 2002). However, tumours with no mitochondrial DNA have also been observed, this may either indicate that mtDNA mutations can have no role in the process of carcinogenesis or that the very absence of this mtDNA was a contributory factor in the tumour's development (Morais *et al.*, 1994).

Although the role of mtDNA in cancer is still under debate, the study of mtDNA mutations is still of value as these may act as a molecular marker to aid the early detection of cancers. The mutational spectra may also be of use in tracking down the mutagens responsible for any damage observed.

3.1.2 Mutation Detection

3.1.2.1 Mutation detection techniques

When examining DNA for mutations there are several different techniques which can be employed, such as, restriction fragment length polymorphism (RFLP), restriction site mutation assay (RSM), single stranded conformational polymorphism (SSCP) and direct sequencing (Cotton, 1993). Both RFLP and RSM require the mutations to occur within restriction sites in order to be detected making these techniques more appropriate for sequences of DNA where there are known mutational hotspots within restriction enzyme sites. As this is not true of the region of the mitochondrial genome to be studied these were not deemed to be suitable techniques for this study. Sequencing obviously provides not just information about the presence or absence of mutations but also the exact nature of the change. This information is expensive and time-consuming to obtain because of high equipment costs, both for initial purchase and maintenance. This makes the technique impractical for preliminary experiments or for tightly budgeted projects as the equipment is costly and expensive to maintain often requiring a dedicated technician. SSCP is a very simple technique for detecting unknown mutations within DNA; as discussed later, it requires only standard laboratory equipment and is both rapid and cost effective with very little loss of sensitivity for mutation detection in comparison to direct sequencing. For these reasons SSCP was chosen for the experiments described within this Chapter.

3.1.2.2 Single Stranded Conformational Polymorphism

Single-strand conformation polymorphism (SSCP) analysis allows detection of unknown point mutations in DNA up to 800bp in length. The technique, first described in 1989 (Orita *et al.*, 1989), is arguably one of the simplest, cost efficient and possibly amongst the most sensitive PCR-based methods for detecting mutations (Hayashi, 1992). The technique, originally developed using radioisotopes, has now been optimised for use with standard mini gel electrophoresis apparatus and conventional silver staining methods without the need for radioactivity making the technique safer, simpler and less expensive (Oto *et al.*, 1993).

The assay is based on the principle that single-stranded DNA molecules form sequence specific conformers under non-denaturing conditions and therefore have different electrophoretic mobilities. The technique is able to detect differences between molecules that differ by even a single base substitution, which alters the secondary and tertiary structure of the DNA molecule and causes a difference in migration through a non-denaturing polyacrylamide gel. It is reported that SSCP detects 99% of mutations in 100 – 300bp fragments and 89% in fragments of 300 – 400bp and has an equal ability to detect transition and transversion mutations (Hayashi 1991; Sheffield *et al.* 1993).

The sensitivity of PCR-SSCP has been assessed in several studies to establish the ability of the technique to detect mutations in mixed cell populations. This is particularly important for studies of mtDNA as not only are there different cells but each cell contains several mitochondria, which in turn contain several copies of the mitochondrial genome. These studies have shown that PCR-SSCP is able to detect mutations found in between 1 in 4 and 1 in 33 copies of the mutated DNA amongst wild type sequences. (Smith *et al.*, 1992; Wu *et al.* 1993) A study of mtDNA demonstrated the ability to detect a defined mutation within the mitochondrial genome was 93% for SSCP alongside 98% for direct sequencing. This study clearly confirms the usefulness and accuracy of this technique used for the study of mtDNA (Jaksch *et al.*, 1995).

It is important to understand that this technique does not always discriminate between mutant and wild-type alleles, even at mutation concentrations of 50%. Furthermore, it is dependant upon the sequence of the DNA to be screened and the position of the mutation within the DNA fragment. This is because in certain locations within a sequence of DNA the base mutated does not effect the folding of the single strand of DNA into it's secondary and tertiary structures making the mutation undetectable by this technique (Hayashi 1992; Hayashi and Yandell 1993; Glavac and Dean 1993). The region of DNA to be amplified by PCR prior to SSCP must therefore be tested after selection to ensure that the technique produces clear banding patterns. The PCR must be carried out using high fidelity enzymes to eliminate any possible artefacts from the amplification stage of the assay.

Occasionally the SSCP bands may appear very diffuse or there may be a heavily smeared background despite the demonstration of a single strongly stained DNA band after PCR purification. The addition of formamide to the PCR product prior to heating and rapid cooling can sharpen the ssDNA bands and ease the detection process. By adding a denaturing agent, the multiple highly similar conformations of ssDNA for a single sequence, which are thought to be the reason for background smearing, migrate in a more uniform manner. Increasing the concentration of acrylamide in the gel and lowering the temperature at which the gel is run can also sharpen the bands. There is though a limit for both of these factors at which the bands do not separate enough to distinguish between them (Yip *et al.*, 1999).

The DNA fragment length is another factor which alters the sensitivity of SSCP, with the optimal DNA fragment length for mutation detection being approximately 150bp (Sheffield, *et al.*, 1993). It is also possible to screen larger fragments by digesting with restriction enzymes before running on a two-dimensional gel, the first dimension of electrophoresis to separate the fragment by size and the second dimension to detect for mutations as in a standard SSCP assay. This technique has been used to successfully analyse fragments of 2.7kb (Kovar *et al.*, 1991).

Electrophoretic conditions such as running time and current intensity also influence the ability of this technique to detect sequence differences. Polyethylene glycol has also been shown to enhance the technique by increasing gel viscosity when added to polyacrylamide gel, especially for fragments of 400-500bp (Markoff *et al.*, 1997).

The technique can also be used for RNA SSCP, which is reported to be more sensitive than standard SSCP due to the larger number of secondary forms RNA can assume. This adaptation of SSCP does lack the simplicity of standard SSCP due to the extra transcription stages involved and the increase in sensitivity of RNA SSCP may not warrant the much more time consuming process (Cotton, 1993).

3.1.3 Rationale of the study

The purpose of the experiments described in section 3.2 was to analyse the control region of the mtDNA genome of colorectal adenocarcinomas, normal colonic tissue and where possible, intermediate stages such as metaplastic polyps and adenomas, as well as lymph node tissue and liver biopsies. The aim of this was to gauge the level of mutation of the mitochondrial genome and search for possible molecular markers for colon cancer. Also, if mutations could be localised to a small fragment of the mitochondrial genome, screening for mutations in the paired bodily fluid samples for patients would become a viable clinical application of this non-invasive, sensitive method. These experiments also aimed to see if mtDNA mutations were an important contributor to the process of carcinogenesis.

3.2 Materials and Methods

3.2.1 Sample collection

65 fresh tissue samples were obtained as described in Chapter 2, section 2.2 from 24 sporadic colorectal cancer patients. The mean age of these patients was 69 years of age at the time of sample collection; the patients consisted of 13 females and 11 males (1.2F : 1M). 12 of these patients had adenocarcinomas of the rectum, 2 of the sigmoid colon, 4 of the transverse colon and 2 of the caecum. 1 patient had a rectal polyp, 3 had sigmoid polyps. 6 of these samples had metastatic tissue samples from liver or lymph node.

3.2.2 DNA extraction

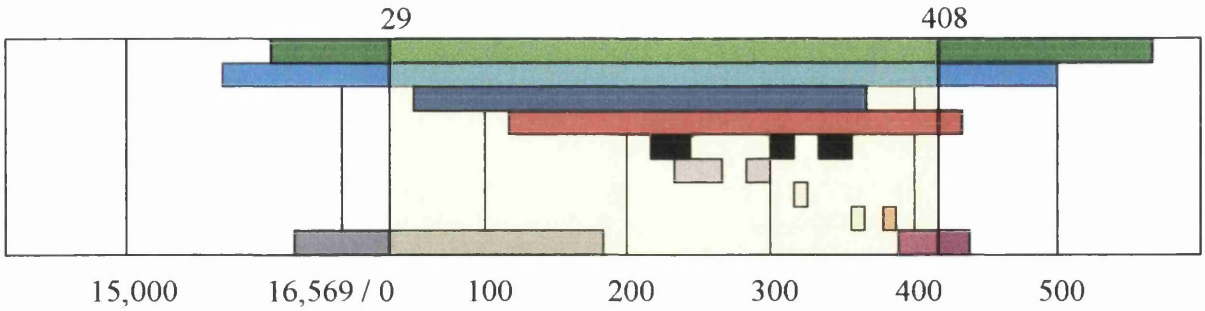
Fresh tissue samples were disrupted as described in Chapter 2, section 2.3.1 prior to DNA extraction using the high salt method described in Chapter 2, section 2.3.3.

3.2.3 DNA quantification

DNA concentration was established as described in Chapter 2, section 2.3.5.

3.2.4 Polymerase Chain Reaction (PCR)

A section of D-loop or control region of the mitochondrial genome from position L29 to H408 was amplified for each sample. This section contained the hypervariable segment 2, the H-strand origin, conserved sequence block 1, mtTF1 binding site, conserved sequence block 2, replication primer, conserved sequence block 3, the mt4 H-strand control element, the mt3 H-strand control element and part of the L-strand promoter. The entire amplified fragment of the mtDNA genome is within the membrane attachment site, which spans from position 15925 – 499 (Albring *et al.*, 1977).



- PCR product (29-408)
- D-loop (16024-576)
- Membrane attachment site (15925-499)
- Hypervariable segment 2 (57-372)
- H-strand origin (110-441)
- Conserved sequence blocks 1, 2, and 3 (213-235, 299-315, 346-363)
- mtTF1 binding site (233-260, 276-303)
- Replication primer (317-321)
- mt4 H-strand control element (371-379)
- mt3 H-strand control element (384-391)
- 7S DNA (16106-191)
- L-strand promoter (392-445)

Figure 3.7

This figure illustrates the locations of various functional sequences of the mitochondrial genome for the section of interest in this Chapter on a linear schematic of human mtDNA. The PCR primers described in section 3.2.4 amplify the region highlighted in pale yellow on this diagram, the resulting PCR product contains all or part of the functional regions listed. The numbers alongside the diagram and following the functional sequence descriptions are the relevant nucleotide positions of the mitochondrial genome according to the Cambridge mtDNA sequence (Mitomap, 2004).

This was carried out as described in Chapter 2, section 2.4 using the following oligonucleotide primers (Vigilant *et al.*, 1989) and under the conditions listed in table 3.1 for 35 cycles in the presence of 1.5µl $MgCl_2$.

Forward primer: GGTCTATCACCCTATTAACCAC

Reverse primer: CTGTTAAAAGTGCATACCGCCA

PCR Stage	Temperature (°C)	Length of stage (seconds)
Denaturation	95	30
Annealing	58	30
Elongation	72	30

Table 3.1

Table of the conditions required for the PCR of the D-loop section

3.2.4 Electrophoresis

PCR products were evaluated by polyacrylamide gel electrophoresis described in Chapter 2, section 2.5.1, the gels stained with silver stain, Chapter 2, section 2.5.3 and images captured as in Chapter 2, section 2.5.5.

3.2.5 PCR purification

Successfully amplified control region PCR products were purified to remove excess primer, free nucleotides and short DNA fragments less than 40bp using the PCR purification protocol described in Chapter 2, section 2.6.

3.2.6 Single Stranded Conformational Polymorphism (SSCP)

SSCP was carried out on the purified PCR products to detect any mutations present in these samples. This was carried out as described in Chapter 2, section 2.7. Each sample was amplified and analysed by this technique a minimum of 4 times.

3.2.7 Sequencing

To establish the nature of the mutations detected by SSCP and to evaluate the sensitivity of the technique in detecting mutations the PCR products were sequenced as described in Chapter 2, section 2.9.

3.3 Results

3.3.1 Results of SSCP analysis

There was an abundance of variation of band migration patterns between individuals in the control region of both the normal and carcinoma samples indicating the presence of many germline variations for this region. A total of 10 different polymorphisms were seen from the 24 patients, these are listed in Table 3.3.

9 out of the 24 (37.5%) patients exhibited tumour specific mutations, that were not present in the paired normal mucosa from the same patient, in the control region of the mitochondrial genome amplified in these experiments, see Table 3.2. These patients did not significantly differ in age (67.6 years of age) or sex ratio (1.24F: 1M) from the overall cohort of patients (68.9 years of age; 1.2F: 1M).

Mutations were only present in the later stages of carcinogenesis, none of the normal mucosa, neither adjacent nor distant, contained mutations in this region. The polyps were also found to be free of mutations within this region.

The samples with SSCP detectable mutations were in the distal sections of the colon, either rectal adenocarcinomas or sigmoid colonic adenocarcinomas, of which 6/9 (67%) were rectal tumour samples. The remaining samples with mutations were either sigmoid colonic tumours or metastatic tumours from liver or lymph node. No mutations were found in the 6 adenocarcinomas of the transverse colon or the caecum. One of the patients (patient 3) had SSCP detectable mutations in both the rectal tumour and the metastatic lymph node, although the bands for these mutations appeared to have migrated to different locations within the gel and therefore maybe different mutations. Another patient, (patient 14) had a detectable mutation in the metastatic liver tissue but not in the rectal adenocarcinoma or metastatic lymph node.

3 of the mutations were present in the heteroplasmic form, this could be seen on the SSCP gel pictures as a further band in addition to the two bands for the original allele (Figure 3.8).

All PCR products were then sequenced to confirm these results and to provide greater detail of the nature of the mutations and polymorphisms detected with SSCP.

Patient no	Sample no	Age	Sex	Sample type	SSCP change	Heteroplasmic
1	4	64	F	Rectal polyp		
	5			Adjacent mucosa		
2	9	80	M	Rectal tumour		
	10			Distant mucosa		
3	11	79	F	Lymph node	Y	
	13			Rectal tumour	Y	Y
	14			Distant mucosa		
4	16	64	M	Rectal tumour	Y	
	17			Adjacent mucosa		
5	19	64	F	Distant mucosa		
	20			Sigmoid polyp		
6	24	71	F	Distant mucosa		
	25			Rectal tumour	Y	
7	28	78	M	Distant mucosa		
	29			Rectal tumour		
8	30	64	M	Rectal polyp		
	31			Liver biopsy		
	33			Distant mucosa		
	34			Rectal tumour	Y	Y
	35			Adjacent mucosa		
9	36	70	F	Sigmoid tumour	Y	Y
	37			Distant mucosa		
	38			Sigmoid polyp		
	39			Adjacent mucosa		
10	40	59	F	Adjacent mucosa		
	42			Transverse polyp		
	43			Transverse tumour		
11	44	67	M	Distant mucosa		
	45			Rectal tumour		
12	46	63	F	Rectal tumour		
	47			Distant mucosa		
13	50	73	M	Sigmoid polyp		
	51			Adjacent mucosa		
14	53	68	M	Distant mucosa		
	54			Lymph node		
	55			Rectal tumour		
	56			Liver biopsy	Y	
15	57	85	M	Transverse tumour		
	58			Distant mucosa		
	60			Caecal polyp		

Table 3.2 continued overleaf

Patient no	Sample no	Age	Sex	Sample type	SSCP change	Heteroplasmic
16	68	68	F	Lymph node		
	69			Distant mucosa		
	70			Rectal tumour		
	71			Adjacent mucosa		
17	72	65	F	Distant mucosa		
	73			Sigmoid polyp		
	74			Adjacent mucosa		
18	76	65	F	Distant mucosa		
	77			Caecal tumour		
	78			Caecal polyp		
19	81	73	M	Distant mucosa		
	82			Lymph node		
	83			Transverse tumour		
20	87	64	F	Rectal tumour	Y	
	88			Adjacent mucosa		
21	99	83	F	Transverse tumour		
	100			Adjacent mucosa		
22	102	58	M	Sigmoid tumour	Y	
	104			Distant mucosa		
	105			Lymph node		
23	122	70	F	Rectal tumour	Y	
	124			Distant mucosa		
24	96	58	M	Caecal tumour		
	97			Adjacent mucosa		
	98			Distant mucosa		

Table 3.1

This shows an overview of the SSCP analysis results. Where a shift in band height, was detected between the samples of the same patient the sample is marked with a Y in the SSCP change column, if the SSCP bands indicated were heteroplasmic this column is marked Y.

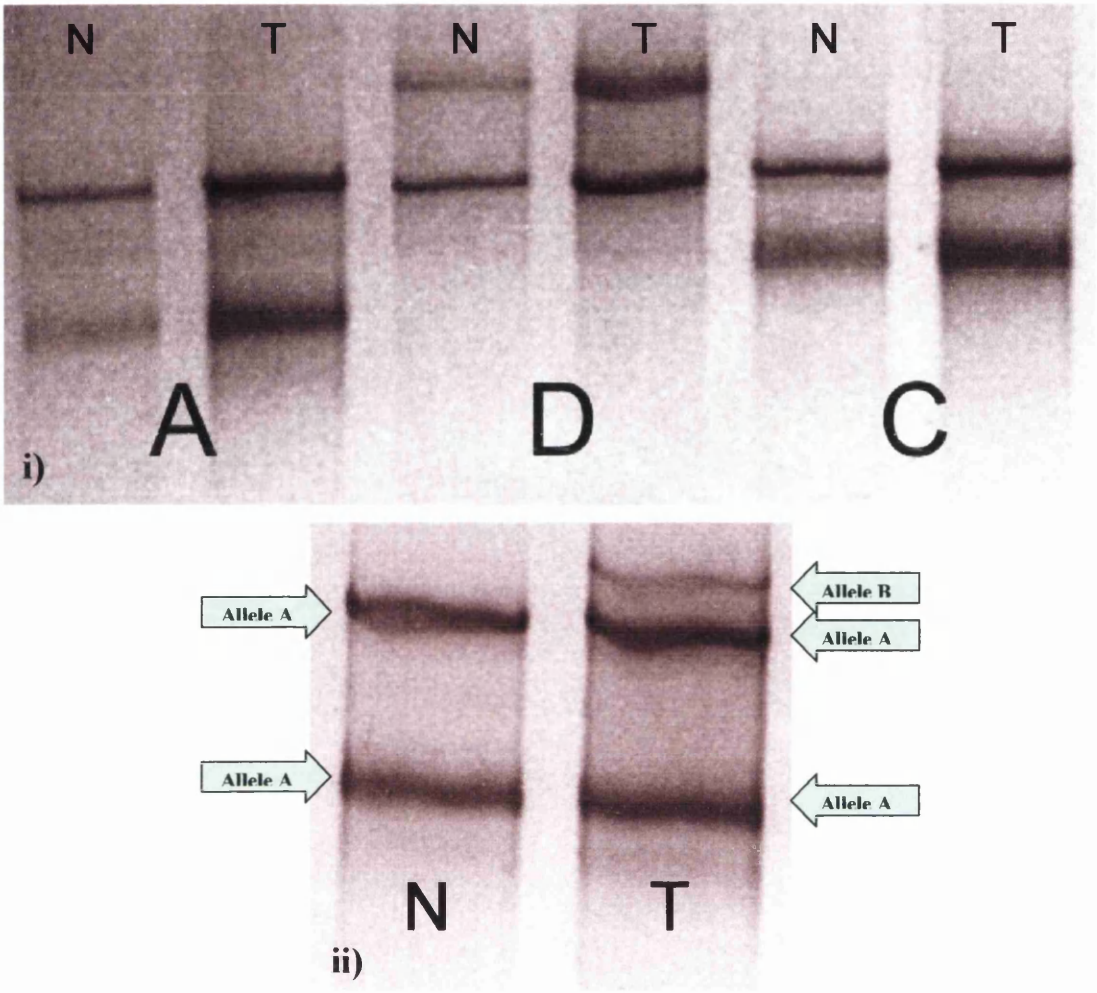


Figure 3.8

(i) Gel image showing SSCP products for the normal (N) and rectal adenocarcinoma tissue (T) for three patients (patient numbers 2, 7 and 11). The alleles A, D and C present in the normal and adenocarcinoma samples can be seen in homoplasmic form, this highlights the variation between individuals for this region of the mtDNA.

(ii) Photograph of a SSCP gel for control region of the normal (N) and sigmoid adenocarcinoma (T) tissue samples from patient number 9, sample 36. The gel image shows the normal tissue from one patient to be homoplasmic for allele A whereas the adenocarcinoma from the same patient is heteroplasmic for both the A and B alleles.

3.3.2 Results of sequencing

The 10 different polymorphisms detected in the 24 patients by SSCP analysis were also detected by sequencing, see Table 3.3. All of the polymorphisms had been previously recorded on Mitomap with the exception of the A-G change at position 178 seen in patient number 9 (Mitomap, 2004). Several samples contained more than one polymorphism. 4 of the polymorphisms were due to deletions or insertions within repeat sequences of G (G_6, position 66-71) or C (C_7, position 303-309; C_5, position 311-315), these altered from the Cambridge sequence in 15/24 (63%) patients with three patients having polymorphisms in more than one of these sequences (figure 3.9).

Nucleotide position	Cambridge sequence	Variation	Frequency
66-71	G_6	G_5	3
114	C	T	1
152	C	T	1
178	A	G	1
222	C	T	1
303-309	C_7	C_6	5
303-309	C_7	C_8	7
310	T	TT	1
311-315	C_5	C_6	3

Table 3.3

The polymorphisms detected across the cohort of 24 patients. Nine polymorphisms are listed, 3 samples had no variation to the Cambridge sequence.

The sequencing also confirmed all of the mutations detected by SSCP, see Table 3.4. One lymph node sample (patient 14, sample 54) contained a homoplasmic G→C transversion mutation which was not detected by SSCP. This gave an overall mutation rate of 9 patients with mutations out of the sample size of 24 (37.5%).

Two of the rectal tumour samples contained more than one mutation (patient number 3, sample 13; patient number 8, sample 34) and two patients had mutations in more than one of the tissues sampled. In total there were 15 mutations from 9 patients out of the cohort of 24 colorectal cancer patients.

Patient Number	Sample Number	Sample type	Sequence alteration	Function of affected mtDNA
3	11	Lymph node	C **del** from C tract **pos 303**	Conserved sequence block 2, mtTF1 binding site,
	13	Rectal tumour	Heteroplasmic G**78**A/G, ATG>GCC, **pos 83,84,85**	Hypervariable region 2
	14	Distant mucosa		
4	16	Rectal tumour	50bp del **pos 298-347**	Hypervariable region 2 MtTF1 binding site H-strand origin Conserved sequence block 2 Replication primer Conserved sequence block 3
	17	Adjacent mucosa		
6	24	Distant mucosa		
	25	Rectal tumour	C del in C-tract **pos 303**	Hypervariable region 2 Conserved sequence block 2, mtTF1 binding site,
8	30	Rectal polyp		
	31	Liver biopsy		
	33	Distant mucosa		
	34	Rectal tumour	Heteroplasmic A**83**G/A, C del in C tract **pos 303**	A83G - Hypervariable region 2 C del - Conserved sequence block 2, C del - mtTF1 binding site,
9	36	Sigmoid tumour	Heteroplasmic (*) G**178**A/G	Hypervariable region 2 H-strand origin
	37	Distant mucosa		
	38	Sigmoid polyp		
	39	Adjacent mucosa		
14	53	Distant mucosa		
	54	Lymph node	G**69**C	Hypervariable region 2
	55	Rectal tumour		
	56	Liver biopsy	C**152**T	Hypervariable region 2 H-strand origin
20	87	Rectal tumour	CT ins in C tract **pos 310**	Hypervariable region 2 Conserved sequence block 2
	88	Adjacent mucosa		
22	102	Sigmoid tumour	C ins **pos 78**	Hypervariable region 2
	104	Distant mucosa		
	105	Lymph node		
23	122	Rectal tumour	C**320**G	Hypervariable region 2 Replication primer
	124	Distant mucosa		

Table 3.4

This table includes all the patients from Table 3.1 which had a sequencing alteration. The nucleotide positions given for each mutation are in reference to the Cambridge sequence. The affected regions for each of the sequence alterations are as listed in Mitomap; C tract refers to the C_7 sequence from position 303-309 (Mitomap, 2004). (*) This sample was heteroplasmic for 1/4 repeats for the change at position 78.

4 of the mutations were C deletions (3) or insertions (1) within the C-tract, Cambridge sequence position 303-309, (figure 3.9). This hotspot was mutated in 4/9 (44%) of the patients with mutations.

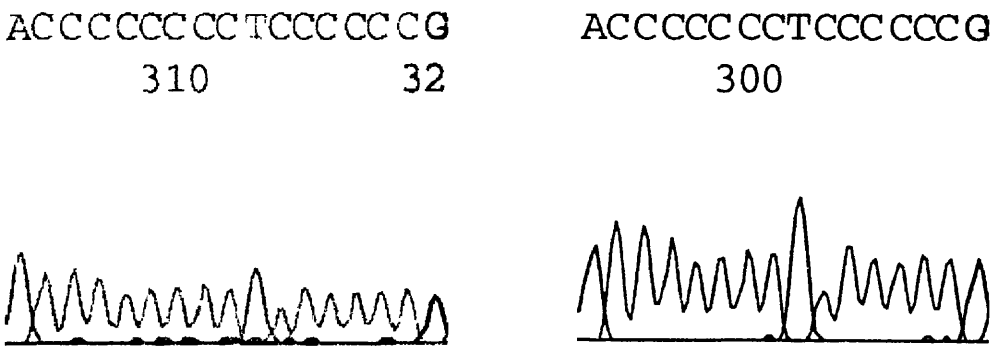


Figure 3.9

These traces show the C-tract region, Cambridge position 303-317 for the normal mucosa and rectal tumour of patient number 6. It can be seen that the normal tissue has C_8TC_6 whereas the rectal adenocarcinoma tissue from the same patient has C_7TC_6 in the same region. This patient had a polymorphism in this sequence in both of the C repeats as the Cambridge sequence is C_7TC_5. The numbers present on the sequencing traces are the number of bases into the sequencing run which vary slightly between runs and do not correlate to the standard Cambridge sequence numbering.

The sequencing also confirmed the heteroplasmy of 2 of the samples (patient 3, sample 13 and patient 8, sample 34). All of the heteroplasmic samples were G→A or A→G transition mutations. The traces showed peaks for both A and G bases and in both cases the heights of these peaks were approximately equal to each other for all repeats. One sigmoid tumour sample (patient 9, sample 36) which was heteroplasmic in all repeats of the SSCP analysis but was less consistent in the sequencing analysis (figure 3.10). The sample was PCR amplified and resequenced four times. One of the sequencing traces showed heteroplasmy with equal peak heights for both the G and A bases, confirming the SSCP analysis. However, three of these repeats showed the mutation to be homoplasmic for G→A although this may be as the smaller peaks on the sequencing traces could not be differentiated from the background levels.

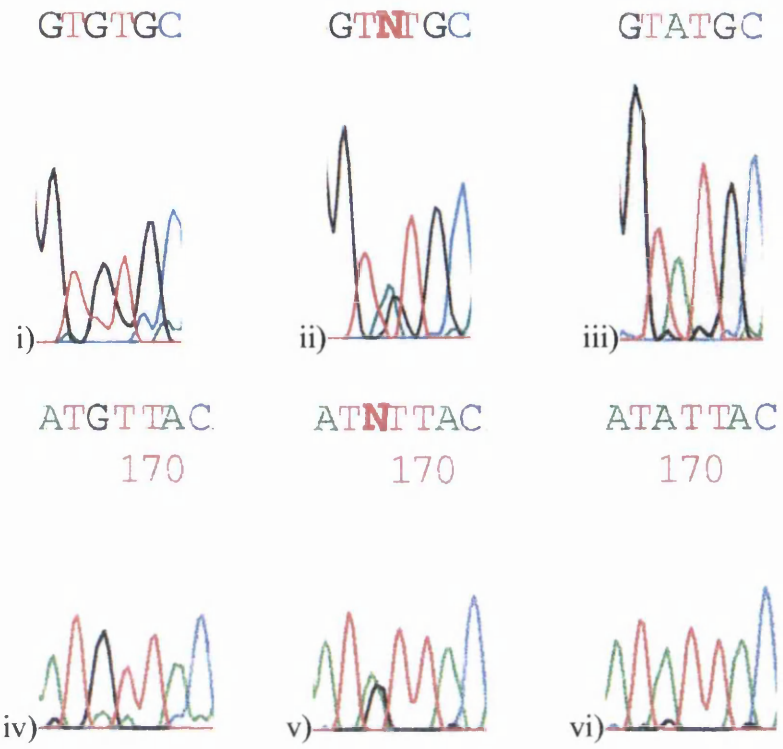


Figure 3.10

These two sets of sequencing traces show the heteroplasmy present in the samples. The top row of traces show the (i) normal mucosa, (ii) rectal adenocarcinoma and (iii) lymph node sample traces from patient 3. It can been seen in these traces that the normal tissue has A at position 78 whereas the rectal adenocarcinoma is heteroplasmic for A/G and the lymph node is homoplasmic for A in this position. The second row show traces from patient 9 for (iv) normal mucosa and (v and repeated in vi) sigmoid adenocarcinoma. The normal tissue has G at position 168, whereas one trace for the adenocarcinoma is heteroplasmic for A/G and a repeat PCR and sequence is homoplasmic for A.

Mutations were present in the rectal adenocarcinoma tissue and also in the metastatic lymph node of one patient (patient number 3). The mutations found in each tissue were different.

From another patient (patient number 14) we were able to obtain sample tissue from normal mucosa, rectal adenocarcinoma and metastatic adenocarcinomas from lymph

node and liver biopsy. Mutations were detected in the metastatic adenocarcinoma samples from liver and also, although not identified by SSCP, mutation in the metastatic lymph node was also detected. The mutations in these metastatic tissues were different from each other.

One patient (patient number 4) had a tumour specific 50bp deletion present in the rectal adenocarcinoma; the fragment deleted included the C tract.

The mutations were predominantly transition mutations (40%), deletion and insertion mutations accounted for a third of the total mutations, transversion mutations made up a fifth and the remaining mutation was a 50bp deletion, see Table 3.5.

Mutations	**Types of mutation**	**Heteroplasmy**
15 mutations from 9/24 patients (37.5%)	6/15 (40%) transition 3/15 (20%) transversion 3/15 (20%) deletion 2/15 (14%) insertion 1/15 (6%) large scale deletion	3/9 (33%)

Table 3.5

A summary of the types of mtDNA mutations found in the 24 colorectal cancer patients

None of the normal mucosa samples from either adjacent to the adenocarcinoma or distant to it contained mtDNA mutations. Likewise, none of the polyps from any of the sampled locations within the colorectum had mutations in the mitochondrial genome. Mutations were found in the adenocarcinoma samples although not from those located within the caecum or transverse colon. Half of the 12 rectal tumours and both of the sigmoid adenocarcinomas contained mtDNA mutations. The metastatic samples were also found to contain mutations, 2/5 lymph nodes and 1/2 of the metastatic liver samples were mutated in their mitochondrial genomes, see Table 3.6.

Tissue Type	Number of samples	Number Mutated (%)
Adjacent mucosa	9	0
Distant mucosa	17	0
Rectal polyp	2	0
Sigmoid polyp	4	0
Transverse polyp	2	0
Caecal polyp	2	0
Rectal tumour	12	6 (50%)
Sigmoid tumour	2	2 (100%)
Transverse tumour	3	0
Caecal tumour	2	0
Metastatic lymph node	5	2 (40%)
Metastatic Liver	2	1 (50%)

Table 3.6

The number of various sample types analysed and the number of these found to contain mtDNA mutations.

3.4 Discussion

3.4.1 Evaluation of techniques

These experiments have proven SSCP to be a valuable quick screening method for identifying previously unknown mutations within the D-loop, especially for the detection of heteroplasmy, as further explained in section 3.4.2. Polyethylene glycol, found in other studies to aid band clarity (Markoff *et al.* 1997), made no discernable alteration to the differentiation of banding patterns in these experiments.

SSCP produced no false positive results for the region amplified. One mutation (sample number 36, A178G) was not detected despite repeated SSCP; this mutation was detected only by sequencing. This false negative result represents 6.7% (1/15) of the mutations detected by sequencing but only 1.7% (1/60) of all the samples analysed. This level of sensitivity is slightly higher than previous studies gauging the sensitivity of SSCP at 89% using PCR products 400bp in length (Hayashi, 1991).

3.4.2 The detection of heteroplasmy

SSCP was more sensitive than sequencing at detecting heteroplasmy, 3 adenocarcinoma samples were found to be heteroplasmic. The 2 rectal tumour samples (patients 3 and 8) were found to have 2 alleles present in their mtDNA in all repeats of SSCP and sequencing. The third sample was a sigmoid tumour (patient 9), this sample showed heteroplasmy in all repeats of SSCP, see Figure 3.8ii, yet was only detected in 1 of 4 sequencing repeats, see Figure 3.10iv-vi. In the other 3 repeats the sequencing trace appeared homoplasmic for the mutant allele. This discrepancy may be due to the inability of sequencing to distinguish between minor allele peaks and background noise.

The intensities of the bands on the SSCP gels varied within each sample, see Figure 3.8, with one band often more intensely stained than the others. This may be an indication of the ratio of alleles present with heteroplasmic samples, with the darker band representing the predominant mtDNA type within a cell with mixed mtDNA. For example, referring to the sigmoid adenocarcinoma tissue of patient 9 seen in

Figure 3.8ii, the darker bands correlates to the wild type mtDNA whereas the lighter band in this heteroplasmic sample is formed from the mutated sequence and as this band is lighter this may indicate this type of mtDNA to be less frequent in the tissue than the wild type. Although these gels have been silver stained which is a non-quantative staining method, this difference in intensities was also observed when the gels were stained with the quantative stain SYBR gold. It may however be an artefact as a result of different efficiencies of various sequences to form a specific conformation. From comparison of the SSCP bands of the homoplasmic samples where one band is frequently more intense than the other for the same allele there is no correlation between band intensity and allele frequency within a sample and this observation is merely an artefact of the technique. The respective peak heights on sequencing traces also do not correlate to the differences seen in SSCP band intensities although this may be due to the sequencing traces themselves not being accurate to measure allele frequencies (Sigurðardóttir *et al.*, 2000).

3.4.3 Polymorphisms detected

A review of several studies indicates that the mutation rate for this region has been estimated at 0.0115 per generation (Sigurðardóttir *et al.*, 2000). The variety of polymorphisms in this study was notable with 10 different germline sequences present in the sample group of 24 patients, see Table 3.3. Several samples had more than one polymorphism in the fragment analysed and only 4/24 (17%) patients exactly matched the Cambridge sequence. The large amount of variation between individuals within the control region is well documented and all of the polymorphisms detected in this study have been previously recorded with the exception of A178G seen in patient number 9 (Mitomap, 2004). This highlights that it is essential to use matched control tissue from the same patient for any mitochondrial genome study.

3.4.4 Mutations detected

3.4.4.1 Age and sex of patients with mutations

As there was no significant difference between the ages of the patients with mtDNA mutations (67.6 years) and that of the overall cohort of patients (68.9 years) this indicates that the somatic mtDNA mutations observed are not purely due to aging. Sex is also not a factor in susceptibility for mutation of the mitochondrial genome as there was no significant difference in the sex ratio of patients with mutations (1.24 F:1 M) compared to the overall cohort of patients (1.18 F:1 M).

3.4.4.2 Mutation rate

The mutation rate of 37.5% was higher than previously published for this region in colorectal samples. These findings differ from those of previous studies of colorectal tumour cells in which no mutations were found within the control region (Heerdt *et al.*, 1994; Polyak *et al.*, 1998). This difference may have arisen because Heerdt's group only analysed 200bp of the control region from nucleotide position 371-570 (Heerdt *et al.*, 1994), which does not include the hypervariable segment 2 within which all of the mutations detected in this present study were found. Since the control region contains hypervariable regions, mutations occur in the control region at a greater rate than the rest of the mitochondrial genome as the remainder coding DNA is well conserved. Despite this relative stability, mutations are well described within the coding region and it is therefore unlikely that the more unstable control region would not contain mutations. It therefore seems unlikely that despite mutations being found in the coding regions of the mtDNA of colorectal tumour cell lines used in Polyak's group that these genomes did not also contain control region mutations (Polyak *et al.*, 1998).

3.4.4.3 Comparison to similar studies

The results from this study do correlate very well with studies of oesophageal, bladder, lung and head and neck tumours which had D-loop mutation frequencies of between 31-36%, see Table 3.5 (Kumimoto *et al.*, 2004; Fliss *et al.*, 2000). The level of heteroplasmy found in this study is at a very similar level to that found in oesophageal tumours at 33% and 31% respectively. The types of mutations found in

the four studies outlined in Table 3.7 followed a similar pattern to each other and, with the exception of the oesophageal study, transition mutations were the most prominent type of mutation. These types of mutation are consistent with ROS related mutation (Cadet *et al.*, 1997).

Study	Tissue	Technique	Mutations	Types of mutation	Hetero-plasmy	D-Loop
Current study	Colorectal tumour surgical resections	PCR-SSCP and sequencing of 400bp region of D-loop	15 mutations from 9/24 patients (37.5%)	40% transition 20% transversion 21% deletion 13% insertion 6% large scale deletion	3/9 (33%)	9/24 (37.5%)
Polyak *et al.*, 1998	Colon cancer cell lines	PCR and sequencing of whole mitochondrial genome	12 mutation from 7/10 patients (70%)	84% transition 8% transversion 8% insertion	2/7 (29%)	0/10 (0%)
Kumimoto *et al.*, 2004	Oesophageal tumour biopsies	PCR and sequencing of hypervariable regions of D-loop	14 mutations from 13/38 patients (34%)	21% transition 50% deletions 29% insertions	4/13 (31%)	13/38 (34%)
Fliss *et al.*, 2000	Bladder tumour surgical resections	PCR of 80% of the genome including the D-loop	20 mutations from 9/14 patients (64%)	75% transition 10% transversion 10% deletion 5% insertion	2/14 (14%)	5/14 (36%)
	Head and neck tumour surgical resections	PCR of 80% of the genome including the D-loop	9 mutations from 6/13 patients (46%)	78% transition 11% deletion 11% insertion	0/13 (0%)	4/13 (31%)
	Lung tumour surgical resections	PCR of 80% of the genome including the D-loop	10 mutations from 6/14 patients (43%)	80% transition 10% transversion 10% deletion	0/14 (0%)	5/14 (36%)
Total of all above studies			80 mutations from 50/112 (45%)	61% transition 9% transversion 18% deletion 11% insertion 1% large scale deletion	11/109 (10%)	36/112 (32%)

Table 3.7

A comparison of mutation frequencies across four studies of mitochondrial mutations in tumourigenic tissues. The table also indicates the proportion of samples with mutations present in the D-loop region.

The level of C tract mutations in this study was 4/9 (44%) patients, this was similar to that of the oesophageal tumour study at 11/38 (29%) patients this reiterates the highly mutable status of this sequence of the control region.

4 of the 9 patients (44%) had mutations within the C tract. Within the nuclear genome insertions or deletions within repeat sequences known as microsatellites indicate a form of genomic instability known as nuclear microsatellite instability (nMSI). These C tract mutations can be described as the mitochondrial equivalent and is termed mitochondrial microsatellite instability (mtMSI). Nuclear MSI occurs as a result of a deficiency in mismatch repair (MMR), little however is known about the role of MMR in the mitochondrial genome. Work using yeast models have identified proteins involved in nuclear MMR within the mitochondria; this had led to the belief that some form of MMR of mtDNA does exist (Reenan and Kolodner, 1992). Studies of mtMSI in oesophageal, breast and colorectal cancer have found no correlation between mitochondrial and nuclear MSI (Kumimoto *et al.*, 2004; Richard *et al.*, 2000; Polyak *et al.*, 1998). A study of gastric cancers has though found a positive correlation for these forms of genomic instability (Habano *et al.*, 2000).

3.4.4.4 Detection of 50bp deletion

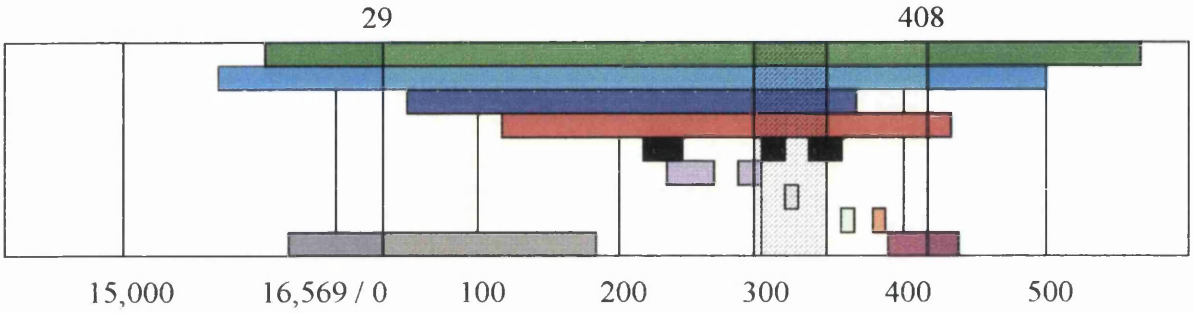
One patient (patient number 4, sample 16) had a rectal tumour containing a homoplasmic mtDNA 50bp deletion, flanked by a 9bp direct repeat, see Figure 3.11.

CCACCAAACCCCCCCTCCCCCGCTTCTGGCCACAGCACTTAAACACATCTCTGCCAAACCCCAA

Figure 3.11

The light strand of the Cambridge sequence for nucleotides 295-358. The sequence highlighted in the green box is the 50bp portion of the mtDNA deleted in the rectal adenocarcinoma of patient 4. The 2 sequences in red are 9bp direct repeat sequences.

The mtDNA molecule had the 4bp replication primer region as well as 50bp of the H-strand origin of replication deleted. The missing fragment also contained 5bp of the mtTF1 binding site, all of the 16bp conserved sequence block 2, 2bp of the conserved sequence block 3 and 50bp of the hypervariable segment 2. It is unlikely that such a large scale deletion would not affect the replication of the mtDNA molecule and in turn the function of the mitochondria and at the higher level of the cell and tissue.



- PCR product (29-408)
- D-loop (16024-576)
- Membrane attachment site (15925-499)
- Hypervariable segment 2 (57-372)
- H-strand origin (110-441)
- Conserved sequence blocks 1, 2, and 3 (213-235, 299-315, 346-363)
- mtTF1 binding site (233-260, 276-303)
- Replication primer (317-321)
- mt4 H-strand control element (371-379)
- mt3 H-strand control element (384-391)
- 7S DNA (16106-191)
- L-strand promoter (392-445)
- 50bp deletion seen in the rectal tumour of patient 4, sample 16 (298-347)

Figure 3.12

This figure illustrates the position of the 50bp deletion within the PCR product of the mtDNA control region. It can be clearly seen that the deleted fragment includes 50bp of the D-loop, membrane attachment site, hypervariable segment 2 and H-strand origin as well as the entire replication primer and conserved sequence block 2 and portions of the mtTF1 binding site and conserved sequence block 3. The numbers alongside the diagram and following the functional sequence descriptions are the relevant nucleotide positions of the mitochondrial genome according to the Cambridge mtDNA sequence (Mitomap, 2004).

Although it has been shown that specific alleles can dominate mitochondrion and become homoplasmic with no selective advantage (Coller *et al.*, 2001) it seems that despite the deletion of the H-strand origin of replication, the 50bp deletion seen in this sample may have an advantage over full length mitochondrial genomes in terms of a shorter replication time. As a result, this smaller molecule came to dominate the mitochondria and cell and through the clonal mechanism of tumorigenesis this has then become homoplasmic for the rectal adenocarcinoma.

This large scale deletion has also been found as a somatic mutation in gastric cancers (Burgart *et al.*, 1995). In this study the mutation was found in 4/77 (5.2%) gastric adenocarcinomas, a similar level to this current study 1/24 (4.2%). Half of these affected tumours were homoplasmic for this deletion whilst the other two cases had the mutation present in the heteroplasmic form accounting for <5% of the mtDNA. This study suggests that the 50bp deletion may have no effect on the function of the mitochondria as the mitochondrial DNA was able to replicate without the H-strand origin of replication and suggested the deletion may have created a new heavy strand promoter (Burgart *et al.*, 1995).

3.4.4.5 Level of heteroplasmy

All of the normal and precancerous tissues in all patients were homoplasmic. The level of heteroplasmy found in this study was 3/9 (33%) patients with mtDNA mutations. This level is very similar to that found within oesophageal tumours 4/13 (31%) and colorectal cell lines 2/7 (29%) (Kumimoto *et al.*, 2004; Polyak *et al.*, 1998). However, a far lower level of heteroplasmy was detected in another study of lung 0/14 (0%), bladder 2/14 (14%) and head and neck 0/13 (0%) tumours (Fliss *et al.*, 2000).

The study of oesophageal tissue found 4/38 normal tissues to be heteroplasmic within the C tract region from Cambridge sequence position 303-309. This was not observed in this research of colorectal cancer patients or in the other studies reviewed in Table 3.4.

Cell fusion experiments have shown that mitochondrial DNA from tumours can have a significant replicative advantage over other types of mtDNA and even give significant selective advantage on a cellular level (Shay and Werbin, 1978; Shay and Ishii, 1990). When two tumour cell lines are fused the mitochondria of one rapidly replace those of the other, it has been suggested that this is due to the mutated mtDNA molecules replicating at a higher rate to compensate for the affect on OXPHOS that deleterious mtDNA mutations may cause (Polyak *et al.*, 1998). These theories can explain the homoplasmy seen in other studies and the homoplasmic mutations seen in this study, such as the 50bp deletion seen in the tumour tissue of patient 4 which would be likely to have a negative effect upon the OXPHOS of the cell. The selective growth advantage may be a result of mutations in either the mitochondrial or nuclear genome (Augenlicht and Heerdt, 2001). This may though also be a result purely of random segregation from the high number of cell generations, around 600, seen in tumour tissue (Coller *et al.*, 2001). This homoplasmy however was not seen in all the samples of this present study, it is possible that the heteroplasmy was not detected in previous studies of lung, bladder and head and neck tumours which relied entirely on sequencing (Fliss *et al.*, 2000), as in this study sequencing also did not always detect the less prominent allele. It has previously been estimated that the minor allele needs to be present at a frequency of $\geq$30% in order to be detectable by sequencing (Nickerson *et al.*, 1997). Other studies have shown respective sequencing trace peak heights to vary according to sequencing direction and concluded that sequencing is not a reliable technique for determining degree or presence of heteroplasmy for the control region of mtDNA (Sigurðardóttir *et al.*, 2000). In the experiments described in this chapter all cases of heteroplasmy were detected on the SSCP gels, which exhibited extra bands for the heteroplasmic tissues whereas only two clear bands could be seen for other homoplasmic tissues. If previous groups have relied on sequencing alone and the sequencing traces not analysed for minor alleles this may explain why little heteroplasmy has been reported in some of the previous studies.

3.4.5 Effect of the mtDNA mutations

There has been much debate as to the role of mtDNA mutations in carcinogenesis. Whereas mutations in the mitochondrial genome have long since been known to cause diseases such as Leber's hereditary optic neuropathy, their role in cancer development

is still uncertain. It is unclear whether these mutations are simply the result of cellular instability due to mutations in nuclear oncogenes which affect the stability of both the nuclear and mitochondrial genomes or whether the damaged mtDNA is directly involved in causing the cancer. One way in which this is possible is if mtDNA mutations cause an increase in the amount of ROS which mutates the mtDNA further and also causes mutations within the nuclear genome. Another possible mechanism for the mtDNA to play a role in carcinogenesis is through apoptosis. Mutations within the mtDNA may affect the membrane potential of the mitochondria making it less able to release the pro-caspases required in the initiation of apoptosis. This makes the cell more resistant to apoptosis and so may replicate with high levels of genetic damage and assist the pathway of carcinogenesis.

Decreased mitochondrial gene expression has been reported in colonic tumours (Heerdt *et al.*, 1990). The majority of the mutations detected in this study would though be unlikely to affect the function of the mitochondria as 73% (11/15) of the mutations lay within the hypervariable region II. 27% (4/15) of the mutations were in the C-tract, which is a variable region across individuals (Mitomap, 2004). This is in accordance with the results for this region previously published (Heerdt *et al.*, 1994). However, there were some mutations lying within sequences of the mitochondrial genome which have a critical role in the function of the mtDNA such as the promoter regions for the L- and H-strand. Despite this it is still difficult to establish the effect of mutations in the mtDNA as it is as yet unknown how the mitochondrial genomes within the individual mitochondria and within the cell interact with respect to genetic dominant and recessive effects (Augenlicht and Heerdt, 2001). Even when the cell is homoplasmic the function of the mitochondria is dependant also upon the nuclear genome as the mtDNA does not code for all of the proteins the organelle requires (Fos *et al.*, 1990; Dunbar *et al.*, 1995). It has also been estimated that up to 80% of the cells mitochondrial genomes can be mutated with no phenotypic effect (Shoffner and Wallace, 1992).

As the mutations detected in this study are unlikely to have an effect on the function of the mitochondria and as these mutations were only found in the later stages of cancer development this suggests that the mutations are an epiphenomenon resulting

from changes in the nuclear genome and are not actually causative of carcinogenesis in their own right.

3.4.6 Location of mutated samples within the colon

All of the mtDNA mutations were found in the sigmoid colon and rectum, with no mutations present in either the ascending or transverse colon samples. The location within the colon of the tumours found with mitochondrial mutations may be a result of the differing functions along the length of the colon. The ascending and transverse sections of colon contain more dilute faecal matter, which is continuously being moved by smooth muscle contractions along the colon known as peristalsis. The sigmoid colon and rectum contain more concentrated faeces where they are stored until expulsion. This results in a prolonged exposure to higher levels of potential carcinogens from endogenous and exogenous agents in the faeces in comparison to other regions of the colon. One such mutagenic agent is bile acid, which is found to be excreted more in those with a higher consumption of fat (Reddy, 1981), another colorectal cancer risk factor. A study has shown an increase in excreted faecal unconjugated bile acids in patients with colorectal cancer and polyps (Imray *et al.*, 1992). After the consumption of a fatty meal, the level of bile in the faeces increases to a concentration which causes DNA damage by the production of ROS. The damaged cells then undergo apoptosis (Glinghammar *et al.*, 2002). After cells in an area of mucosa have been killed, surviving cells from nearby areas migrate to produce a new epithelium. Cells are more likely to survive exposure to cytotoxic agents if they have a higher resistance to apoptosis. Therefore, over time, the colonic mucosa of those eating many high fat meals may become populated by an increased number of cells resistant to apoptosis and so the burden of irreparable DNA damage increases giving a higher colorectal cancer risk (Bernstein *et al.*, 1999). This can also explain why 50% of colorectal tumours are found in this region despite this region being less than a third of the total colon and that different mechanisms for tumorigenesis are responsible for the majority of cancers found in the different regions of the colon (Spence and Johnston, 2001). Tumours which exhibit nuclear microsatellite instability are predominantly located on the right side of the colon; yet none of the tumours exhibiting mitochondrial microsatellite instability (mtMSI) were in this location.

It is possible that other exogenous factors are responsible for an increased risk of causing mtDNA mutations; for example, mtDNA has been shown to be mutated by carcinogens such as benzo(a)pyrene, polycyclic aromatic compounds and aflatoxin at a higher rate than nuclear DNA (Backer and Weinstein, 1980; Allen and Coombs, 1980; Niranjan *et al.*, 1982). Studies on the parotid gland found mtDNA mutations in the histologically normal tissue samples from 22% of the smokers analysed (Lewis *et al.*, 2002). However, an evaluation of smoking status and alcohol consumption levels of patients with oesophageal cancers found no significant relationship between these lifestyle factors and presence of mtDNA mutation (Kumimoto *et al.*, 2004). Unfortunately data on diet, smoking status and alcohol consumption levels were not obtained for the colorectal cancer patients in this study.

3.4.7 Assessment of mutation level within coding regions of the mtDNA

In a scan using SSCP of a 473bp fragment of the conserved gene NADH dehydrogenase subunit 2 (ND2) of 34 of the patient samples used in the above described experiments I found no detectable mutations. In a previous study of this ND2 region of the mtDNA in the histologically normal parotid tissue of smokers, 21.7% (5/23) of the samples were found to contain point mutations compared with none of the 16 non-smokers (Lewis *et al.*, 2002). Although the parotid tissue mtDNA mutations could be detected before any histological changes could be seen, in colorectal adenocarcinoma tissue, which is clearly different from normal mucosa, no such mutations in the ND2 gene were detected by SSCP.

3.4.8 Samples and sample numbers

This is one of the largest studies of its kind with 65 samples from 24 patients; it is also the only study to look at mtDNA mutations within pre-cancerous polyp samples and metastatic secondary cancers in the liver and lymph node. The sex ratio and the age range of the cohort also typical for colorectal cancer in the general population. There was a bias in the locations of the tumours within the colon with the majority of cancers from the rectal region of the colorectum with 12/20 (60%) of the adenocarcinoma samples from the rectum. The samples were collected over 12 months simply as they became available through surgery and as such they are

representative of the occurrence of these cancers within South Wales. Patients with left-sided tumours generally present earlier as these affect bowel function sooner and may result in fresh bleeding par rectum. Right-sided tumours are less likely to affect bowel function but patients present with more vague symptoms such as chronic tiredness due to anaemia. These latter tumour types are therefore more likely to be left until much later stages of carcinogenesis where the cancer becomes inoperable and are therefore not seen as often in surgery although the rate of incidence may be higher than the number of these sample types in this study suggest.

Another point that must be clarified is that all of the mtDNA mutations seen in primary tumours were found in either rectal or sigmoid adenocarcinoma tissue samples. This is 50% of all the rectal adenocarcinomas sampled and all of the sigmoid tumours. However, whilst there were 12 rectal tumours sampled there were only 2 sigmoid tumours in the sample group. The small number of sigmoid adenocarcinomas in this study means that whilst the two samples may be representative of tumours from this region this is unlikely due to the possibility of sampling error and it is therefore impossible to extend the claim that all sigmoid adenocarcinomas contain mtDNA control region mutations

3.4.9 Diagnostic potential of mitochondrial DNA mutations

This study found tumour specific mitochondrial DNA mutations in 37.5% (9/24) of patients and of these 44% (4/9) were in a short sequence of the control region, the C-tract spanning from nucleotide position 303-309. One purpose of these experiments was to discover if there were any mutational hotspots within the mitochondrial genome which could be used to screen for colorectal cancer as screening the entire genome is not a cost effective method, at least not with currently available techniques. Mutations in the tumour suppressor gene p53 have been detected in the faeces of patients whose tumours contained these mutations (Dong *et al.*, 2001). These mutations occur late in the progression of carcinogenesis and so an earlier marker would be a significant advance in the field of colorectal cancer diagnostics. The results from these experiments show that 17% (4/24) of patients have a tumour specific mutation within a 7 base sequence of their mitochondrial genomes. As this is such a small region, it is a realistic target for screening. Since these C-tract mutations

were not detected in all of the patients, this would have to be used as part of a bank of screening methods to detect all colorectal cancers. This bank of markers could include APC which is mutated early on in cancer progression and, to detect other cancers developed from other pathways, a marker for nMSI such as transforming growth factor β-type receptor type II (TGFβRII) which again occurs early in the development of carcinomas which have deficiencies in mismatch repair (Atkin and Martin, 2001; Grady *et al.*, 1998; Samowitz *et al.*, 2001b).

The other notable point about these results in terms of the potential for this region to be used for screening is the stages at which these mtDNA alterations were detectable. The perfect screening method for colorectal cancer would be non-invasive, cost-effective, accurate and sensitive to the earliest detectable changes as cancers form where treatment less radical, often requiring surgery alone and if detected at Dukes' stage A or B the overall survival rate is 80-95%. Whilst the results in this study provide scope for a non-invasive screening technique that is cost effective the mutations were only detected in the adenocarcinoma and metastatic carcinoma stages of progression. At these stages the cancers are rarely asymptomatic and, for example, if detected in Dukes stage C or D, where 63% of colorectal cancers are detected, there is a 5 year survival rate of between 5-20% and as such this proposed screening method has little clinical value. Whether the cancers with these mutations respond differently to various forms of treatment such as chemotherapy or are particularly aggressive or indeed have a higher rate of survival than the cancers without mitochondrial genome aberrations is an area worthy of further investigation.

3.4.10 Discussion summary

This is the first study of its kind to analyse precancerous lesions such as polyps as well as colorectal carcinomas and normal mucosa from distant and adjacent positions to the adenocarcinomas. This is also the largest mtDNA study of colorectal cancer patients and used surgical samples rather than cell cultures which tend to obtain mutations as they undergo the many generations typical of cell culture. This has provided valuable information as to when the mtDNA mutations occur against the progression of carcinogenesis within the colorectum.

As no mutations were present in any of the normal, adenoma and hyperplastic polyp samples and in one patient found in metastatic adenocarcinomas tissue but not in matched adenocarcinoma tissue this suggests that these mitochondrial mutations occur at a late stage of progression. These results suggest that mtDNA mutations are an epiphenomenon rather than a driving factor of colorectal carcinogenesis. These late occurring mutations would therefore be of little clinical significance in terms of an early molecular biomarker for the detection of colorectal cancer. Despite this, these mutations, which were found in 9/24 (37.5%) patients, could still be valuable as a late biomarker. These could be valuable in detecting fully formed carcinomas in, for example, the faeces of patients post operational to detect recurrence of the disease. These results are also of importance in the context of elucidating more about the different mechanisms in the carcinogenesis of colorectal tumours in different locations within the colon. It may also be possible to identify environmental agents responsible for causing colorectal cancer by comparing the mutational spectra of the tumours against those recorded for various carcinogens. Due to the nature of the mutations, predominantly in microsatellites within the mtDNA, these results also may add insight into the mismatch repair mechanisms of the mitochondrial genome. To try to establish any connection between mtMSI and nMSI I have assessed all the samples analysed in this chapter for nMSI. This research is the content of Chapter 4.

Chapter 4

The evaluation of nuclear microsatellite stability status using fragment analysis

4.1 Introduction

Colorectal cancer arises when epithelial cells within the colorectum become mutated so that the normal controls regulating cell growth and cell death are no longer able to balance these processes leading to tumour development. There are several proposed pathways by which carcinogenesis can occur (Narayan and Roy, 2003). The majority of colorectal cancers arise from the chromosome instability (CIN) pathway which is discussed further in Chapter 6. However, a significant proportion of sporadic colorectal cancers and also those cancers which develop in patients with the familial disease hereditary nonpolyposis colorectal cancer arise from a different route known as the microsatellite instability (MSI) pathway (Minamoto and Ronai, 2001). The proposed pathways of colorectal carcinogenesis are illustrated in Figure 4.1 with the MSI pathway highlighted in blue. In Chapter 3, mutations were found in a polyC microsatellite present within the control region of the mitochondrial DNA of tumour tissue in 17% of patients studied. The purpose of this current chapter is to analyse these same patients to establish whether there is a link between microsatellite instability (MSI) in the mitochondrial and nuclear genomes.

4.1.1 Microsatellites

The term microsatellite is derived from the term for satellite DNA from the observation that fragments of DNA with tandemly-repeated sequences form 3 'satellite' bands when genomic DNA is fractionated by density gradient centrifugation as these fragments of DNA have atypical buoyant densities in comparison to the average density of human DNA (Brown, 1999; Gall *et al.*, 1973). Microsatellites are repetitive sequences of nucleotide repeats usually less than 100 bases in length (Lodish *et al.*, 1995). These tandem repeated sequences are usually between 1bp, such as mononucleotide repeats of cytosine (poly(C) tracts) and pentanucleotide motifs of

5bp repeats. These types of sequences are frequent throughout the genome and are randomly distributed (Sutherland *et al.*, 1995). The function of microsatellites is as yet unknown (Brown, 1999).

4.1.2 Microsatellite instability

Microsatellite instability (MSI) is characterised by alterations to these simple repeat sequences, including insertions and deletions both of which result in frameshift mutations and in turn, where these lie in coding regions, in truncated proteins. Microsatellites are prone to these mutations which occur as a result of slipped strand mispairing. This mispairing predominantly occurs during DNA replication when the normal pairing between complementary DNA strands is altered by staggering of the repeats on the 2 strands. Deletions or insertions are a result of the newly synthesised strand slipping forward or backwards respectively (Strachan and Read, 1998).

MSI arises due to defects in the system responsible for maintaining genomic stability known as mismatch repair (MMR) (Wheeler *et al.*, 2000). MMR increases the fidelity of DNA replication by identifying and removing single-base mismatches and insertion-deletion loops that may arise from strand slippage. MMR involves replication, repair and recombination stages (Narayan and Roy, 2003). This repair system is the reason why, despite their more vulnerable sequences, the mutation rate at these microsatellite sites is only slightly higher than at other genomic sites at around 5×10^{-4} to 5×10^{-5}. However, in cells that are MMR deficient the overall mutation rate is 100-1000 fold higher than in normal cells (Hearne *et al.*, 1992; Narayan and Roy, 2003). A germline or somatic mutation in any of the genes encoding for the proteins involved in any part of this complex process, such as hMSH2, hMSH6, MLH3 and hMLH1, can give rise to MSI (Wu *et al.*, 2001; Wu *et al.*, 1999; Wheeler *et al.*, 2000). MMR is discussed further in Chapter 1, section 1.1.1.3. MSI tumours are also termed mismatch repair deficient (MMR^-) or replication error positive (RER^+) as they are unable to repair errors occurring during DNA replication.

Throughout their development, MSI tumours accumulate over 10^5 clonal somatic deletions and insertions in the microsatellite repeats with at least 10 occurring in functional sequences (Ionov *et al.*, 1993; Ionov *et al.*, 2000; Duval *et al.*, 1999). MSI

tumours often have frameshift mutations within the repeat sequences in the MMR genes hMSH3 and hMSH6 (Malkhosyan *et al.*, 1996; Wu *et al.*, 1999). Transforming growth factor-β receptor II (TGF-βRII), Bax, insulin-like growth factor II receptor gene (IGFIIR), phosphatase and tension homologue deleted on chromosome 10 gene (PTEN) and caspase 5 are all known gene targets of MMR gene inactivation as these have microsatellite regions prone to mutation and are also all involved in cell growth regulation (Souza *et al.*, 1996; Narayan and Roy, 2003). Associations have been established between MMR deficiency and loss of normal cell cycle control, particularly G_2 arrest as a result of the disruption of these targeted genes (Fallik *et al.*, 2003).

As well as the length of the microsatellite and the preceding nucleotide sequence, the nature of the base repeat sequence (such as mono- or dinucleotide repeats) and the chromatin structures surrounding the repeat sequence are also important factors for determining the rate of mutation within the microsatellite (Zhang *et al.*, 2001; Boyer *et al.*, 2002). For example, mononucleotide repeat sequences of G have been shown to be significantly more prone to mutation than A tracts (Zhang *et al.*, 2001; Boyer *et al.*, 2002). The mutation of coding microsatellites in MMR^- tumours also appear to be affected by the gene function as these mutations are non-random and several genes are targeted (Duval and Hamelin, 2003). This is explained in further detail later in this chapter.

4.1.3 Microsatellite instability and colorectal cancer

4.1.3.1 Hereditary non-polyposis colorectal carcinoma (HNPCC)

The hereditary disease HNPCC is caused by inherited dominant germline mutations of mismatch repair genes and is responsible for between 5-13% of colorectal carcinomas (Wheeler *et al.*, 2000; Minamoto and Ronai, 2001). The resulting carcinomas have a mutator phenotype due to replication errors that are not repaired with these mutations mainly occurring in microsatellites. hMLH1 or hMSH2 mutations account for the majority of germline mutations in HNPCC families (Ward *et al.*, 2001). As HNPCC polyps develop to malignancy at a faster rate than familial adenomatous polyposis (FAP) polyps it is thought that the MSI mutator phenotype

may be responsible for this acceleration of tumour development (Chung, 2000). This disease has also provided invaluable insight into this MSI pathway of carcinogenesis which also occurs in around 6-18% of sporadic colorectal cancer cases (Young *et al.*, 1993; Thibodeau *et al.*, 1993; Ionov *et al.*, 1993; Chao *et al.*, 2000).

4.1.3.2 Microsatellite instability and sporadic colorectal cancer

Sporadic colorectal cancers with MSI occur as a result of hypermethylation of the promoter region or new somatic mutation of mismatch repair genes (Herman *et al.*, 1998). Mutations within poly(A) tracts were first used to define MSI in human tumours. This study of sporadic colorectal cancers suggested that 12% of colorectal carcinomas contained these mutations and the instability of simple repeat sequences was greater in one subset of colon tumours than others, suggesting a different mechanism of tumourigenesis (Ionov *et al.*, 1993). This research promoted further studies and higher order microsatellites (such as di-, tri- and tetra-nucleotide repeats) were analysed in sporadic and familial colorectal cancers and again alterations were found (Young *et al.*, 1993).

Studies have shown that 93% of MSI sporadic tumours are located in the proximal colon; but proximal colonic tumours are not predominantly microsatellite unstable (Thibodeau *et al.*, 1998; Ward *et al.*, 2001, Chung, 2000). Sporadic MSI tumours have a mucinous phenotype, are generally at Dukes' stage B (stages of colorectal cancer are described in Chapter 1, section 1.2.1) and more frequently occur in women (Ward *et al.*, 2001; Thibodeau *et al.*, 1998; Samowitz *et al.*, 2001b; Chung, 2000). Tumours with MSI also have distinctive clinical features such as poor differentiation, reduced invasiveness, prominent infiltration of lymphocytes and a Crohn's-like reaction in surrounding tissue where there is an aggregation of lymphoid cells (Chung, 2000). Although originally no correlation with MSI tumours and age was found this may have been because the mean age is the same for MSI and microsatellite stable (MSS) patients. The distribution of ages is though very different for MSI tumours which have a bimodal distribution with most of these tumours developing in younger patients as well as in patients over 75 years (Thibodeau *et al.*, 1993; Chao *et al.*, 2000).

MSI tumours have been linked to cigarette smoking, with a linear relationship between risk of MSI tumours and amount smoked in terms of cigarettes per day and number of years of smoking (Slattery *et al.*, 2000; Samowitz *et al.*, 2001b). From a study of over 1500 patients, it has been estimated that smoking ≥20 cigarettes a day doubles your risk of MSI colon cancer, a risk that is only removed after having stopped smoking for over 15 years. A recent study has suggested that 21% of MSI colon cancers can be attributed to cigarette smoking (Slattery *et al.*, 2000).

4.1.3.2.1 MSI and prognosis in sporadic colorectal cancer

MSI tumours were also shown to be linked to a better prognosis, especially in patients under 50 years old, with a reduction of approximately 60% in the risk of death due to colorectal cancer (Gryfe *et al.*, 2000; Samowitz *et al.*, 2001a). This increase in patient survival is possibly due to an induction of an antitumour response, as lymphocytic infiltrates have been detected in these tumour types (Chung, 2000; Ward *et al.*, 2001). Another possible explanation for the enhanced survival of patients with these cancer types could be the tendency of MSI-H tumours to fail to progress from localised to metastatic tumours as the mutational load of these tumours becomes detrimental to the growth of the tumour and limits the metastatic potential (Shibata *et al.*, 1994). This reduction in the ability to progress through the carcinogenesis pathway could be a result of the correlation of MSI-H tumours with wild type p53. The lack of p53 mutation may also explain why many MSI-H tumours in stages II and III are larger in size than MSS tumours and therefore may have an increase in severity of symptoms prompting the patient to present earlier than those with MSS tumours (Ward *et al.*, 2001). MSI tumours have been thought to have a reduced risk of liver metastasis (Ionov *et al.*, 1993; Gryfe *et al.*, 2000). A more recent study has challenged this, finding their series of liver metastatic tumours to have a similar proportion of MSI as colorectal carcinomas which did not metastasise (Fallik *et al.*, 2003).

4.1.4 Microsatellite instability and choice of chemotherapeutic treatments

As well as mutation, RER^+ has been shown to be caused by biallelic hypermethylation of the promoter regions of MMR genes especially in sporadic cases (Herman *et al.*, 1998; Cunningham *et al.*, 1998). This hypermethylation of MMR gene promoter regions may explain why MMR^- cells have been found to be highly tolerant to methylating chemotherapeutic drugs and therefore less effective at treating these types of tumour (Claij and Riele, 1999). Methylating chemotherapy agents also work by producing O^6-methylG which then mispairs with thymine. This is then excised by MMR but is then replaced by the same mutation, MMR occurs again and so long-standing DNA breaks occur which trigger apoptosis (Ma *et al*, 2002). In cells that lack MMR, these methylating agents can simply cause further mutation. Studies have also shown that metastatic colorectal tumours with MSI have a better response to the chemotherapeutic agent irinotecan than those that are not MSI (Fallik *et al.*, 2003). Therefore, MMR status plays a crucial role in the toxicity of DNA-damaging drugs used in cancer chemotherapy and is important in the choice of treatments for colorectal cancer, especially where first-line treatments such as 5-fluorouracil have failed (Carethers *et al.*, 2003). 5-fluorouracil has been found to be less effective in MMR^- cell lines and more importantly has been shown to actually reduce the survival of patients with MSI-H stage II and III colorectal tumours. This is because patients lose their improved survival if treated with 5-fluorouracil with the 5 year survival dropping from >85% if untreated to only 70% following treatment (Carethers *et al.*, 2003; Ribic *et al.*, 2003). Other drugs such as the topoisomerase-I inhibitor chemotherapeutic agent camptothecin have been shown to successfully kill MMR^- cancer cells exhibiting MSI-H (Bras-Goncalves *et al.*, 2000). These studies have highlighted the importance of establishing the MSI status of a tumour before deciding the most appropriate chemotherapeutic treatment.

4.1.5 Pathway of tumourigenesis for MMR^- tumours

Studies analysing patients with the familial disease hereditary nonpolyposis colorectal cancer (HNPCC) have provided insights into not only this disease but also into the subset of MSI sporadic colorectal cancers. One of the earliest studies to suggest a different pathway for these cancers was published by Konishi in 1996. It found that replication error positive status occurs at an early stage of carcinogenesis in HNPCC

but at a later stage in non-HNPCC and suggested that this may indicate that HNPCC tumours develop through a different adenoma-carcinoma sequence (Konishi *et al.*, 1996).

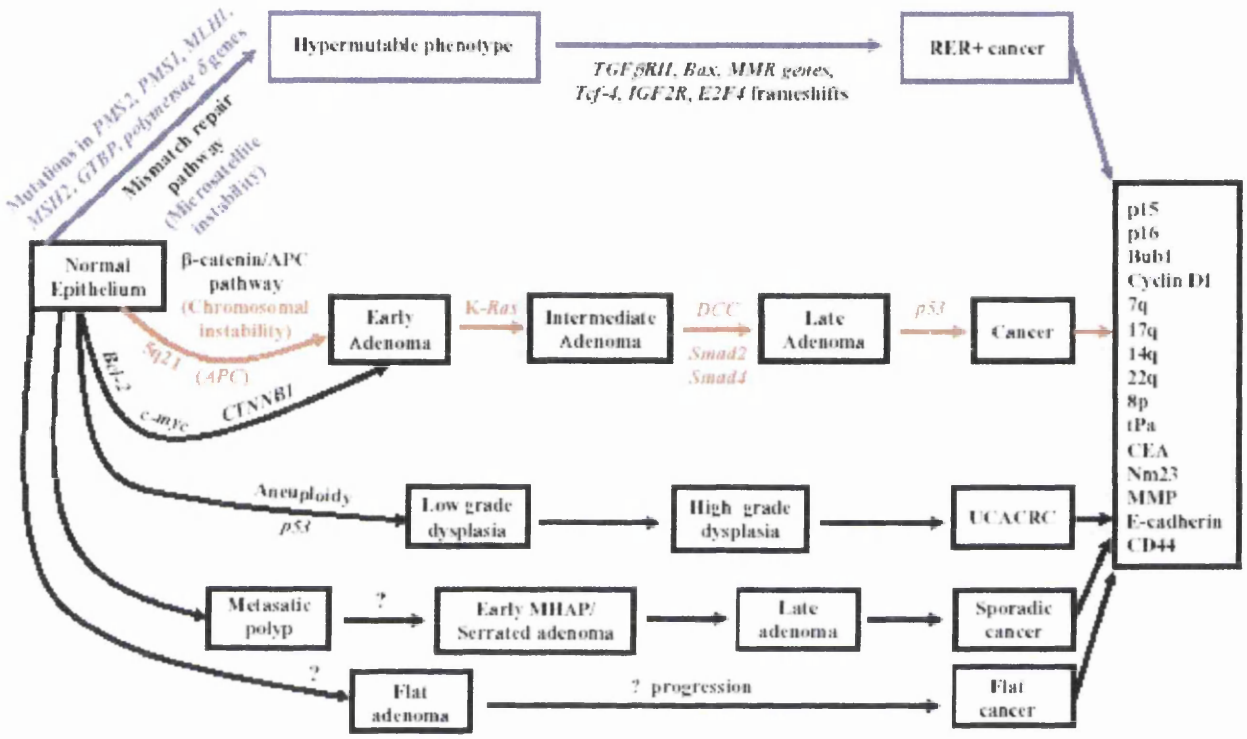


Figure 4.1

This diagram illustrates the proposed pathways to colorectal cancer that currently exist. The original pathway based on Vogelstein's model is shown as the chromosome instability (CIN) pathway and has red arrows with the key changes required for cancer progression labelled. The microsatellite instability (MSI) pathway is at the top of the diagram and is highlighted in blue listing the main genes involved in mismatch repair (MMR) which, once mutated, cause the hypermutable phenotype. The main target genes mutated in MSI colorectal cancers are then listed, frameshifts within these causing the MSI tumour to develop into a carcinoma. The remaining three pathways have several question marks to indicate the ambiguity surrounding these pathways and it is possible these pathways may overlap in several respects. UCACRC is the initialisation used for ulcerative colitis-associated colorectal cancer. Diagram from Narayan and Roy, 2003.

The pathway for sporadic cancers with MSI-H differs from the adenoma-carcinoma pathway seen in the majority of colorectal cancers, see Figure 4.1 (Jass *et al.*, 2000). There are many studies, with many different findings to back up this hypothesis. Experiments have shown that MMR^- tumours and carcinomas with TGF-βRII mutations are less likely to have mutations in the tumour suppressor gene K-ras (Slattery *et al.*, 2000). Another study has shown MSI to be three times more prevalent in tumours with wild type p53, APC and K-ras, all of which are key genes in the standard Vogelstein model of carcinogenesis (Iacopetta *et al.*, 1998; Fearon and Vogelstein, 1990). To add support to the theory that MSI tumours develop through a different mechanism to other carcinomas, an inverse relationship was found between MSI tumours and those with APC and p53 mutations and tumours exhibiting LOH for chromosomes 5q (APC), 17p (p53) and 18q (DCC) (Ionov *et al.*, 1993; Thibodeau *et al.*, 1993; Aaltonen *et al.*, 1993; Konishi *et al.*, 1996).

Although the genes involved in HNPCC and sporadic MSI colorectal cancer may be the same, the mode in which these MMR genes become inactivated is different, with hMSH2 and hMLH1 being mutated in hereditary cases but are, in contrast, inactivated through hypermethylation in the majority of sporadic cases (Jass, 2000; Herman *et al.*, 1998; Cunningham *et al.*, 1998). Another difference between the heritable and sporadic disease is the lack of sporadic adenomas with high levels of MSI (MSI-H) despite 80% of HNPCC adenomas having a MSI-H phenotype (Iino *et al.*, 2000). There is also an increased frequency of MSI-H carcinomas in sporadic rather than inherited cancers. This may be due to a rapid evolution of adenoma to carcinoma, the sporadic MSI-H adenomas being flat and therefore missed at endoscopy, an alternative pathway or a combination of these ideas (Pawlik *et al.*, 2004). The possibility of small adenomas being missed is very dependent upon the experience of the surgeon and is critical with adenomas of less than 5mm which have been found with MSI-H (Iino *et al.*, 2000). A new technique known as chromoendoscopy has now been adopted by many endoscopy units to facilitate the detection of small or flat lesions which may otherwise be missed. This technique involves applying a dye to the colonic mucosa which stains these growths so that far fewer are missed at colonoscopy even by less experienced surgeons (Kiesslich *et al.*, 2001).

Even within the classification of sporadic MSI-H colorectal cancers, differences between reports of sporadic MSI from groups based in Western countries and those from Asia have been highlighted, and it is suggested that MSI-H tumours may arise from two pathways, one via serrated polyps and one via flat adenomas (Shitoh *et al.*, 2000; Jass, 2001a)

4.1.6 Detecting microsatellite instability

MSI assays have focused on the effects of the mutations of MMR genes in terms of microsatellite alterations as these has been found to be a more efficient method of measuring instability than looking directly at the MMR genes as there are so many involved in the process (Minamoto and Ronai, 2001). As opposed to HNPCC, mutations in the MMR genes of sporadic cancers are relatively infrequent, with the wild type genes becoming inactivated by the process of biallelic hypermethylation of the promoter regions (Jass, 2001b). This is especially true of the MMR gene hMLH1, the altered expression of which is thought to be responsible for the RER^{+} phenotype in the majority of MSI-H tumours (Herman *et al.*, 1998; Veigl *et al.*, 1998; Cunningham *et al.*, 1998).

Immunohistochemistry has been used to assess for altered protein levels and the method has demonstrated hMLH1 protein expression is absent in 90% of MSI colorectal cancers (Thibodeau *et al.*, 1998). Whilst PCR-based analysis of microsatellites is recognised as the most sensitive method of MSI identification, immunohistochemical staining for MLH1 and hMSH2 is a technically simpler, inexpensive, rapid and accurate method of MSI-H detection with a predictive value for MSI-H tumours of over 96% (Ward *et al.*, 2001).

Hundreds of MSI assays studying microsatellite alterations have now been undertaken and the occurrence of MSI in colorectal tumours is well documented, with rates of MSI ranging from 85-90% in HNPCC tumours (Aaltonen *et al.*, 1993) to between 6-30% in sporadic cases (Ionov *et al.*, 1993; Thibodeau *et al.*, 1993; Watatani *et al.*, 1996). The majority of sporadic cancers occurring in young people below the age of 36 are MSI with a rate of around 64%, much higher than in patients over 36 for which the rate of MSI is around 12%. In the younger cohort of patients 42% had germline

mutations of a MMR gene. By contrast, in MSI cases in older patients, MMR germline gene mutations are only very rarely found (Liu *et al.*, 1995a; Liu *et al.*, 1995b). In a study of familial adenomatous polyposis (FAP) tumours MSI was found in only 5% of tumours (Konishi *et al.*, 1996). Overall, taking into account heritable diseases such as HNPCC and sporadic cases, 25% of colon cancers are microsatellite unstable (Coleman and Tsongalis, 2002).

The frameshift mutations that arise from unrepaired slipped strand mispairing, generate nonsense mutations, the RNA transcripts for which are usually targeted for degradation through the process of nonsense mediated decay (NMD). NMD is a very rapid process which recognises and breaks down mRNA molecules which contain premature termination codons (Holbrook *et al.*, 2004). This process can also be utilised to identify genes with tumour suppressor functions. For this technique NMD is blocked and stress response genes are suppressed so that the increased levels of mutant mRNA molecules containing premature termination codons can be detected. Using this technique, the gene p300 has been shown to be inactivated in MSI tumours; the function of this gene is as yet unknown (Ionov *et al.*, 2004).

4.1.6.1 The Bethesda panel of MSI markers

The National Cancer Institute recently devised a system to classify tumours by their degree of MSI. Three categories were made, microsatellite stable (MSS), low level of MSI (MSI-L) and high level of MSI (MSI-H) and tumours categorised according to how many PCR marker sets were unstable (Liu *et al.*, 1995a; Boland *et al.*, 1998; Thibodeau *et al.*, 1998). A panel of five microsatellite markers for MSI analysis were proposed by the International Workshop on MSI and RER phenotypes in Cancer Detection and Familial Predisposition in 1997 in an attempt to standardise the MSI markers used. These markers consisted of two mononucleotide repeats (BAT25 and BAT26) and three dinucleotide repeats (D2S123, D5S346 and D17S250) and are collectively known as the Bethesda markers (Boland *et al.*, 1998). Potential genes for the screening of MSI in colorectal cancers should have a high mutational frequency, biallelic inactivation, a role in a growth suppressor pathway, alterations also in MSI negative tumours and there should be functional evidence (Boland *et al.*, 1998). Two of these criteria, a high mutational frequency and functional evidence are generally

agreed to be the most important of these factors, especially in the search for novel MSI target genes (Vilkki *et al.*, 2002).

The BAT26 locus contains a A_{26} tract within the hMSH2 gene and is the most sensitive of these markers (Ward *et al.*, 2001, Loukola *et al.*, 2001) and has been used alone in several studies (Samowitz *et al.*, 1999; Zhou *et al.*, 1998; Hoang *et al.*, 1997) and been shown to detect MSI in 97-99.5% of MSI-H tumours (Loukola *et al.*, 2001; Zhou *et al.*, 1998). Although polymorphisms in Caucasian populations are rare (0.08%), in African-American populations germline variation for BAT26 has been found to be between 7.7 - 12.6% and therefore this marker should be used along side a matched normal control tissue (Pyatt *et al.*, 1999; Samowitz *et al.*, 1999). This also applies to the T_{25} tract within the c-kit gene known as MSI marker BAT25 which exhibits allelic variation in over 18% of African-Americans (Pyatt *et al.*, 1999). Matched normal tissues are also required for the dinucleotide markers D2S123, D5S346 and D17S250 (Loukola *et al.*, 2001).

The Bethesda markers, especially the dinucleotide repeats, have also been criticised for frequently requiring reamplifications to obtain successful amplifications and reproducible unambiguous results. The ambiguity of scoring these markers has also been commented on, with these markers deemed only suitable for those with experience in interpretation (Loukola *et al.*, 2001) and may be a reason for the wide range of prevalence of MSI tumours reported in different studies despite the attempt to standardise MSI analysis.

4.1.6.2 Mononucleotide coding region MSI markers

Further discussion of potential target genes for the screening of MSI in colorectal cancers has occurred and it has been suggested that markers for MSI should meet several criteria such as a high mutational frequency, biallelic inactivation or imprinting of one allele and a role in cell growth suppression, apoptosis, cell differentiation, cell senescence or the escape from immune surveillance (Boland *et al.*, 1998; Perucho, 1999). Transforming growth factor-β receptor II (TGF-βRII), BAX, human *Saccharomyces cerevisiae* MutS homolog 3 (hMSH3) and insulin-like growth factor II receptor gene (IGFIIR) all meet these criteria and have previously been used

in MSI studies and the approximate timing of these mutations within the MSI tumour progression pathway are shown in Figure 4.2 (Souza *et al.*, 1996; Rampino *et al.*, 1997; Ikeda *et al.*, 1998; Molenaar *et al.*, 1998).

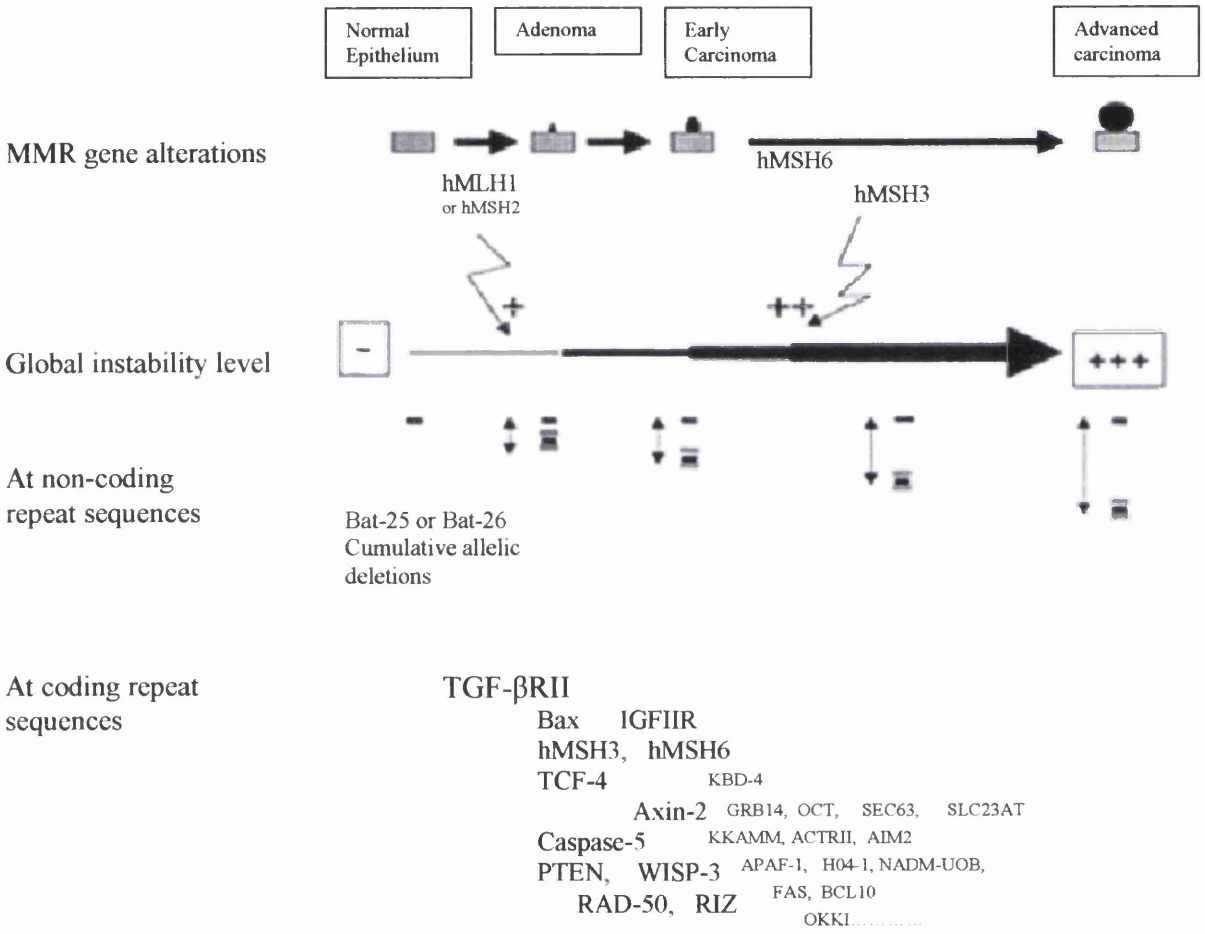


Figure 4.2

Diagram showing the evolution of the microsatellite instability during MSI-H tumour progression from normal epithelium to advanced carcinoma. The inactivation of MMR genes hMLH1 or hMSH2 triggers the mutator phenotype causing the total number of mutations to increase at short coding repeat sequences such as TGF-βRII and Bax. Meanwhile, long non-coding mononucleotide repeats such as Bat-25 or Bat-26 are more and more deleted by a multistep cumulative unstable process. Adapted from Duval and Hamelin, 2002.

4.1.6.2.1 Transforming growth factor-β receptor II (TGF-βRII)

A microsatellite in the gene sequence of the tumour suppressor gene transforming growth factor-β receptor II (TGF-βRII) is frequently mutated in colorectal tumours. Transforming growth factor β (TGF-β) inhibits the growth of endothelial and most epithelial cell types and induces cells to enter a post-mitotic state (Massague, 1990). TGF-β induces G_1-phase cell cycle arrest and induces apoptosis through the expression of the COX2 gene (Hannon and Beach, 1994; Wang *et al.*, 1995a). There are three polypeptide variant forms. Mutations in TGF-βRII form are found in HNPCC carcinomas and sporadic MMR^- colorectal carcinomas. TGF-βRII is inactivated by mutation within A_{10} tract in the coding region of the gene from nucleotide position 709-718. This mutation, which occurs at the beginning of the coding sequence, inactivates the gene which in turn disables the receptor and makes the cell resistant to the growth inhibitory effects of transforming growth factor-β (Markowitz *et al.*, 1995). The gene is often considered a tumour suppressor as restoring the mutated gene to the cell's wild type state cancels the tumourigenicity of colon cancer cell lines containing mutated TGF-βRII (Wang *et al.*, 1995b).

These mutations of TGF-βRII have been proven to be excellent markers for MSI as they are found mutated in around 90% - 100% of MMR deficient sporadic colorectal tumours, 57% of HNPCC adenomas and 85% of HNPCC carcinomas (Parsons *et al.*, 1995; Souza *et al.*, 1996; Iacopetta *et al.*, 1998; Zhang *et al.*, 2001; Akiyama *et al.*, 1997a). TGF-βRII mutations are thought to be the earliest mutational event in MSI tumours since they are so widespread and have also been found in metaplastic polyps, adding to the evidence that these growths are in fact a form of neoplasm and not simply benign polyps (Duval *et al.*, 2001; Markowitz *et al.*, 1995; Young *et al.*, 1995; Konishi *et al.*, 1996). The level of MSI in sporadic adenomas has been very varied between studies, with some stating it is very rare (Young *et al.*, 1995) whilst others detecting a level of 27% of colorectal adenomas to be MSI, using markers which are relatively specific for MSI-H (Grady *et al.*, 1998). This discrepancy may be due to different proportions of early and late adenomas, as TGF-βRII mutations, which occur in nearly all MSI tumours, occur in advanced adenomas and have not been found in early adenomas, linking this mutation with the progression of these adenomas to

cancer (Young *et al.*, 1995; Abdel-Rahman *et al.*, 1999; Grady *et al.*, 1998; Duval *et al.*, 2001).

The presence of TGF-βRII mutations has no discernible effect upon survival for patients with the earlier stages of colorectal cancer. However, mutations in this gene are associated with a significantly better prognosis in patients who have tumours with distant metastasis (Iacopetta *et al.*, 1998; Ionov *et al.*, 2000; Samowitz *et al.*, 2001a).

4.1.6.2.2 BAX

BAX is a tumour suppressor gene which, when transactivated by p53, promotes apoptosis. A lower level of functional BAX protein alters the balance of this protein with the anti-apoptotic protein Bcl2 which in turn prevents cells from undergoing apoptosis (Korsmeyer, 1995). A diagram of the role of BAX in apoptosis by creating channels within the mitochondrial membranes through which cytochrome C is released, beginning the cascade of apoptosis is in Chapter 3, figure 3.5. Around 50% - 58% of MSI colorectal tumours are found to have mutations in the G_8 microsatellite between codons 38-41 of the 3rd exon of BAX (Abdel-Rahman *et al.*, 1999; Rampino *et al.*, 1997, Ionov *et al.*, 2000; Zhang *et al.*, 2001). BAX mutations occur at a later stage in cancer progression, apparent due to a lack of clonality in tumours with these mutations (Abdel-Rahman *et al.*, 1999; Rampino *et al.*, 1997). BAX inactivation confers a selective advantage during clonal evolution by enhancing survival and may be involved in metastatic progression (Abdel-Rahman *et al.*, 1999; Fallik *et al.*, 2003; Ionov *et al.*, 2000). For these reasons BAX inactivation, which rarely occurs in MSS tumours, is an indicator of poor prognosis for patients with MSI tumours (Rampino *et al.*, 1997; Ionov *et al.*, 2000). Along with MSI, loss of BAX expression is a marker for patients that respond well to the chemotherapeutic agent irinotecan (Fallik *et al.*, 2003).

4.1.6.2.3 Human *Saccharomyces cerevisiae* MutS homolog 3 (hMSH3)

hMSH3 (also known as DUG, DUT or DUC-1) is a gene involved in the hMSH2-dependent mismatch repair process repairing di- and tetranucleotide repeats by forming a heterodimer with hMSH2 and binding to insertion-deletion loops (Risinger

et al., 1996; Palombo *et al.*, 1996; Acharya *et al.*, 1996). hMSH3 also itself contains an A_8 microsatellite within exon 7 of the gene's open reading frame that is frequently mutated in MSI tumours. Mutations in hMSH3 have been found in 50% - 58% of HNPCC cancers (Yin *et al.*, 1997; Akiyama *et al.*, 1997b) and around 30% - 53% of sporadic MSI colorectal cancers of which virtually all the mutations reported were 1bp deletions (Yin *et al.*, 1997; Malkhosyan *et al.*, 1996; Jeong *et al.*, 2003; Abe and Masuda, 2000; Yamamoto *et al.*, 1997). Another study reported a level of 37.5% of MSI tumours containing hMSH3 mutations. Yet when these MSI tumours were categorised into MSI-H and MSI-L the levels of mutation were 67% and 0% respectively (Ikeda *et al.*, 1998). This again provides support that MSI-H and MSI-L colorectal tumours arise from different pathways and grouping these subsets together only confuses the observable genetics patterns and phenotypes of these groups. Mutations in hMSH3 have not been found in MSS colorectal tumours (Abe and Masuda, 2000).

4.1.6.2.4 Insulin-like growth factor II receptor gene (IGFIIR)

IGFIIR is essential for the degradation of the potent growth stimulant IGFII and also activates TGF-βRI which then acts as a growth suppressant (Wang *et al.*, 1997; Dennis and Rifkin, 1991). IGFIIR is not only therefore important in epithelial cell growth regulation, but is also prone to mutation within its coding sequence as it contains several microsatellites. Historically, IGFIIR was the second gene after TGF-βRII discovered to have mutations in the microsatellites within its coding region (Souza *et al.*, 1996). The G_8 microsatellite from nucleotide position 4089-4096 is the site which is most frequently mutated within IGFIIR in MSI colorectal cancers. Tumour samples which contained mutations within the G_8 microsatellite were also examined at another G_8 locus but no mutations were found at this site, supporting the theory that these mutations are non-random, but are in fact targeted mutations within the mutator phenotype (Souza *et al.*, 1996). IGFIIR has been reported to be mutated in around 13% of HNPCC colorectal cancers and approximately 10% - 36% of MSI sporadic colorectal cancers. No mutations have been reported in the tissues of MSS colorectal cancer patients (Souza *et al.*, 1996; Jeong *et al.*, 2003; Woerner *et al.*, 2001).

4.1.7 Degree of MSI in colorectal tumours

There has been argument to merge the classifications MSS and MSI-L because it is thought that with enough markers all colorectal cancers may exhibit some level of MSI and the clinical features of these groups are so similar that it is not a useful differentiation to make (Thibodeau *et al.*, 1998; Jass *et al.*, 1998; Loukola *et al.*, 2001; Ward *et al.*, 2001). For example, there is an increased expression of specific mucins with MSI-H cancers as opposed to MSI-L or MSS cancers (Thibodeau *et al.*, 1993; Jass, 2000).

It has been proposed that MSI-L tumours may develop from metaplastic polyps by a distinctive pathway and clinically may behave differently from both MSS and MSI-H tumours (Iino *et al.*, 1999; Ward *et al.*, 2001). It is also a possibility that serrated polyps and MSI-L colon cancers are related histogenetically as there is a similarity in early morphogenesis for MSS and MSI-H, but differences for MSI-L cancers (Jass, 2001a). With these differences of opinion and with very little known about hyperplastic and serrated polyps and the possible pathways of carcinogenesis involved with these aberrations, it is still of value to differentiate between MSI-L and MSI-H in studies, especially while there is such uncertainty (Shitoh *et al.*, 2000; Jass, 2001a, Jass 2001b).

4.1.8 Rationale of the study

As the experiments from Chapter 3 showed, the tumour specific mutations in the mitochondrial genome were predominantly in poly-C microsatellites. This chapter describes studies which aimed to analyse the same samples as the previous chapter in order to establish whether there is a link between MSI in the mitochondrial and nuclear genomes. Each sample will be examined at four nuclear microsatellites which have been proved in research from other groups to be frequently mutated in MSI tumours, in order to assess the level of nuclear microsatellite instability. The results of these experiments will provide more information regarding the repair mechanisms of the mitochondrial genome and whether there is a correlation between nMSI and mtMSI. If such a correlation can be shown, this would provide evidence of a mtDNA mismatch repair system which utilises some of the same repair proteins as nDNA or even a shared pathway of mismatch repair.

4.2 Materials and Methods

4.2.1 Sample collection

The 65 tissue samples from 24 patients used in these experiments were the same as those used in Chapter 3. These are detailed in appendix I. The mean age of these patients was 69 years of age at the time of sample collection; the patients consisted of 13 females and 11 males (1.2F : 1M). 12 of these patients had adenocarcinomas of the rectum, 2 of the sigmoid colon, 4 of the transverse colon and 2 of the caecum. 1 patient had a rectal polyp, 3 had sigmoid polyps. 6 of these samples had metastatic tissue samples from liver or lymph node. DNA extraction and quantification was previously carried out as described in section 3.2.2 and 3.2.3

4.2.2 Polymerase Chain Reaction (PCR)

4.2.2.1 Choice of microsatellites to analyse

Four microsatellites were chosen for this study on the basis that they had been widely used in previously published studies of MSI and are all mononucleotide repeats which are generally more sensitive to MSI than more complex repeat sequences. These microsatellites lie within TGF-βRII, BAX, IGF and hMSH3; details of these microsatellite markers can be seen in Table 4.1. TGF-βRII has also been shown to be at least as sensitive to MSI as a panel of markers and mutations within this gene occur in the vast majority of MMR deficient tumours (Zhang *et al.*, 2001; Iacopetta *et al.*, 1998; Guanti *et al.*, 2000).

4.2.2.2. PCR conditions and primer labels

PCR was optimised using un-labelled primers and, once optimised, oligonucleotides (Invitrogen) labelled with WellRed dye (Beckman) were used for forward primers. The primer sequences and dye colour for each primer set are detailed in Table 4.1. PCR conditions for these primer sets are listed in Table 4.2. Each PCR reaction began with 1 min at 94°C to fully denature the target DNA and ended with a 5 min stage at 72°C to ensure full length PCR products were produced.

For each sample the PCR reaction and subsequent fragment analysis was repeated at least once to confirm results.

Gene	Primers	WellRed labelling dye	Product length (bp)	Micro-satellite	Reference
IGFIIR	Forward – GCAGGTCTCCTGACTCAGAA Reverse – GAAGAAGATGGCTGTGGAGC	Dye4PA Blue	109	$(G)_8$	Souza *et al.*, 1996
BAX	Forward – ATCCAGGATCGAGCAGGGCG Reverse – ACTCGCTCAGCTTCTTGGTG	Dye3PA Green	92	$(G)_8$	Rampino *et al.*, 1997
hMSH3R	Forward – GAGATAATGACTGATACTTCTACC Reverse – CATTTGTTCCTCACCTGCAAAG	Dye2PA Black	131	$(A)_8$	Ikeda *et al.*, 1998
TGF-βRII	Forward – ATGCTGCTTCTCCAAAGTGCATTA Reverse – GCACTCATCAGAGCTACAGGAACA	Dye2PA Black	85	$(A)_{10}$	Molenaar *et al.*, 1998

Table 4.1

PCR primer sequence and labels for the microsatellites used for fragment analysis.

Primer set	$MgCl_2$ amount (µl)	Denaturation		Annealing		Elongation		Number of cycles
		Temp (°C)	Time (s)	Temp (°C)	Time (s)	Temp (°C)	Time (s)	
IGFIIR	2.0	94	30	58	30	72	30	35
BAX	2.0	94	30	62	30	72	30	35
hMSH3R	2.5	94	30	64	30	72	40	38
TGF-βRII	1.4	94	30	62	30	72	30	35

Table 4.2

PCR conditions for each microsatellite primer set.

4.2.3 Fragment analysis

This was carried out as described in Chapter 2, section 2.10.

4.2.4 Sequencing

Several samples were sequenced as described in Chapter 2, section 2.9

4.3 Results

4.3.1 Fragment analysis results

A full table of the values obtained for fragment length for all 4 of the analysed microsatellites for all the samples from the 24 patients studied can be seen in appendix I. Figure 4.3 shows a typical fragment analysis trace and details how these results are read.

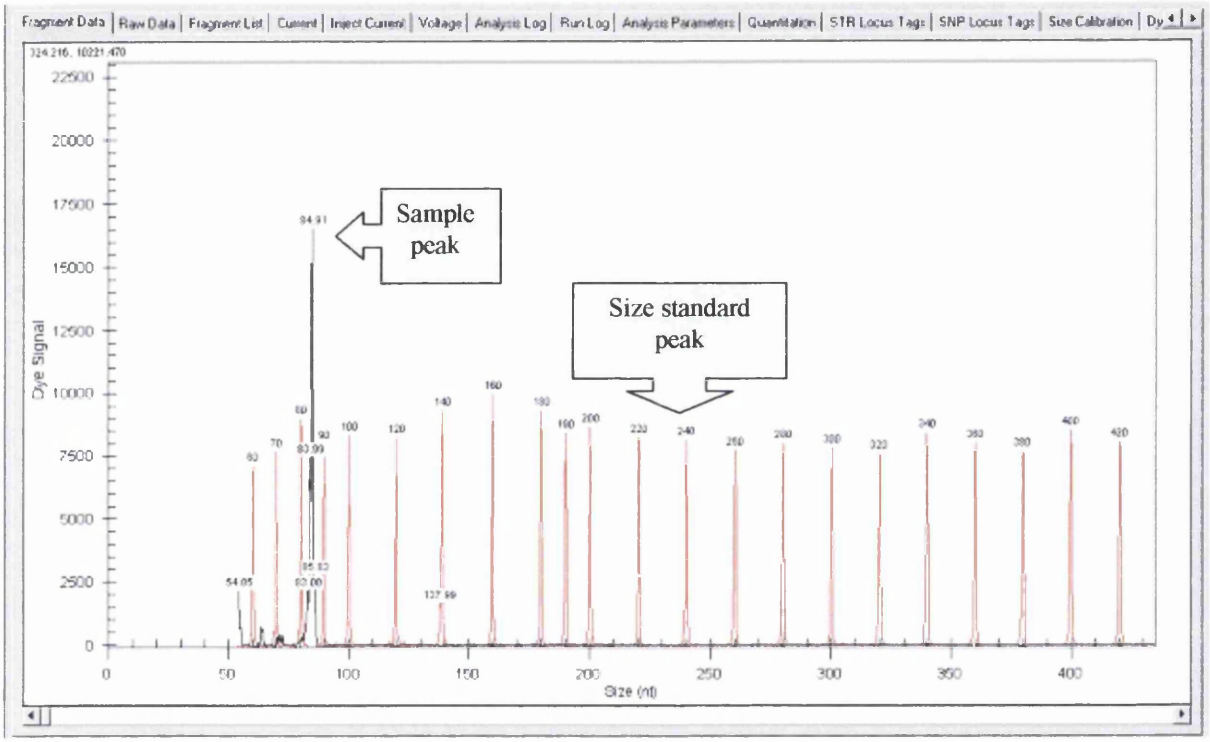
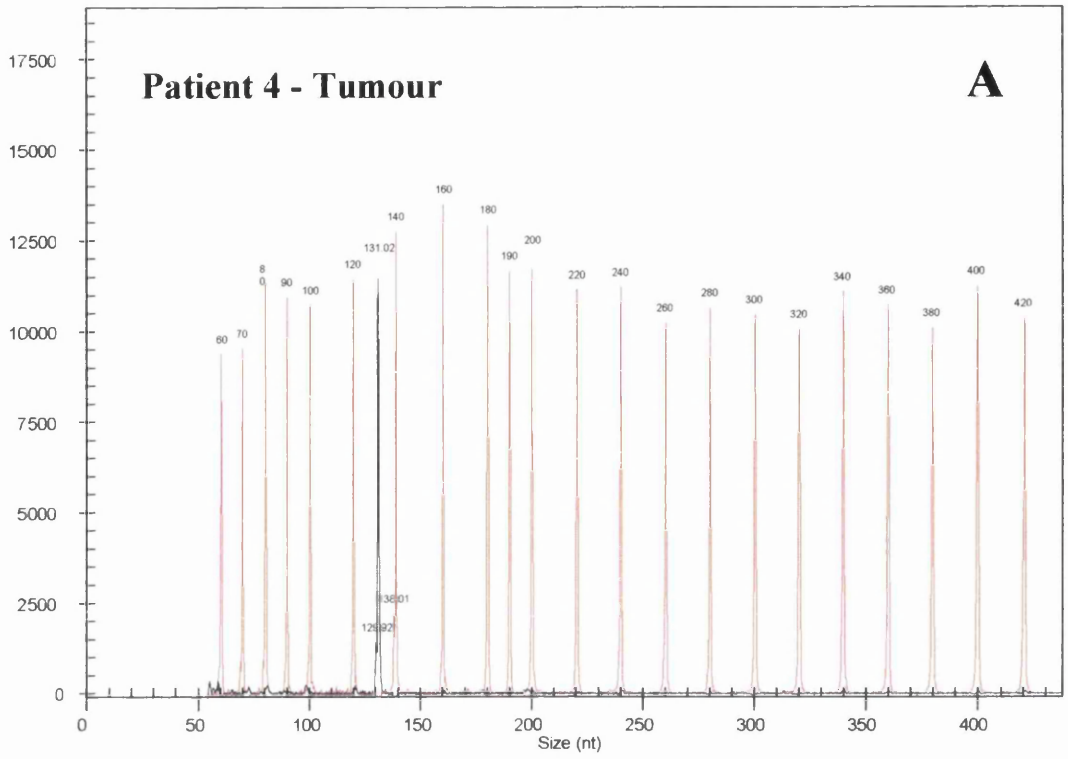


Figure 4.3

This figure shows a typical fragment analysis trace. The x axis illustrates the size, in bp, of fragments within the sample and the y axis shows the intensity of the dye signal of each dye labelled fragment. The red peaks result from the red labelled size standard which is added to the sample to give an accurate scale from 60bp to 420bp against which the PCR product labelled with a different coloured dye can be measured. The black peak corresponds to the labelled PCR product and the size of this is accurately measured and written at the apex of the peak.



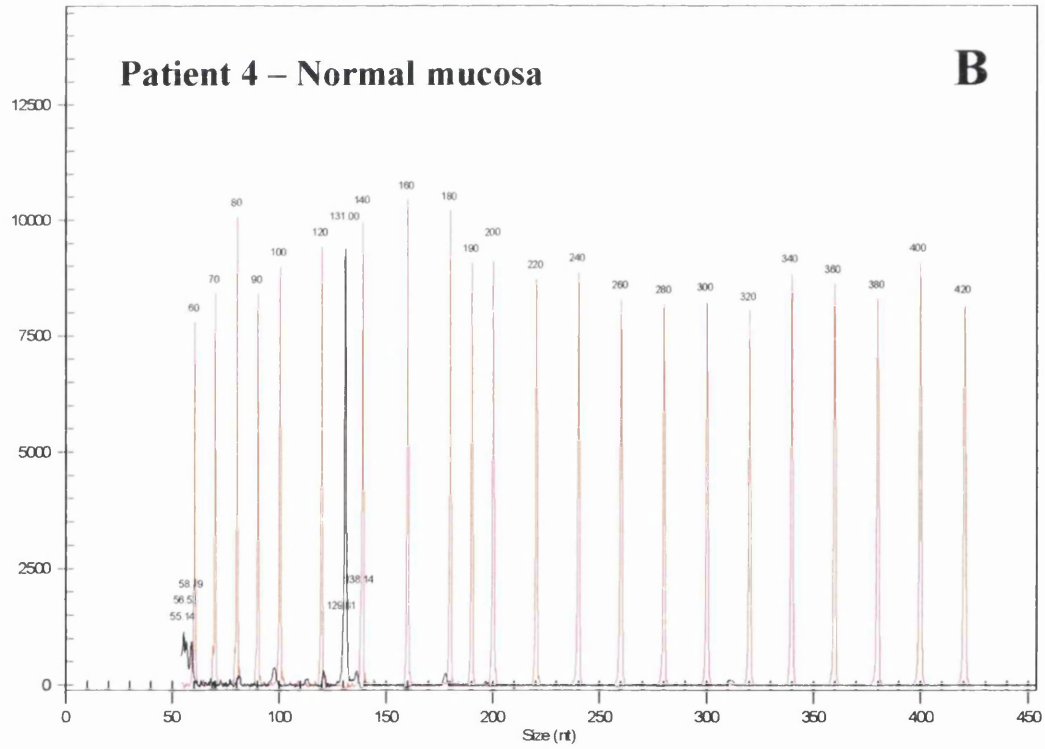
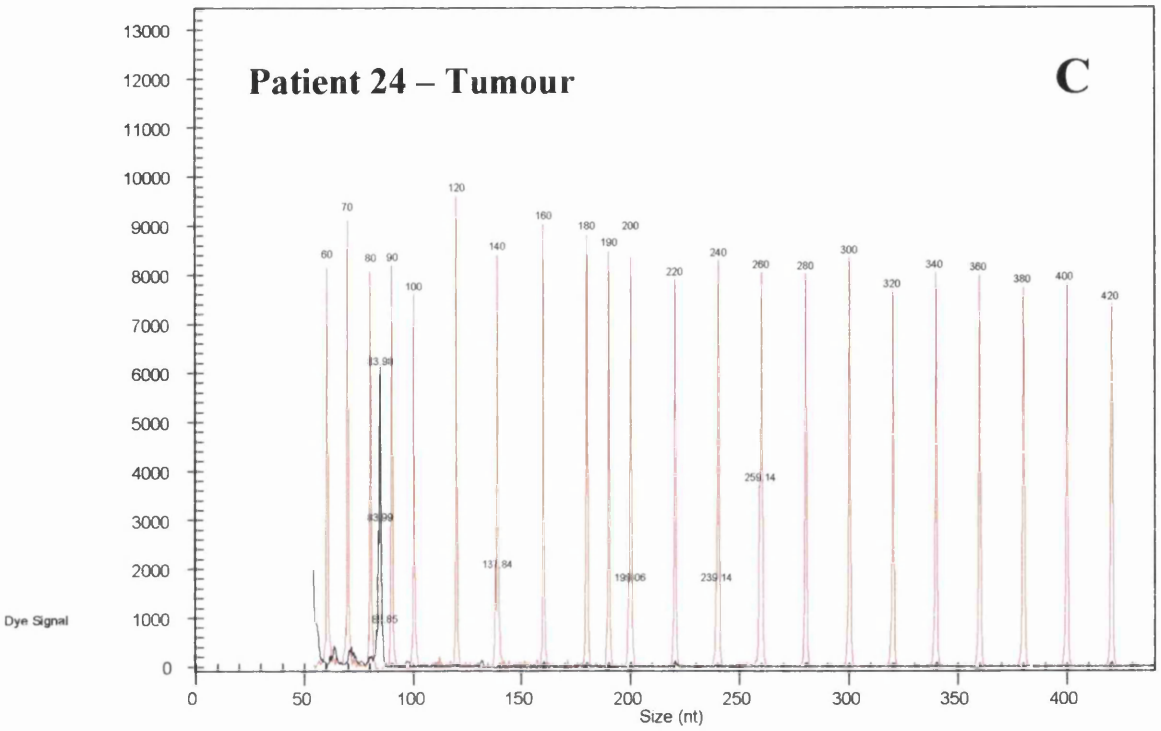


Figure 4.4

Fragment analysis traces showing the length of the PCR products for the hMSH3 microsatellite for patient 4 represented by black peaks. **A** is the resulting trace for amplifications from sample 16, rectal tumour and **B** is the trace from the normal adjacent mucosa sample number 17. Both of these samples have fragment lengths corresponding to wild type sequence for this microsatellite.



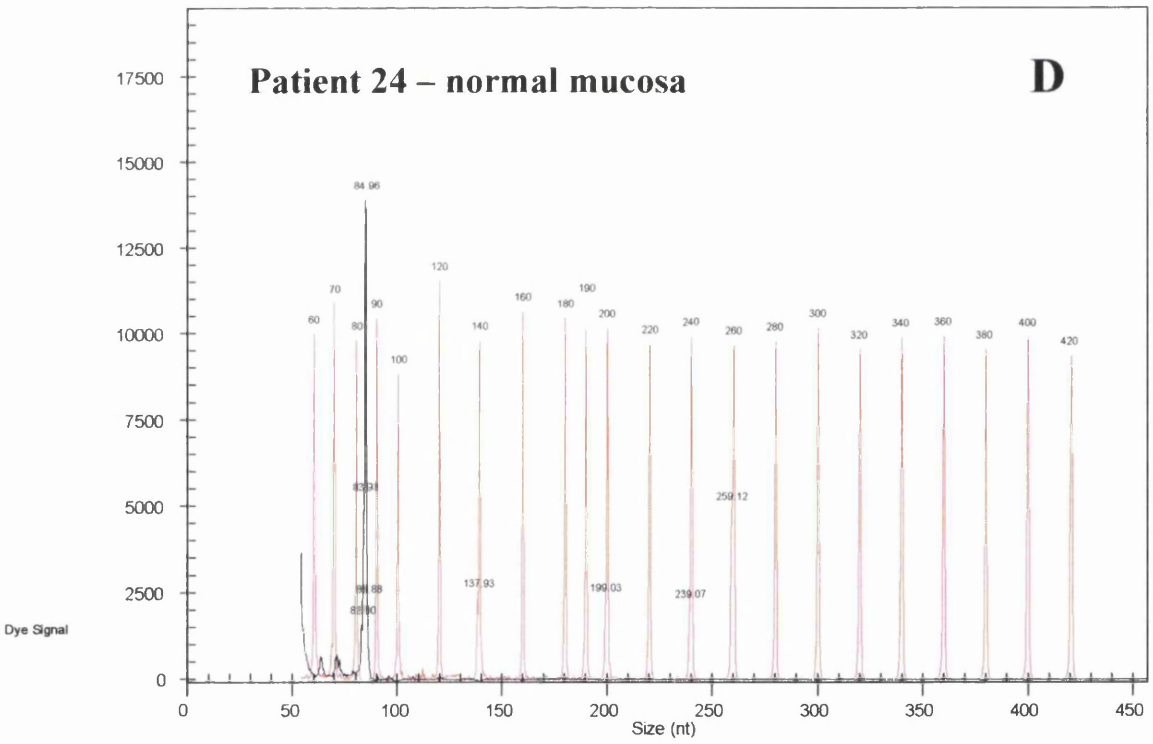


Figure 4.5

Fragment analysis traces showing the length of the PCR products for the TGF-RII microsatellite for patient 24 represented by black peaks. **C** is the resulting trace for amplifications from sample 96, caecal adenocarcinoma and shows a fragment length of 1bp less than the wild type sequence. **D** is the trace from the normal adjacent mucosa sample 97 and has a fragment length corresponding to the wild type sequence for this microsatellite.

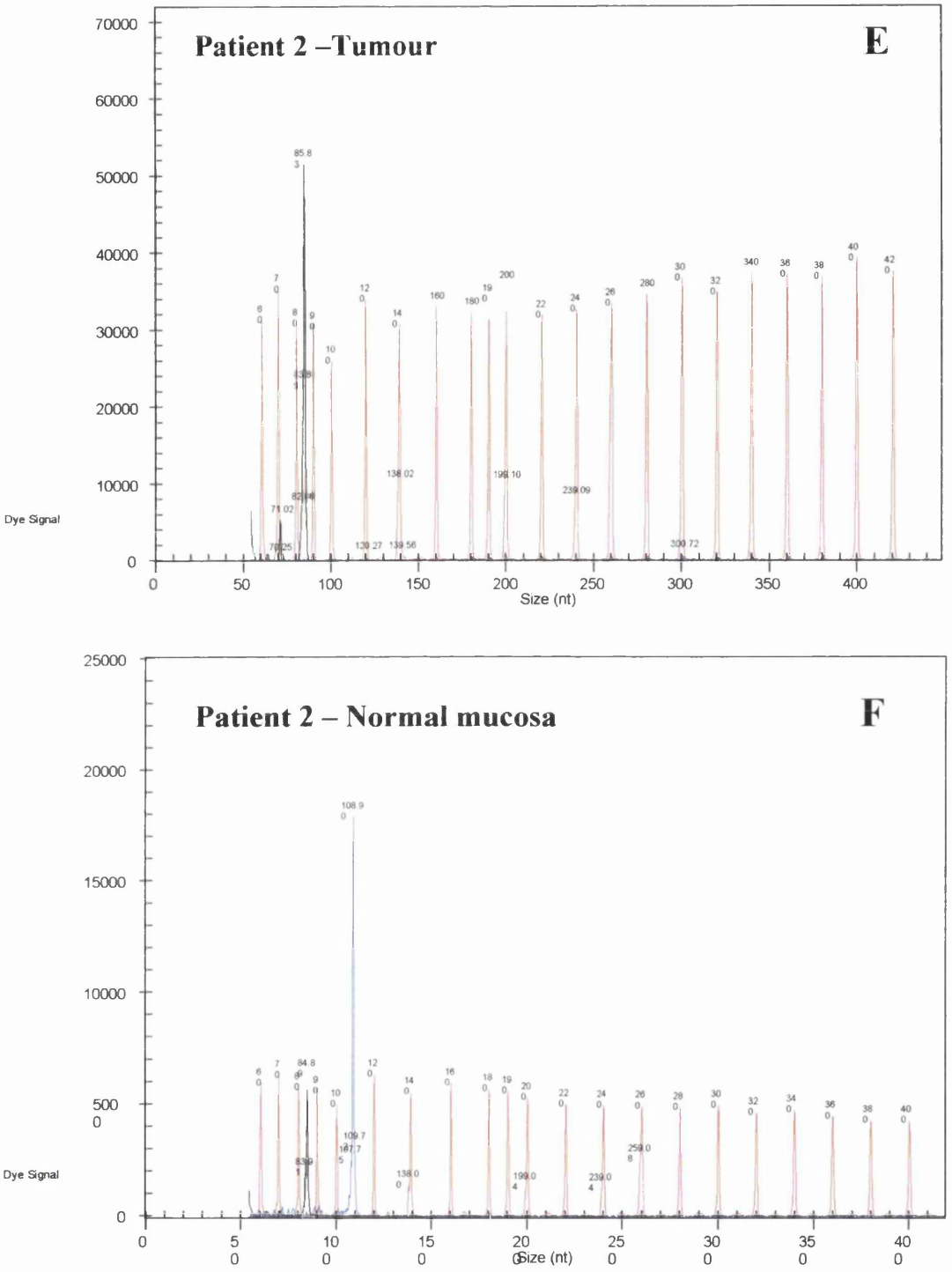


Figure 4.6

Fragment analysis traces showing the length of the PCR products for the TGF-RII microsatellite for patient 2 represented by black peaks. **E** is the resulting trace for amplifications from sample 9, rectal tumour and shows a fragment length of 1bp more than the wild type sequence. **F** is the trace from the normal distant mucosa sample 10 and has a fragment length corresponding to the wild type sequence for this microsatellite. Trace **F** also has a blue peak representing the length of the PCR fragment amplified from the IGFIIR microsatellite, again this has the same length as the wild type sequence.

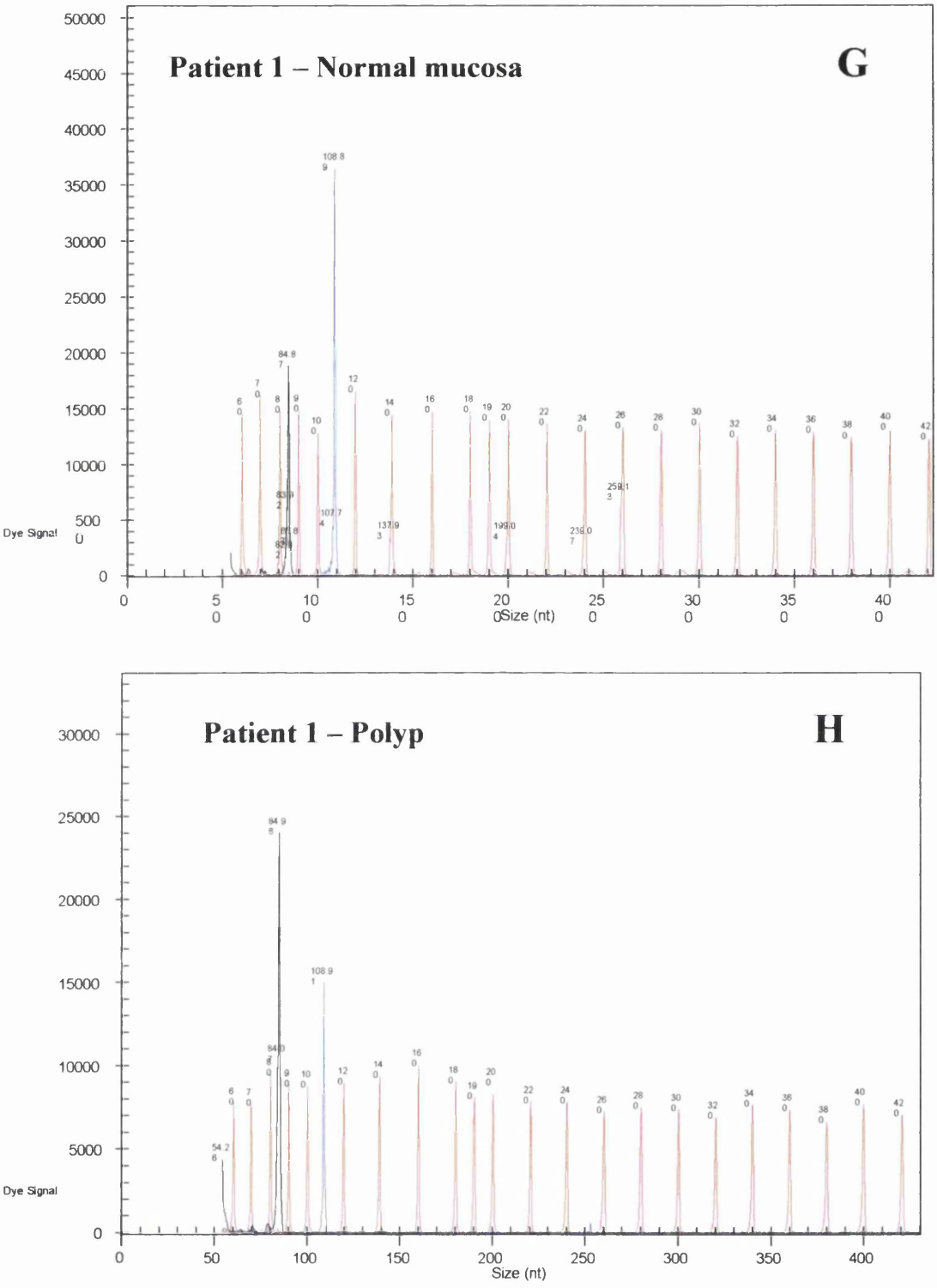


Figure 4.7

Fragment analysis traces showing the length of the PCR products for the TGF-RII microsatellite for patient 1 represented by black peaks and the products of IGFIIR by blue peaks. **G** is the resulting trace for amplifications from sample 5, normal adjacent mucosa and **H** is the trace from the rectal polyp sample 4 and both traces show fragment length corresponding to the wild type sequence for both microsatellites.

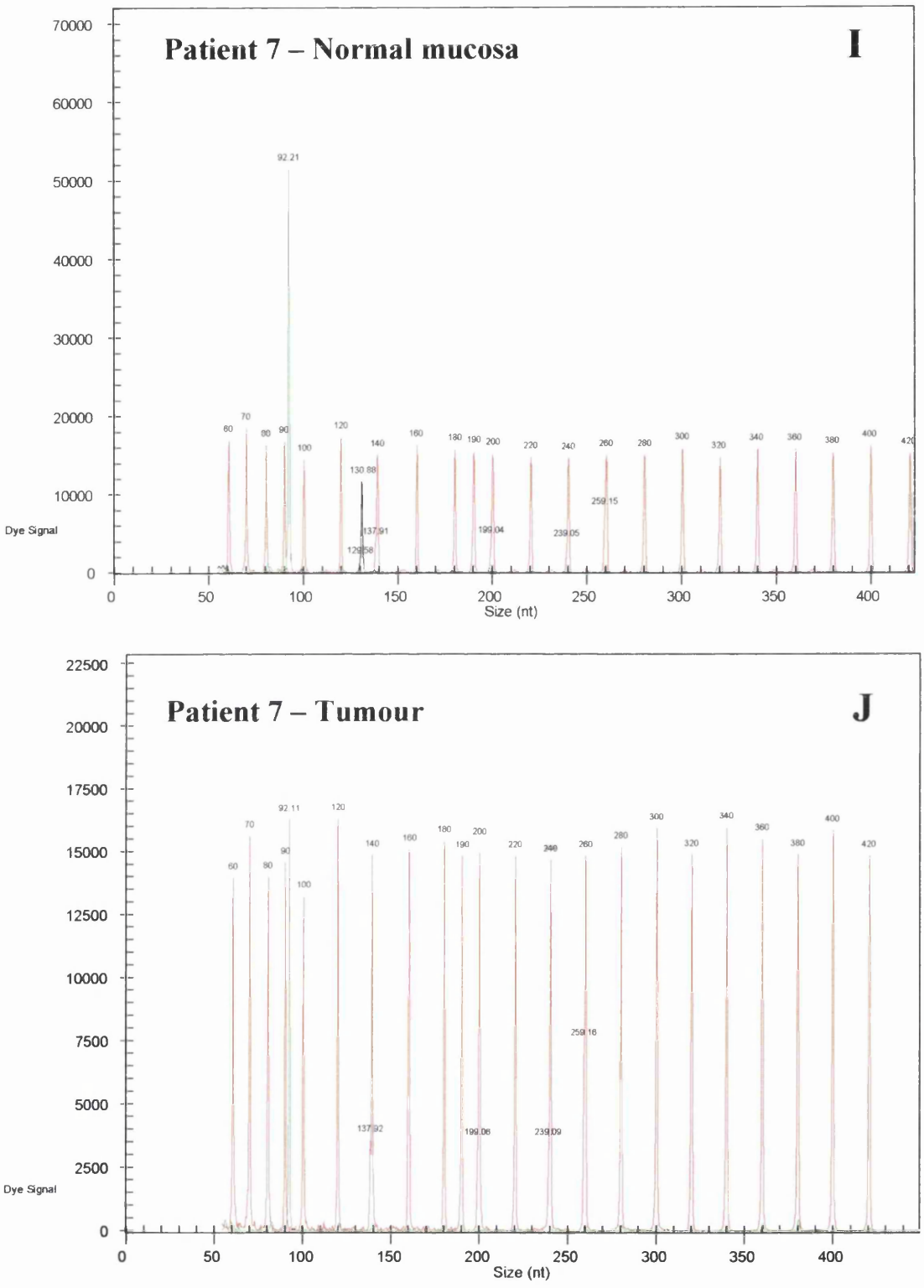


Figure 4.8

Fragment analysis traces showing the length of the BAX PCR products for patient 7 represented by green peaks and, on trace **I,** the products of hMSH3 by black peaks. **I** is the resulting trace for amplifications from sample 28, normal distant mucosa and **J** is the trace from the rectal adenocarcinoma sample 29 and both traces show fragment length corresponding to the wild type sequence for the BAX microsatellite and trace **I** shows a wild type sequence length for hMSH3.

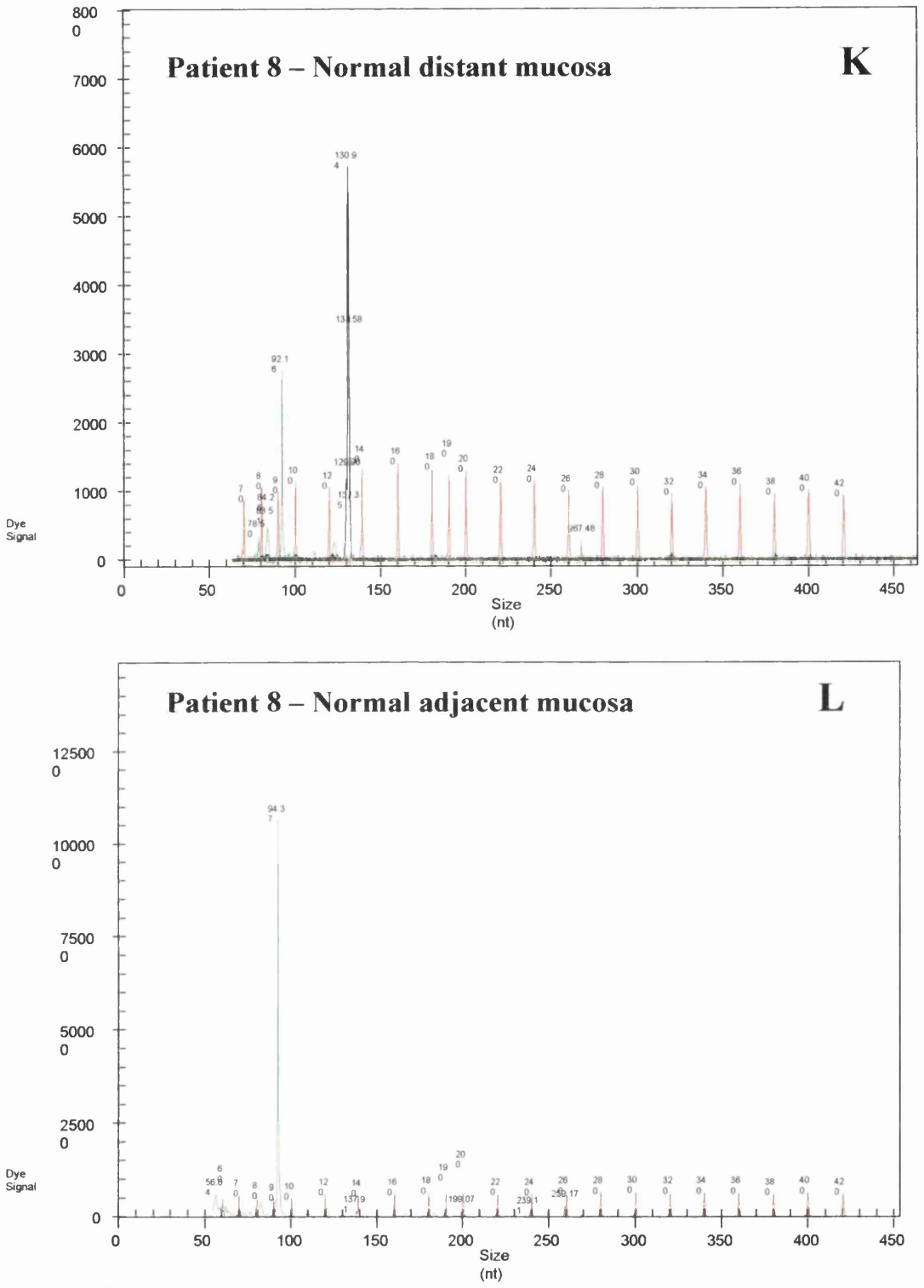


Figure 4.9

Fragment analysis traces showing the length of the BAX PCR products for patient 8 represented by green peaks and, on trace **K,** the products of hMSH3 by black peaks. **K** is the resulting trace for amplifications from sample 33, normal distant mucosa and shows wild type sequence lengths for both the BAX and hMSH3 PCR products. **L** is the trace from the normal adjacent mucosa sample 35 and shows fragment length corresponding to a sequence length 2bp longer than the wild type sequence.

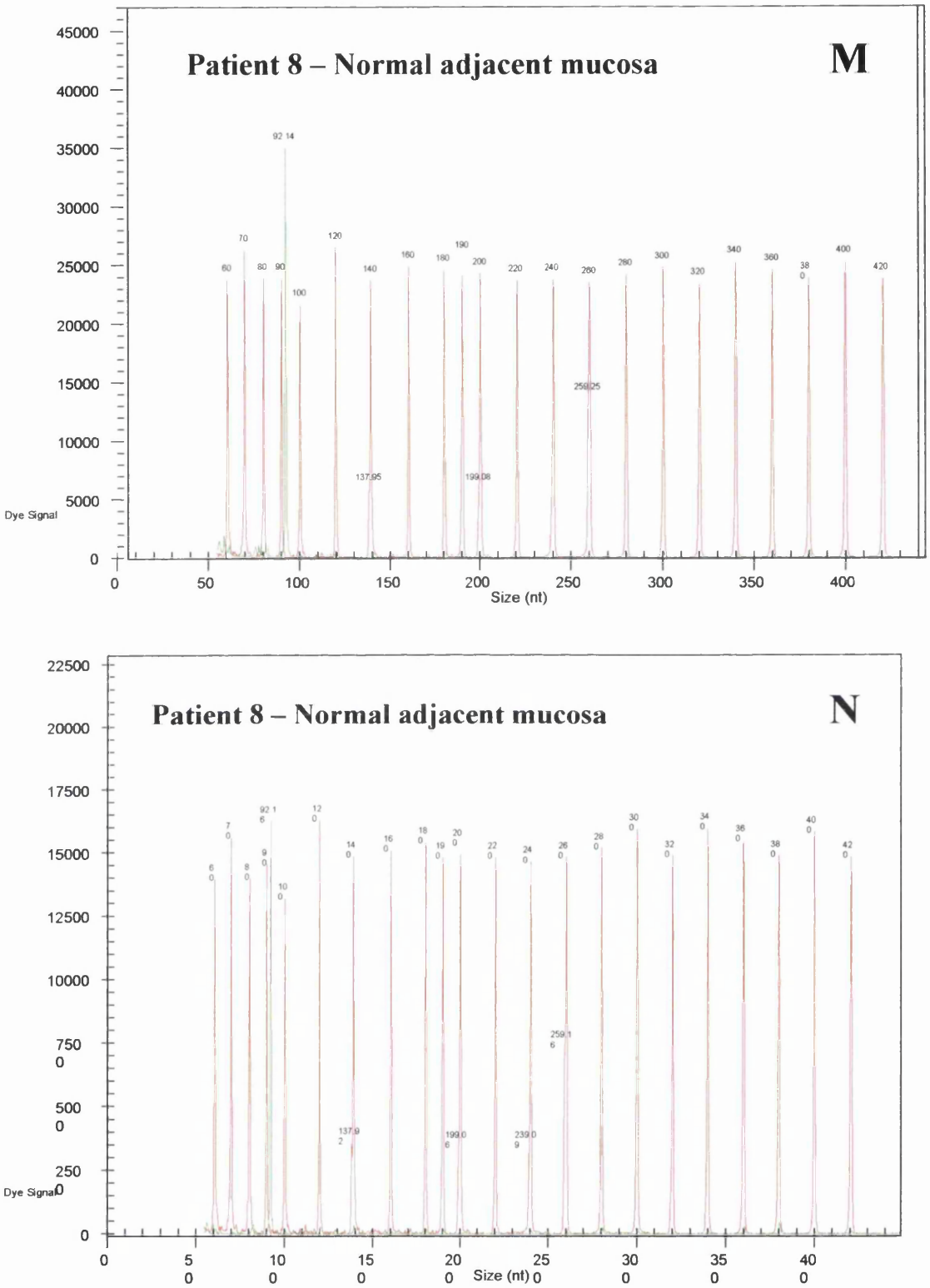


Figure 4.10

Fragment analysis traces showing the length of the BAX PCR products for patient 8 represented by green peaks. **M** and **N** are the resulting traces for repeat amplifications from sample 35, normal adjacent mucosa both traces show fragment length corresponding to the wild type sequence for the BAX microsatellite.

			PCR fragment length (bp)							
Patient No.	Sample No.	Sample Type	TGF-βRII		BAX		hMSH3		IGF	
2	9	Rectal tumour	**86**	**86**	92	92	131	131	109	109
	10	Distant mucosa	85	85	92	92	131	131	109	109
8	30	Rectal polyp	85	85	92	92	131	131	109	109
	31	Liver biopsy	85	85	92	92	131	131	109	109
	33	Distant mucosa	85	85	92	92	131	131	109	109
	34	Rectal tumour	85	85	92	92	131	131	109	109
	35	Adjacent mucosa	85	85	**94**	92	92	131	131	109	109
24	96	Caecal tumour	**84**	**84**	92	92	131	131	109	109
	97	Adjacent mucosa	85	85	92	92	131	131	109	109
	98	Distant mucosa	85	85	92	92	131	131	109	109

Table 4.3

Table of fragment analysis data for patients which showed an anomaly in fragment length. The figures give the length in base pairs (bp) of each of the PCR fragments containing the microsatellite. Figures in bold represent fragment lengths that were different from the wild type (WT) sequence length for that PCR product. The remaining patients were all WT for all microsatellites tested, this data is included in appendix I.

Microsatellite marker	Number of patients with alteration	Frequency
TGFß-RII	2/24	8.3%
BAX	0/24	0%
hMSH3	0/24	0%
IGF	0/24	0%

Table 4.4

Table showing the number of each microsatellite marker found altered in the patients analysed in this study.

4.3.2 The results of sequencing

All the samples from patients showing an anomaly in any of the MSI marker PCR products by differing from the wild type sequence length were sent for sequencing to elucidate more about these changes. Samples from patients 1, 3, 4, 5 and 6 were also all sequenced to confirm these sequences were wild type. Table 4.4 details all the changes that were detected by sequencing, all other sequences were confirmed as wild type. Sequencing confirmed the fragment analysis results.

Patient No.	Sample No.	Age	Sex	Sample Type	Dukes' Stage	Microsatellite change
2	9	80	M	Rectal tumour	C	A_1 insertion in TGF-βRII
	10			Distant mucosa		
24	96	58	M	Caecal tumour	C1	A_1 deletion in TGF-βRII
	97			Adjacent mucosa		
	98			Distant mucosa		

Table 4.5

Table detailing the patient information for patients exhibiting microsatellite instability and the details of the microsatellite change as confirmed by sequencing.

4.4 Discussion

4.4.1 Choice of MSI markers

There have been many studies assessing sporadic colonic cancers for MSI and the levels detected have ranged from 6% to 29% (Young *et al.*, 1993; Thibodeau *et al.*, 1993; Ionov *et al.*, 1993; Chao *et al.*, 2000; Watatani *et al.*, 1996). This wide range is most likely to be a result of the different loci studied, ranging from mononucleotide repeats to more complex tetranucleotide microsatellites. The loci also vary in their genomic location in various genes and non-coding regions on different chromosomes, making it difficult to establish the variation in MSI levels of colorectal tumours. It was for this reason the standard markers were established in 1998. However, these are not used in this current study as these markers contain polynucleotide repeats which were not present in mtDNA and there have also been reported problems using the Bethesda markers in terms of amplification difficulties and problems with interpreting the results (Boland *et al.*, 1998; Loukola *et al.*, 2001). The markers selected for this study were chosen as the microsatellite altered in the mitochondrial genome was a mononucleotide repeat and so the markers in this study are also mononucleotide repeats. These have all been used in studies by other groups to assess for MSI (Souza *et al.*, 1996; Rampino *et al.*, 1997; Ikeda *et al.*, 1998; Molenaar *et al.*, 1998). These microsatellites also meet the revised Bethesda criteria set for choosing target genes as opposed to so-called bystander genes which, although may be mutated, have no significant effect upon tumourigenesis (Boland *et al.*, 1998; Perucho, 1999). The criteria include: a high mutational frequency, biallelic inactivation or imprinting of one allele and, a role in cell growth suppressing, apoptosis, cell differentiation, cell senescence or the escape of immune surveillance (Perucho, 1999).

Microsatellites with mononucleotide repeats were chosen as these would be altered not only by deficiencies in the hMSH2 and hMLH1 genes but also in tumours deficient in hMSH6 (Fallik *et al.* 2003; Loukola *et al.* 2001). These microsatellites have also been found to be more sensitive than more complex repeat sequences at detecting MSI (Loukola *et al.*, 2001; Ward *et al.*, 2001; Chen *et al.*, 1995). In a study comparing the effectiveness of all 4 of the microsatellites (hMSH3, BAX, IGFRII and TGF-βRII) analysed in this study with the Bethesda consensus markers, there was

found to be no significant difference between the two sets of markers (Samowitz *et al.*, 2001b). Another 2001 study of MSI colorectal tumour tissue found all 24 of these samples to contain mutations within the TGF-βRII microsatellite, compared with 96% of samples having mutations within the non-coding Bethesda BAT25 marker (Zhang *et al.*, 2001).

Microsatellite instability for each marker site was defined as an increase or decrease in size by one or more bases in comparison to the normal DNA sample. These criteria have been used in several studies (Samowitz *et al.*, 2001b).

4.4.2 Levels of mutations found in this study

4.4.2.1 Transforming growth factor-β receptor II (TGF-βRII)

Mutations within the A_{10} microsatellite within TGF-βRII have been found to occur very early in tumour progression, and mutations within this gene have been found in around 90% - 100% of MSI colorectal cancers (Parsons *et al.*, 1995; Zhang *et al.*, 2001; Guanti *et al.*, 2000). In the study described in this chapter 2/24 (8.3%) of patients were found to have mutations within this microsatellite within their tumour tissue. No normal tissues were found to contain mutations within the section of TGF-βRII analysed, confirming the accuracy of this locus to detect MSI mutations.

There is another microsatellite within TGF-βRII that was not amplified in the PCR reactions in these experiments, as this fell outside the primer set used. This microsatellite is a dinucleotide repeat of $(GT)_3$ located at nucleotides 1931-1936 and has been found to contain an insertion in 1/11 (9%) MSI colorectal tumour cell lines examined (Markowitz *et al.*, 1995). This microsatellite was not examined in this study as in comparison to the A_{10} tract it is relatively infrequently mutated in MMR deficient tumours and therefore not as suitable as a molecular marker for MSI.

4.4.2.2 BAX

Previous studies have found BAX to be mutated within the G_8 microsatellite in around 50% of MSI colorectal tumours (Abdel-Rahman *et al.*, 1999; Rampino *et al.*,

1997, Ionov *et al.*, 2000). Because of this, alterations in the microsatellite within BAX are not enough alone to diagnose a tumour as MSI. However, they are effective as part of a panel of markers and may also provide prognostic information about the degree of severity of the tumour. In this study, there were no BAX mutations detected in any of the patients including those tumours mutated within the reliable MSI marker TGF-βRII microsatellite. As there were only 2 such tumours in this study it is not possible from these figures to challenge the levels of BAX mutations found in previous studies. It has been reported that BAX mutations are very rare in MSS tumours (Rampino *et al.*, 1997; Ionov *et al.*, 2000) and this was also found in this study where no MSS tumours (0/22) were found to have mutations within the BAX microsatellite.

Neither of the 2 patients deemed as MSI by the presence of TGF-βRII microsatellite mutations had metastatic carcinomas. Previous studies have found BAX mutations occurring at a later stage in cancer progression and as being involved in metastasis, it would be expected that these patients would be unlikely to have BAX mutations, as indeed they did not (Abdel-Rahman *et al.*, 1999; Rampino *et al.*, 1997; Fallik *et al.*, 2003; Ionov *et al.*, 2000). Together, TGF-βRII and BAX may act as a good marker set for colorectal cancers with MSI as these mutations are likely to be detectable in the faeces of patients with tumours which contain these mutations. These markers would detect adenomas progressing to carcinomas and also give an indication of prognosis (Rampino *et al.*, 1997; Dong *et al.*, 2001).

4.4.2.3 Human *Saccharomyces cerevisiae* MutS homolog 3 (hMSH3)

There were no mutations found within the A_8 microsatellite of hMSH3 of any of the samples from any of the patients examined in this study. Levels of hMSH3 mutation in previous studies have ranged between 30-53% of sporadic MMR^- tumours (Yin *et al.*, 1997; Malkhosyan *et al.*, 1996; Jeong *et al.*, 2003; Abe and Masuda, 2000; Yamamoto *et al.*, 1997; Ikeda *et al.*, 1998). As there were very low overall levels of MSI in the patient cohort, it is impossible to draw any conclusions from the lack of hMSH3 mutations found in this study other than, if it is assumed 22 of these patients are MSS then, there have been no false positives from this MSI marker.

hMSH3 mutations have been linked with MSI-H tumours (Duval *et al.*, 2001; Ikeda *et al.*, 1998) and the inactivation of this gene is thought to be, after the alteration of a first MMR gene, a second critical event during MSI-H tumour progression by increasing the overall instability from MSI-L to MSI-H (Iino *et al.*, 2000; Duval *et al.*, 2001). It may be that the 2 patients with TGF-RII mutations only had low levels of MSI and so mutations within hMSH3 would be unlikely. Also as BAX mutations have been associated with hMSH3 mutations in MSI tumours (Duval *et al.*, 2001) the absence of BAX mutations again suggest that it would be unlikely that these samples would have hMSH3 mutations as these mutational events seem to occur late in the MSI carcinogenesis pathway.

4.4.2.4 Insulin-like growth factor II receptor gene (IGFIIR)

There were no mutations detected within the section of the IGFIIR gene amplified in any of the samples analysed in this study. In previous studies, levels of IGFIIR mutations in sporadic MSI tumours are between 10% - 24% (Souza *et al.*, 1996; Jeong *et al.*, 2003). As there were only 2 patients found to have MSI colorectal tumours in this study it would have been approximately 20%-50% chance for either of these to have been mutated in this microsatellite based on levels found in previous studies. It is however not possible with such a small number to draw conclusions from this data, other than there were no samples from the 22 patients with MSS tumours with mutations at these loci. Therefore this gene would be effective as a marker in respect that there are no MSS colorectal cancers with false positive results for the IGFIIR microsatellite.

4.4.2.5 Overall MSI level in the sample group

If the assumption is made that tumours with mutations within the TGF-βRII microsatellite are indicative of MSI, as these mutations are found in 90-100% of MSI+ tumours, then the level of MSI in the patients of this study is 2/24 (8.3%). This is further qualified as there are no reported cases of mutations within the A_8 microsatellite of the gene in MSS tumours and therefore these results are unlikely to be false positives. There is however the possibility that there may be false negative

results within this group since up to 10% of MSI+ tumours may lack mutations within the TGF-βRII A_8 microsatellite.

The level of 8.3% of sporadic colorectal tumours with MSI falls well within the previously reported levels of 6-18% (Young *et al.*, 1993; Thibodeau *et al.*, 1993; Ionov *et al.*, 1993; Chao *et al.*, 2000).

4.4.3 Types of mutation found in microsatellites analysed

The 2 mutations found in this study were 1bp deletion and 1bp insertion within the TGF-βRII microsatellite. These are entirely consistent with previous studies such as a study which analysed the types of mutation within 29 microsatellites containing mononucleotide repeats and have found that 41% of mutations are 1bp insertions, 55% bp deletions and 4% involved shifts of 2bp (Zhang *et al.*, 2001).

4.4.4 Profile of patients with MSI tumours

The details of the two patients which had MSI tumours are listed in Table 4.3. The details of the overall cohort are tabulated in appendix I.

4.4.4.1 Sex ratio of patients with MSI tumours

Although no statistical significance can be determined from these results as the sample size of patients with MSI tumours is very small, there was no apparent sex bias with one male and one female patient with MSI tumours. A female bias of MSI tumours has been repeatedly reported (Ward *et al.*, 2001; Samowitz *et al.*, 2001b; Thibodeau *et al.*, 1998)

4.4.4.2 Ages of patients with MSI tumours

The ages of these two patients (80, 58, average age 69) were typical of those with MSI tumours as it has been reported that although the average age of patients with MSI tumours is not significantly different to those with MSS tumours (66 and 64 years respectively), the spread across age groups is significantly different following a

J-shaped distribution with peaks of incidence at the younger and older age groups (Samowitz *et al.*, 2001b; Chao *et al.*, 2000). The highest proportion of patients with MSI tumours are aged 80 years and older (Chao *et al.*, 2000). This statistic is in agreement with this study where one of the two patients with MSI tumours was 80 years at the time of surgery. There are also a large number of patients diagnosed at a younger age although the age of cut-off for this cohort varies between studies from under 50 years (Chao *et al.*, 2000) to under 55 years (Samowitz *et al.*, 2001b). From these age groupings the 58 year old male patient displaying MSI in this study is 3 years older than the cut off point for those patients regarded as young in previous studies.

4.4.4.3 Anatomical locations of MSI tumours with the colorectum

MSI tumours are predominantly found in the proximal colon (Thibodeau *et al.*, 1993; Samowitz *et al.*, 2001b; Ward *et al.*, 2001; Chao *et al.*, 2000). There have been few studies assessing the level of MSI in rectal as opposed to colorectal tumours; however, those that have differentiated between colon and rectum, report lower levels of MSI in rectal tumours than studies which group these cancer types together (Nilbert *et al.*, 1999; Kapiteijn *et al.*, 2001).

The locations of the 2 MSI tumours found in this study were the proximal colonic region, the caecum, and the distal colorectal region, the rectum. As there were only 6 proximal tumours included in this study this gives a level of 1/6 (16.7%) of proximal tumours with MSI and 1/2 (50%) of caecal tumours. There were 14 distal colorectal adenocarcinomas, of which 1/14 (7.1%) displayed MSI; of these 12 were rectal tumours of which 1 was found to have MSI. This level of 1/12 (8.3%) higher than the 3/165 (1.8%) level found to have MSI in a previous study of rectal tumours and it has been suggested by other groups that rectal MSI is a result of a hereditary predisposition (Nilbert *et al.*, 1999). The patients included in this study were selected as they were sporadic cases with no family history of colorectal cancers. Although it is feasible that the patient with a rectal MSI tumour had an undiagnosed inherited condition, it is more likely that the patient was a sporadic case. More studies examining specifically rectal tumours for MSI need to be undertaken to assess levels of MSI in this region of the colorectum. The study of rectal tumours by Nilbert and

colleagues examined 165 patients, a much larger study of these tumour types than this current study of 13 rectal tumours and can therefore be considered a more accurate measure of MSI in rectal tumours, with this current study including a non-typical population.

The level of MSI found in this study 2/24 (8.3%) is slightly lower than reported in some studies and may be a result of the majority of the polyps and tumours in this sample group 18/24 (75%) originating from a distal location and only 6/24 (25%) from a proximal location. None of the 5 lymph node samples analysed in this study showed MSI consistent with the observations that this pathway of carcinogenesis is linked with an improved prognosis.

4.4.4.4 Tumour stages of MSI tumours

The tumours of the patients were both at Dukes stage C1, at which point the tumour has spread to lymph nodes but has not at this point metastasised to distant organs such as the liver. This stage is more advanced than the Dukes stage B, at which there is no lymph node involvement, which, according to some studies is highly associated with MSI tumours (Samowitz *et al.*, 2001b; Thibodeau *et al.*, 1998). There are also studies where MSI has been linked to a higher grade of tumour (Ward *et al.*, 2001; Chao *et al.*, 2000) although there are also many studies which have shown a negative correlation with metastatic disease (Shibata *et al.*, 1994; Ionov *et al.*, 1993; Gryfe *et al.*, 2000).

Neither of the patients with MSI tumours had mutations in the microsatellite within the BAX gene which is an indicator of poor prognosis with the MSI subset of colorectal cancers and is also linked to metastatic progression of colorectal cancer (Abdel-Rahman *et al.*, 1999; Fallik *et al.*, 2003; Ionov *et al.*, 2000). BAX inactivation confers a selective advantage during clonal evolution by enhancing survival advantage and may be involved in metastatic progression (Abdel-Rahman *et al.*, 1999; Fallik *et al.*, 2003; Ionov *et al.*, 2000).

4.4.5 Is there a link between mtMSI and nMSI?

In Chapter 3 mutations within a microsatellite in the mitochondrial genome were found in several patients and one of the goals of the experiments in this chapter was to establish whether or not there is a link between microsatellite instability in the nuclear genome (nMSI) and mitochondrial genome (mtMSI). Neither of the two patients with nMSI tumours had mutations within the control region of the mtDNA; likewise none of the patients with mtDNA microsatellite mutations had nMSI mutations.

These findings are in agreement with those of Heerdt *et al.* (1994) in whose study of 24 colonic tumours it was reported that none of the tumours exhibiting nuclear MSI showed coincident mtMSI at a $(CA)_n$ locus. A study by Habano *et al.*, (1998) also detected no mtMSI in the colorectal tumours which were unstable in the nuclear microsatellite loci analysed. Also, a study of microsatellite instability of the nuclear and mitochondrial genomes of gastrointestinal cancer found no association between these types of mutations (Schwartz and Perucho, 2000). From these sets of results it was reasoned that there were separate mechanisms for genomic instability between the nuclear and mitochondrial genomes with different enzymes responsible for the mismatch repair of the two genomes (Heerdt *et al.*, 1994; Habano *et al.*, 1998).

The same conclusions have also been drawn from similar studies examining different tumour types such as a study of breast tumour tissue which found 17/40 (42.5%) of the tumours to have mutations within microsatellites in the mitochondrial genome and 20/40 (50%) to have nMSI but with no correlation between the mtMSI and nMSI. From this study it was thought that this is a result caused by abnormalities at different stages of the pathway leading to cell transformation (Richard *et al.*, 2000).

The presence of mtMSI may be a result of higher levels of strand slippage mispairing in the mitochondrial genome compared to that of nuclear genome rather than a difference in mismatch repair capabilities. Mutation rates in microsatellites within mtDNA may be so high that even with MMR the microsatellite mutation level observed is still higher than that in the nuclear genome where the rate of mutation in

microsatellites may have been lower, allowing for repair to be complete. It is thought that mtMSI is more dependent upon polymerase-γ mismatch repair than the damage caused by ROS (Richard *et al.*, 2000). Therefore the difference between mitochondrial and nuclear MSI status may hinge on the efficiency of the MMR function of polymerase-γ which may be altered through the pathway in which mtMSI is observed.

4.4.6 Discussion Summary

Mutations in microsatellites were found in 2/24 patients, both of which were found within the TGF-βRII gene. As this gene has been shown to be an exceptionally sensitive marker of MSI in colorectal cancers, it was assumed from this that these 2 patients were MSI and that, although it cannot be ruled out that there may be other patients of this phenotype within this sample, there have been no recorded incidences of TGF-βRII microsatellite mutation within patients with effective MMR. None of the other 3 microsatellites, BAX, IGFIIR or hMSH3 were mutated in any of the samples. These levels of mutation are in line with previous studies; however, as there were only 2 patients within the sampling group with MSI, this is too small a sample size to draw statistically significant results from. The overall level of MSI in this study is 2/24 (8.3%); this level correlates with previous studies. This level is at the lower end of previously recorded MSI levels in sporadic colorectal cancer and may be a result of the sample consisting predominantly of distal colorectal cancers. Despite this bias towards distal tumours within the sample population, proximal tumours were shown to contain proportionately more microsatellite mutations with 1/6 (16.7%) being MSI as opposed to 1/14 (7.1%) distal tumours.

None of the patients with mtDNA MSI had nMSI mutations and, although there was only a small number of patients showing nMSI, this data suggests there is no link between mtMSI and nMSI, and that these probably occur through different pathways.

Despite the lack of correlation between mtMSI and nMSI, these may still be important factors when considering the treatment of the cancers and prognosis for specific patients. The more that is known about the differences in types of colorectal tumour, the more chance there is of developing treatments which are specific to the cells with defects in specific pathways. For example colorectal tumours with defective mismatch repair could be treated with chemotherapeutic agents which do not affect normal cells where such repair is functional (Parsons *et al.*, 1993). One such pathway specific cancer treatment for sporadic MSI-H cancers suggests treating with demethylating agents which may reverse the hypermethylation of promoters for MMR genes such as hMLH1 and either treat or help prevent the development of these colorectal tumours (Veigl *et al.*, 1998).

Chapter 5

Detection of tumour suppressor gene expression changes with tumour progression using array analysis and real time PCR

5.1 Introduction

5.1.1 Gene expression

Gene expression is the term used to describe how different genes within a cell are turned on or off to varying extents depending upon the requirements of the cell. It is because of differing gene expression patterns that cells from different tissues that contain the same DNA are able to take on completely different roles such as the cells of the colorectal mucosa and cells of the underlying muscle. Until recently gene expression has primarily been analysed by histochemistry with anti-bodies and stains defining the proteins expressed. These techniques are still of great value especially when examining the localised patterns of expression, for example, by studying the varying levels of proteins required for the homeostatic balance between cell proliferation and cell differentiation in a single crypt foci of the colonic epithelium. Patterns of APC and Rb expression vary within a single crypt foci with higher levels of Rb detected at the base of the crypt than at the top of the same crypt, an opposite pattern is found for APC which is predominantly expressed at the top of each crypt (Ali *et al.*, 1993; Smith *et al.*, 1993). Techniques such as RT-PCR and cDNA arrays have now been developed which can analyse the cellular levels of mRNA, the precursor to proteins.

5.1.1.1 Gene expression changes in colorectal cancer

As well as adding to the understanding of cancer development, the study of gene expression can act as an indicator of prognosis. For example, a lack of expression of the cyclin dependent kinase inhibitor p57 in colorectal cancers is linked to poor prognosis for the patient (Li *et al.*, 2003). The expression levels of several tumour endothelial markers have been associated with colorectal cancer progression and nodal involvement and therefore may be useful as prognostic indicators (Rmail *et al.*, 2005). Gene expression in a specific tumour can also give indications on how a

particular patient may respond to certain chemotherapeutic agents. For example, colon cancer cells with low levels of Bax are resistant to 5-flurouracil, the most commonly used drug in colon cancer treatment (Violette *et al.*, 2002). Gene expression analysis of individual patients may also be important in determining the dose of chemotherapy for each patient. Irinotecan is an anti-cancer drug which interacts with DNA-topoisomerase 1, the effectiveness of this drug in patients varies widely between different patients. A recent study of colorectal tumours has shown the expression level of the genes CES1 and CES2 which are involved in the irinotecan pathway vary greatly between patients. This information can be used to decide not only whether this drug would be appropriate in each individual case but also to establish a dosage which would have less of a toxic effect on the patient's normal tissues (Yu *et al.*, 2005).

5.1.2 DNA Arrays

The advent of array technology has made it possible to examine the levels of gene expression of many genes in one experiment. Arrays are now commercially available as gene chips with several thousand DNA sequences printed on a glass slide, these require expensive and specialist equipment for data reading and further software and expertise for the data mining required for such large data sets. Arrays are also available as nylon membranes containing between 18 and 96 genes which have advantages over the gene chips in terms of being less expensive in terms of consumables and specialised equipment needed and allows a more focused approach to gene expression analysis as the arrays contain genes which are grouped in terms of function such as DNA repair and tumour suppressor genes. The disadvantages of these types of nylon membrane arrays are that there can be more background noise in comparison to the glass slide arrays and the membrane can become distorted, both of these disadvantages are due to the material of the medium on which the genes are printed (Cheung *et al.*, 1999). These negative aspects can be overcome using the software designed for the analysis of these specific types of array. The array selected for these experiments is the GEArray (Superarray, USA) human cancer/tumour suppressor array which is composed of 23 genes that inhibit cell or tumour growth including known tumour suppressor genes, cyclin-dependent kinase inhibitors and genes which regulate tumour suppressors, these genes and their functions listed in Table 5.1. Further details including full gene names and Genebank accession numbers are listed in Table 5.2.

5.1.3 Introduction to genes present on the GEArray tumour suppressor array

Gene	Function
APC	Regulates epithelial homeostasis processes such as cell cycle progression and apoptosis
BRCA1	Transcription factor, activates genes such as p53 following DNA damage
BRCA2	Transcription factor, involved in the repair of double strand breaks
CBP	Modulates DNA damage response by transcriptional activation
DPC4	Involved in the TGF-β signalling transduction pathway
IRF-1	Transcriptional activator and collaborates with p53 to induce cell cycle arrest
MSH2	Mismatch repair gene
NF2	Stabilizes cell membrane
p18	Cyclin-dependent kinase inhibitor, inhibits cell cycle progression
p19	Cyclin-dependent kinase inhibitor, inhibits cell cycle progression
p21	Cyclin-dependent kinase inhibitor, inhibits cell cycle progression
p27Kip1	Cyclin-dependent kinase inhibitor, inhibits cell cycle progression
p300	Modulates DNA damage response by transcriptional activation
p53	Suppresses cell growth, mediates cell cycle arrest, activates apoptosis inducing genes
p57Kip2	Cyclin-dependent kinase inhibitor, inhibits cell cycle progression
PTEN	Encodes a dual-specificity phosphatase
Rb	Regulates cell cycle progression from G1 to S-phase
TGF-βRI	Acts as a signal transducer after binding the epithelial cell growth inhibitor TGF-β
TGF-βRII	Acts as a signal transducer after binding the epithelial cell growth inhibitor TGF-β
TSC1	Involved in cell cycle control, endocytosis, cell adhesion and transcription
TSC2	Involved in cell cycle control, endocytosis, cell adhesion and transcription
VHL	Down regulator of transcription elongation
WT1	Transcription factor involved in growth, differentiation and cell cycle arrest
GAPDH	Constitutively expressed housekeeping gene used on this array as a positive control
β-actin	Constitutively expressed housekeeping gene used on this array as a positive control. Codes for protein which forms part of the cells cytoskeletal structure
PUC18	Bacterial plasmid used on this array as a negative control

Table 5.1

Brief description of the function of the genes present on the tumour suppressor array used in these experiments.

Abbreviated Gene name	Full gene name	Genebank code
APC	Adenomatous polyposis coli	M74088
BRCA1	Breast cancer 1, early onset	U68041
BRCA2	Breast cancer 2, early onset	X95177
CBP	Human CREB-binding protein	U85962
DPC4	Human deletion target in pancreatic carcinoma	U44378
IRF-1	Interferon regulatory factor 1	NM_002198
MSH2	MutS homolog 2 (nonpolyposis colon cancer)	U03911
NF2	Neurofibromin 2	L11353
p18	Cyclin-dependent kinase inhibitor 2C	U17074
p19	Cyclin-dependent kinase inhibitor 2D	U40343
p21	Cyclin-dependent kinase inhibitor 1A	L47233
p27Kip1	Cyclin-dependent kinase inhibitor 1B	U10906
p300	E1A binding protein p300	NM_001429
p53	Tumor protein p53	M14694
p57Kip2	Cyclin-dependent kinase inhibitor 1C	U22398
PTEN	Phosphatase and tensin homolog	U96180
Rb	Retinoblastoma 1	M15400
TGF-βRI	Transforming growth factor, beta receptor I	L11695
TGF-βRII	Transforming growth factor, beta receptor II	D50683
TSC1	Tuberous sclerosis 1	AF013168
TSC2	Tuberous sclerosis 2	X75621
VHL	Von Hippel-Lindau syndrome	AF010238
WT1	Wilms tumor 1	X51630
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	M33197
β-actin	Beta Actin	X00351
PUC18	PUC18 bacterial plasmid DNA	

Table 5.2

Abbreviations and descriptions of the genes and control sequences on the tumour suppressor array used in these experiments.

5.1.4 Rationale of the study

The previous chapters described experiments designed to detect and evaluate any differences between the various stages of colorectal cancers in terms of mitochondrial mutations and microsatellite instability. Alterations were found in several of the carcinoma samples which were informative in terms of pathways and mechanisms of carcinogenesis and therefore possible treatment avenues. However, these mutations were present at a late stage and therefore were not useful candidate markers for early diagnosis. Rather than look for possible markers and see if these were present early on, the experiments in this chapter look at early stage samples and see if there are any changes present that may have potential as a molecular marker or may appear important in the early stages of progression of carcinogenesis and therefore to add data to the Vogelgram of cancer progression from adenoma to carcinoma as well as to try to discover why some polyps progress to cancer whilst others do not.

The purpose of this study was to address these issues by exploiting the new technologies available to study gene expression of several genes in a single tissue sample in one experiment, particularly as some samples were very small, limiting the quantity of RNA available for analysis.

5.2 Materials and methods

5.2.1 Sample collection

16 samples from 5 patients were collected from surgery, resected with the assistance of a pathologist and stored at 4°C in RNA*later* (Ambion, UK) until use as described in Chapter 2, Section 2.2. Tumour samples were taken from patient five from the central and peripheral regions of the carcinoma to allow analysis of the clonality of the tumour. Table 5.3 lists the patient details for this sample cohort.

Patient Number	Sex	Age	Dukes' Stage	Location of tumour
1	67	M	C	Rectal
2	61	M	A	Rectal
3	63	M	C	Sigmoidal
4	57	F	A	Rectal
5	74	F	C	Rectosigmoidal

Table 5.3 Patient details for the tissue samples used in the array analysis described in this chapter.

5.2.2 RNA extraction and quantification

RNA was extracted from the tissues as described in Chapter 2, Section 2.11.1 and quantified as described in Chapter 2, Section 2.11.2. The purity of the RNA was also assessed to detect any protein contamination, this is also described in Chapter 2, Section 2.11.2

5.2.3 Arrays

The GEArray original series human cancer/ tumour suppressors gene array (SuperArray, USA) with 23 tumour suppressor genes was selected for these experiments. The genes on this array are listed in Tables 5.1 and 5.2. The location of

these genes on the array is illustrated in Figure 5.1. Gene expression levels were measured for the tumour suppressor and housekeeping genes described in section 5.1.3 utilising nylon membrane arrays using the protocol described in Chapter 2, section 2.12.

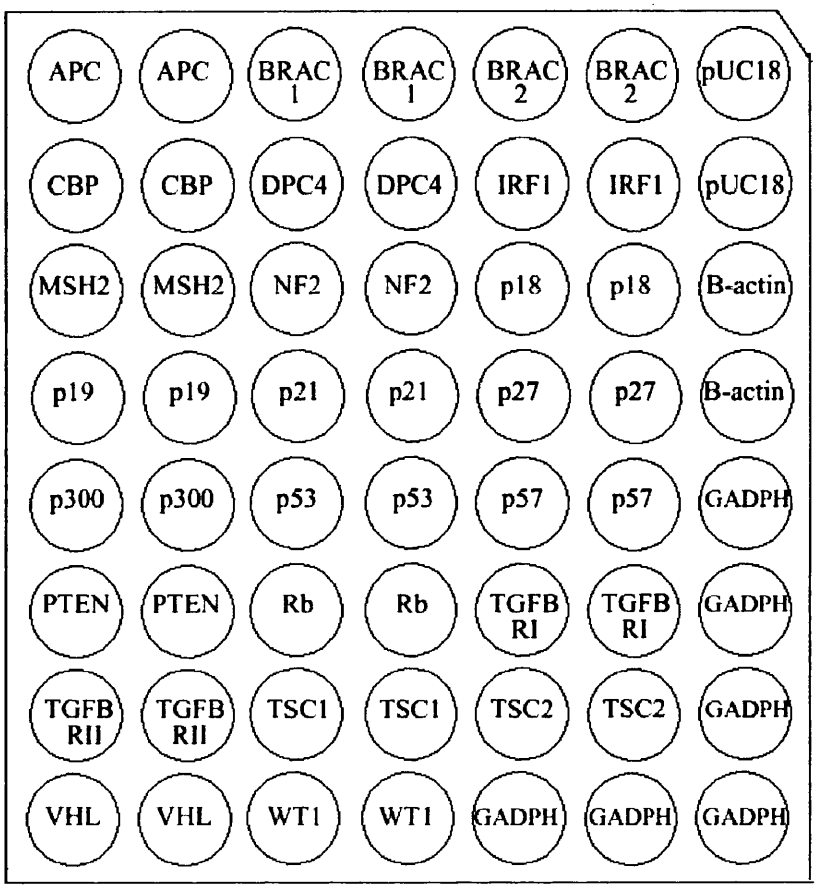


Figure 5.1

Locations of the genes on the tumour suppressor membrane array. Full gene names for each abbreviation are listed in Table 5.2.

5.2.3.1 Array analysis

Images of each array were captured as described in Chapter 2, section 12.1. Spot intensities were consolidated prior to background subtraction and normalisation as described in Chapter 2, section 12.2. Data was filtered to remove results from genes being expressed at levels less than 10% of the housekeeping gene β-actin as this is

lower than can be accurately measured using these arrays. Expression ratios were transformed to produce a continuous range of fold changes; this is explained in more detail in Chapter 2, Section 2.12.2. Fold changes over ±2-fold are considered significant changes in gene expression (Quackenbush, 2002). Arrays from each tissue type were analysed independently against the remaining tissue types from the same patient, for example the array from the normal mucosa was analysed against that of the polyp and the tumour and the array from the polyp was separately analysed against the tumour array. This was carried out so that an accurate assessment of the gene expression changes at each stage could be determined and genes that were for example down-regulated from normal mucosa to polyp but up regulated from polyp to mucosa could be detected even though these may give no overall change in expression from normal mucosa to tumour.

5.3 Results

5.3.1 Quantity and quality of RNA extracted

The RNA quality and quantity extracted from each sample depended upon the size of the sample and the sample type. Some adenoma samples measured <3mm and so a limited amount of RNA could be extracted. The tissue from the adenoma and carcinoma samples was far easier to break up and so a higher amount of RNA per gram of tissue was extracted with the normal mucosa yielding lower amounts. The quality of the RNA for all the samples used in this study was high as determined by spectrophotometry with an A_{260}/A_{240} ratio of over 1.8 as described in Chapter 2, Section 2.11.2. Repeat array analysis was undertaken where RNA quantities allowed. RNA quality and quantity values are included in Table 5.4.

Patient Number	Sample No	Sample Type	Ratio	Conc. µg/ml
1	12	Rectal adenocarcinoma	1.97	1834
	15	Polyp	1.89	1595
	13	Normal mucosa	1.85	690
2	28	Rectal adenocarcinoma	1.88	2587
	31	Large Polyp	1.82	1012
	33	Normal mucosa	2.10	716
3	38	Sigmoid adenocarcinoma (Peripheral region)	1.96	1140
	39	Sigmoid adenocarcinoma (Central region)	1.93	1364
	40	Normal mucosa	1.95	753
	42	Polyp	2.00	692
4	16	Rectal adenocarcinoma	1.94	1917
	19	Polyp	1.82	687
	17	Normal mucosa	1.91	672
5	86	Normal mucosa	1.89	686
	88	Rectosigmoid adenocarcinoma	1.90	902
	90	Polyp	1.81	840

Table 5.4

Table of RNA sample details including the A_{260}/A_{240} ratio and the concentration of RNA extracted for each tissue sample.

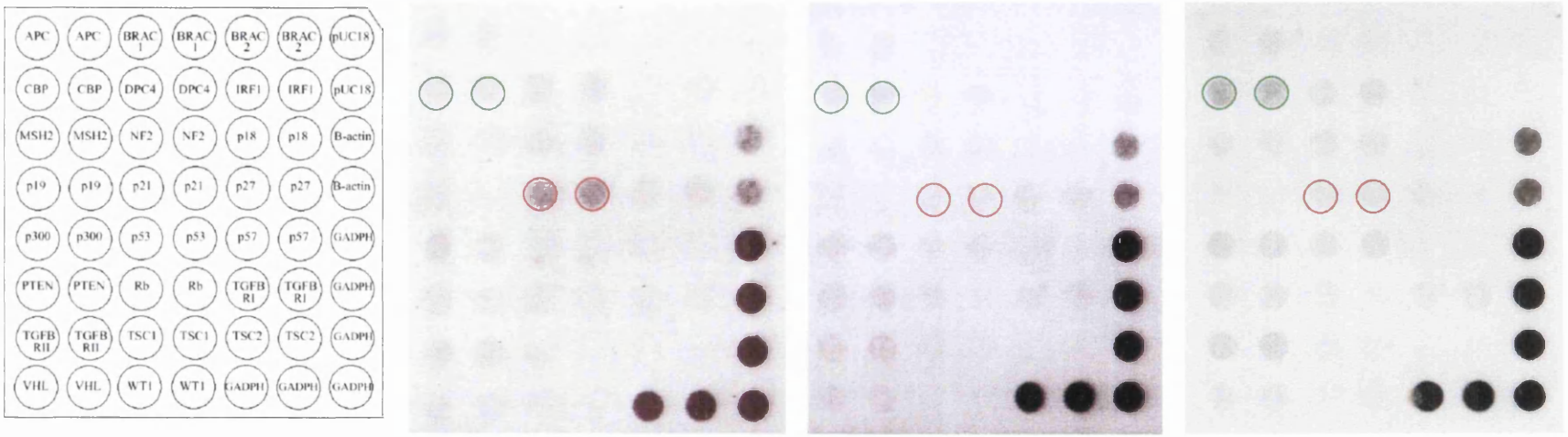
5.3.2 Array analysis and results

For each patient RNA was extracted from normal mucosa, polyp and an adenocarcinoma as described in Chapter 2, Section 2.11.1. cDNA was produced and used to carry out the array analysis, see Chapter 2, Section 2.12. Analysis of the spot intensity data obtained for each gene was carried out as described in Section 5.2.3 to give an average result for the two spots per gene on each array, to remove background noise levels and to normalise the data against housekeeping gene β-actin to allow for meaningful comparisons to be made between each array.

5.3.2.1 Array results from patient one

Patient one was a 67 year old male with Dukes' stage C rectal cancer. Photographs of the array images for this patient can be seen in Figure 5.2 The darker spots on these images correspond to genes with comparatively higher levels of gene expression than the other genes within the same array. The experiments were repeated and the average fold changes between tissue types are listed in Table 5.5 and are depicted graphically in Figure 5.3.

Images taken of the cDNA arrays obtained from patient one for the RNA extracted from normal, polyp and tumour tissue.



Map of gene locations on array Normal mucosa Polyp Adenocarcinoma

Figure 5.2

These photographs show arrays from the same patient for the normal mucosa, a polyp sample and from an adenocarcinoma sample. Green circles highlight the cDNA corresponding to the tumour suppressor CBP, it can be seen that this spot on the array is darker through the stages of carcinogenesis suggesting an up regulation of this gene. The red circles highlight the cDNA corresponding to the tumour suppressor p21, these are darker in relation to the control genes on the array from the normal mucosa than they are from the polyp. This corresponds to a higher level of cDNA, and therefore mRNA, for p21 in normal mucosa compared to polyp and tumour tissue in this patient.

Gene	Fold change in gene expression		
	Normal to polyp	Normal to tumour	Polyp to tumour
APC	0.00	0.13	0.00
BRCA1	0.00	1.02	0.00
BRCA2	0.00	1.41	0.00
PUC18	0.00	1.00	0.50
CBP	1.53	3.27*	2.14*
DPC4	-2.75*	0.16	2.96*
IRF-1	0.00	-1.59	0.00
MSH2	0.00	1.23	0.00
NF2	-2.08*	-0.12	1.89
p18	0.00	-3.18*	0.00
GAPDH	1.03	1.08	0.05
p19Ink4d	0.00	-1.70	0.00
p21Waf1	-7.01*	-3.64*	2.00*
p27Kip1	1.23	2.31*	1.88
p300	-1.28	2.01*	2.52*
p53	-1.82	0.00	1.86
p57Kip2	-1.30	-6.75*	-4.87*
b-actin	1.00	1.00	1.00
PTEN	-1.33	1.45	1.87
Rb	0.00	-2.54*	0.00
TGFBR1	-1.37	-0.07	1.29
TGFbR2	-0.05	2.53*	2.64*
TSC-1	0.00	0.12	0.00
TSC2	0.00	-5.31*	0.00
VHL	0.00	1.76	0.00
WT1	0.00	-5.61*	0.00

Table 5.5

Gene expression fold changes between tissue samples for patient one. A value of 0.00 has been used where data for the gene was not above 10% of B-actin and is therefore not included as this is below the threshold recommended by Superarray, by these same recommendations, changes above 2 fold are significant and are marked with an asterisk.

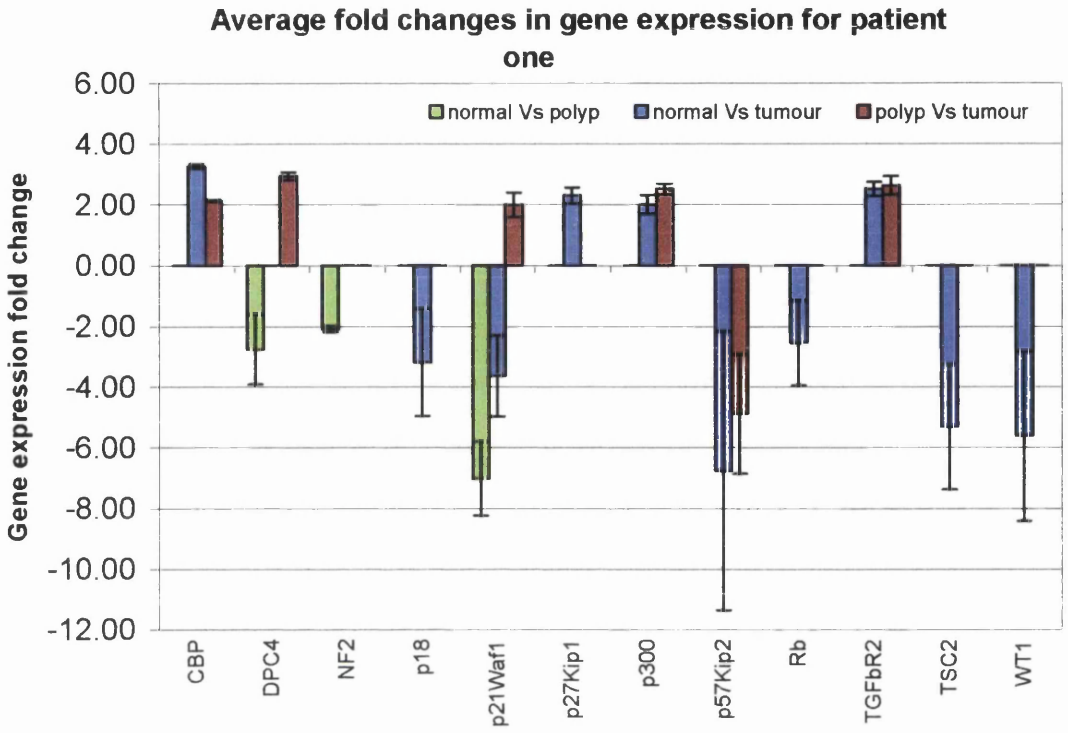


Figure 5.3

This graph shows the fold changes between the tissue types for the genes where the changes in gene expression are over 2 fold. The error bars for each gene indicate +/- standard deviation between repeat experiments.

5.3.2.2 Array results from patient two

Patient two was a 61 year old male with Dukes' stage A rectal cancer. The average fold changes between tissue types as determined by array analysis are listed in Table 5.6

	Fold change in gene expression		
Gene	Normal to polyp	Normal to tumour	Polyp to tumour
APC	-2.69*	0.00	0.00
BRCA1	0.00	0.62	2.18*
BRCA2	0.00	0.00	0.00
PUC18	0.00	0.00	0.00
CBP	0.25	-0.77	-0.59
DPC4	2.09*	-1.15	-1.40
IRF-1	-0.95	0.00	0.00
MSH2	-0.94	0.62	1.95
NF2	0.85	-0.63	0.52
p18	-2.78*	-0.72	2.29*
GAPDH	1.07	-1.31	-1.39
p19Ink4d	-8.18*	-0.77	2.96*
p21Waf1	-3.96*	-2.72*	0.47
p27Kip1	-0.37	-1.44	-0.11
p300	-1.91	-1.25	1.59
p53	-0.08	0.05	1.24
p57Kip2	-2.27*	0.88	0.61
b-actin	0.00	1.00	1.00
PTEN	-11.31*	-1.06	2.09*
Rb	-5.97*	-0.16	4.11*
TGFBR1	-6.98*	1.62	12.90*
TGFbR2	-10.76*	-0.75	2.21*
TSC-1	-3.78*	-1.51	4.42*
TSC2	0.00	1.35	1.23
VHL	0.00	-0.87	2.85*
WT1	0.00	0.00	0.00

Table 5.6

Gene expression fold changes between tissue samples for patient two. A value of 0.00 has been used where data for the gene was not above 10% of B-actin and is therefore not included as this is below the threshold recommended by Superarray, by these same recommendations, changes above 2 fold are significant and are marked with an asterisk.

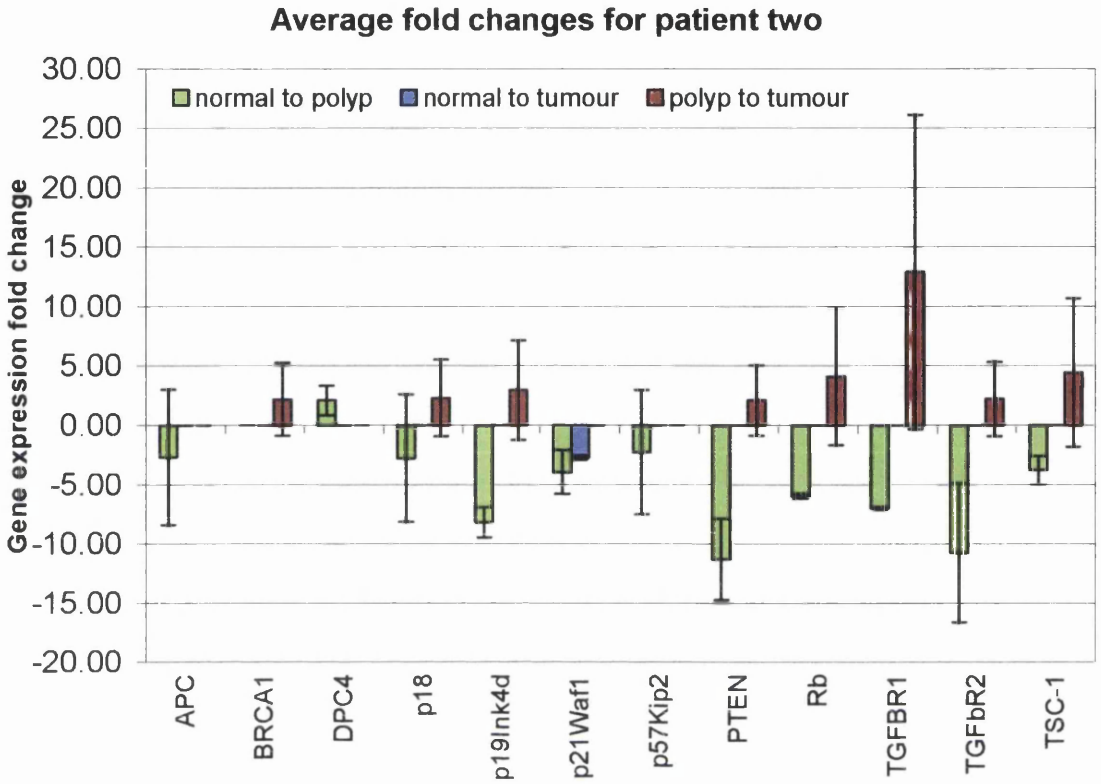


Figure 5.4

This graph shows the fold changes between the tissue types for the genes where the changes in gene expression are over 2 fold. The error bars for each gene indicate +/- standard deviation between repeat experiments.

5.3.2.3 Array results from patient three

Patient three was a 63 year old male with Dukes' stage C sigmoidal cancer. From patient three RNA was extracted from normal mucosa, polyp and two samples from the same adenocarcinoma, one from the centre of the tumour and one from the peripheral region. cDNA was produced and used to carry out the array analysis. There was unfortunately insufficient RNA to carry out a repeat array experiment for the peripheral tumour. The data recorded in Table 5.6 is the average fold changes between the tissue types excluding peripheral tumour, but observations from this experiment are discussed in Section 5.4.5.

Gene	Fold change in gene expression		
	Normal to polyp	Normal to central tumour	Polyp to central tumour
APC	-2.30*	-0.69	0.59
BRCA1	0.00	-0.75	0.63
BRCA2	-0.80	-0.68	0.59
PUC18	1.00	1.00	1.00
CBP	-11.48*	-3.28*	3.73*
DPC4	-2.26*	-3.90*	-1.37
IRF-1	-1.62	-1.50	-2.67*
MSH2	-0.84	-2.62*	-0.87
NF2	-2.04*	-2.92*	-1.44
p18	-0.01	-0.88	-0.68
GAPDH	-1.07	0.30	0.40
p19Ink4d	-1.55	-0.95	-0.15
p21Waf1	-4.97*	-8.44*	-1.71
p27Kip1	2.05*	3.70*	1.29
p300	-2.54*	-1.92	0.52
p53	0.38	3.24*	2.89*
p57Kip2	-1.33	-0.92	-0.56
b-actin	1.00	1.00	1.00
PTEN	-2.56*	-2.18*	0.51
Rb	-1.31	2.20*	2.39*
TGFBR1	-3.62*	-1.30	2.57*
TGFbR2	-2.66*	-1.86	1.61
TSC-1	0.00	1.56	-1.43
TSC2	0.00	4.43*	0.00
VHL	0.00	7.80*	0.61
WT1	0.00	3.12*	3.79*

Table 5.7

Gene expression fold changes between tissue samples for patient three. A value of 0.00 has been used where data for the gene was not above 10% of B-actin and is therefore not included as this is below the threshold recommended by Superarray, by these same recommendations, changes above 2 fold are significant and are marked with an asterisk.

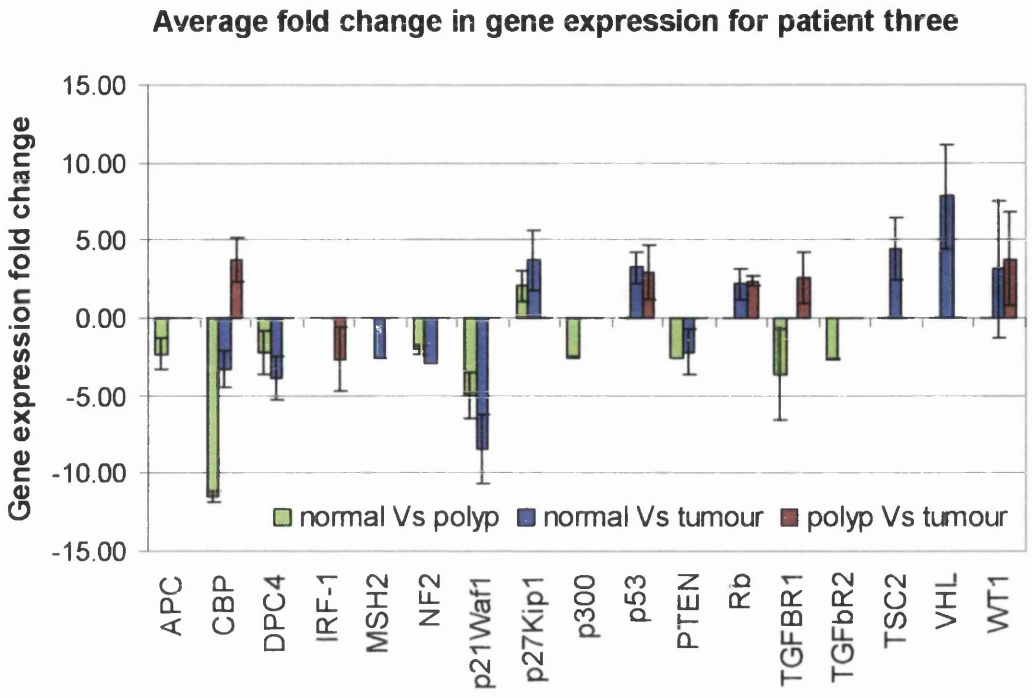


Figure 5.5

This graph shows the fold changes between the tissue types for the genes where the changes in gene expression are over 2 fold. The error bars for each gene indicate +/- standard deviation between repeat experiments.

5.3.2.4 Array results from patient four

Patient 4 was a 57 year old female with Dukes' stage A rectal cancer. The average fold changes between tissue types as determined by array analysis are listed in Table 5.8

Gene	Fold change in gene expression		
	Normal to polyp	Normal to tumour	Polyp to tumour
APC	-1.65	-2.54*	-1.54
BRCA1	-2.48*	-1.11	2.25*
BRCA2	-2.12*	-1.99	0.08
PUC18	0.00	1.00	1.00
CBP	-1.88	-2.16*	-0.16
DPC4	-3.03*	-27.06*	-8.95*
IRF-1	-2.53*	-4.93*	-1.92
MSH2	-1.86	-3.80*	-2.04*
NF2	-3.17*	-6.75*	-2.09*
p18	-1.28	-2.07*	-1.60
GAPDH	-1.12	-1.14	-0.01
p19Ink4d	-1.85	-2.68*	-0.45
p21Waf1	-3.73*	-5.07*	-1.35
p27Kip1	0.05	-1.66	-1.76
p300	-1.71	-1.91	-0.10
p53	-1.55	-1.93	-0.19
p57Kip2	-15.35*	0.00	-8.99*
b-actin	1.00	1.00	1.00
PTEN	-3.33*	-8.29*	-2.43*
Rb	-1.38	-2.56*	-1.77
TGFBR1	-3.01*	-7.95*	-2.74*
TGFbR2	-2.61*	-4.40*	-1.73
TSC-1	-0.01	1.79	-1.31
TSC2	-0.06	-15.57*	-31.76*
VHL	0.50	1.26	-1.65
WT1	-4.56*	0.23	3.17*

Table 5.8

Gene expression fold changes between tissue samples for patient four. A value of 0.00 has been used where data for the gene was not above 10% of B-actin and is therefore not included as this is below the threshold recommended by Superarray, by these same recommendations, changes above 2 fold are significant and are marked with an asterisk.

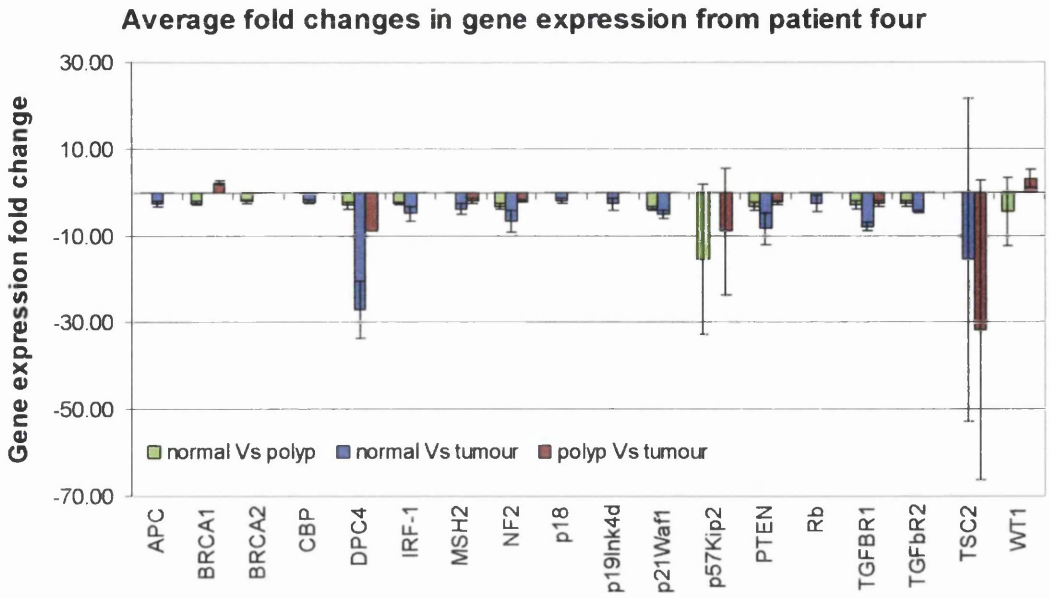


Figure 5.6

This graph shows the fold changes between the tissue types for the genes where the changes in gene expression are over 2 fold. The error bars for each gene indicate +/- standard deviation between repeat experiments.

5.3.2.5 Array results from patient five

Patient 5 was a 74 year old female with Dukes' stage C rectosigmoidal cancer. The average fold changes between tissue types as determined by array analysis are listed in Table 5.9

	Fold change in gene expression		
Gene	Normal to polyp	Normal to tumour	Polyp to tumour
APC	1.50	-1.98	-2.90*
BRCA1	0.00	-1.93	-3.02*
BRCA2	0.00	0.00	0.00
PUC18	0.00	0.00	0.00
CBP	-1.57	-0.47	0.20
DPC4	-1.42	-1.42	0.06
IRF-1	0.00	0.00	0.00
MSH2	-2.28*	0.22	2.65*
NF2	-3.14*	-0.09	2.94*
p18	0.00	0.00	0.42
GAPDH	-1.16	-1.32	-1.14
p19Ink4d	-0.10	-1.49	-1.41
p21Waf1	-3.58*	-3.38*	0.11
p27Kip1	-1.18	-0.08	0.13
p300	-1.87	-0.20	1.64
p53	-0.41	3.79*	3.24*
p57Kip2	-1.24	-6.00*	-4.25*
b-actin	1.00	1.00	1.00
PTEN	-1.48	-3.74*	-2.52*
Rb	-0.09	2.54*	2.74*
TGFBR1	-2.33*	-9.49*	-5.13*
TGFbR2	-1.72	-2.96*	-1.73
TSC-1	0.28	-1.96	-2.73*
TSC2	0.00	0.00	-2.32*
VHL	2.47*	-6.46*	-15.75*
WT1	0.00	0.00	0.00

Table 5.9

Gene expression fold changes between tissue samples for patient five. A value of 0.00 has been used where data for the gene was not above 10% of B-actin and is therefore not included as this is below the threshold recommended by Superarray, by these same recommendations, changes above 2 fold are significant and are marked with an asterisk.

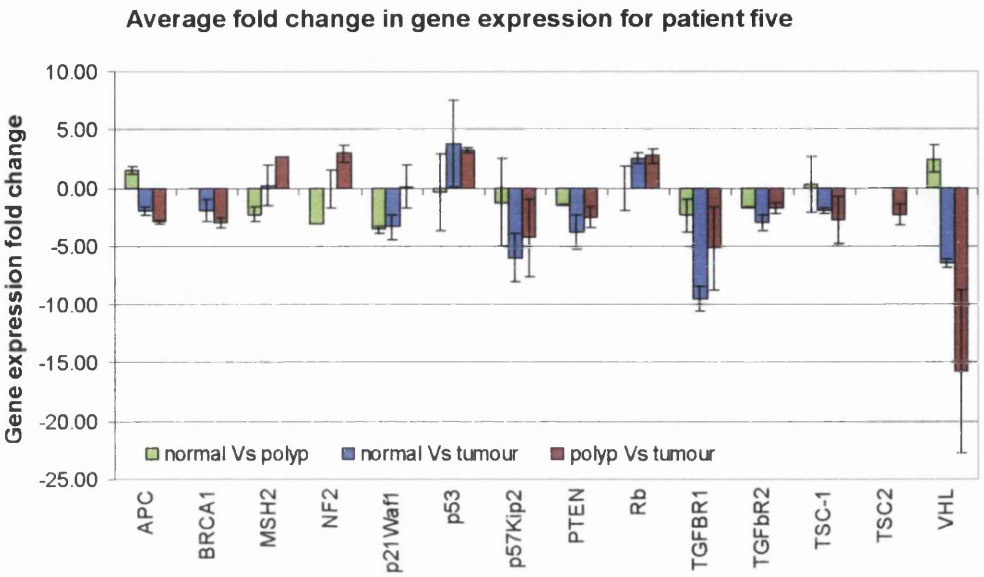


Figure 5.7 This graph shows the fold changes between the tissue types for the genes where the changes in gene expression are over 2 fold. The error bars for each gene indicate +/- standard deviation between repeat experiments.

5.3.2.6 Overall array results

To obtain an overall picture of the gene expression changes during colorectal carcinogenesis the mean changes across all the analysed patients for each gene were calculated. These results are listed in Table 5.10

Gene	Fold change in gene expression		
	Normal to polyp	Normal to tumour	Tumour to polyp
APC	-1.28	-1.27	-1.12
BRCA1	-1.71	-0.43	-0.09
BRCA2	-1.46	-0.42	0.15
PUC18	1.00	1.00	1.00
CBP	-2.63*	-0.68	1.33
DPC4	-1.47	-6.67*	-1.83
IRF-1	-1.70	-2.68*	-1.98
MSH2	-1.48	-0.87	-0.17
NF2	-1.92	-2.10*	0.24
p18	-1.35	-1.71	-0.68
GAPDH	-0.25	-0.48	-0.18
p19Ink4d	-2.92*	-1.52	-0.69
p21Waf1	-4.58*	-4.65*	-0.45
p27Kip1	0.35	0.57	0.55
p300	-1.86	-0.66	1.10
p53	-0.70	1.03	1.85
p57Kip2	-4.30*	-10.76*	-5.30*
b-actin	1.00	1.00	1.00
PTEN	-4.00*	-2.76*	-0.60
Rb	-2.19*	-0.11	0.83
TGFBR1	-3.46*	-3.44*	-1.02
TGFbR2	-3.56*	-1.49	0.27
TSC-1	-0.66	0.00	-1.83
TSC2	-0.06	-3.77*	-11.99*
VHL	1.73	0.70	-4.11*
WT1	0.31	-0.75	2.20*

Table 5.10

Mean gene expression fold changes between tissue samples for all five patients. Averages for each gene were taken for data above 10% of B-actin, data below this level was excluded as this is below the threshold recommended by Superarray. By these same recommendations, changes above 2 fold are significant and are marked with an asterisk.

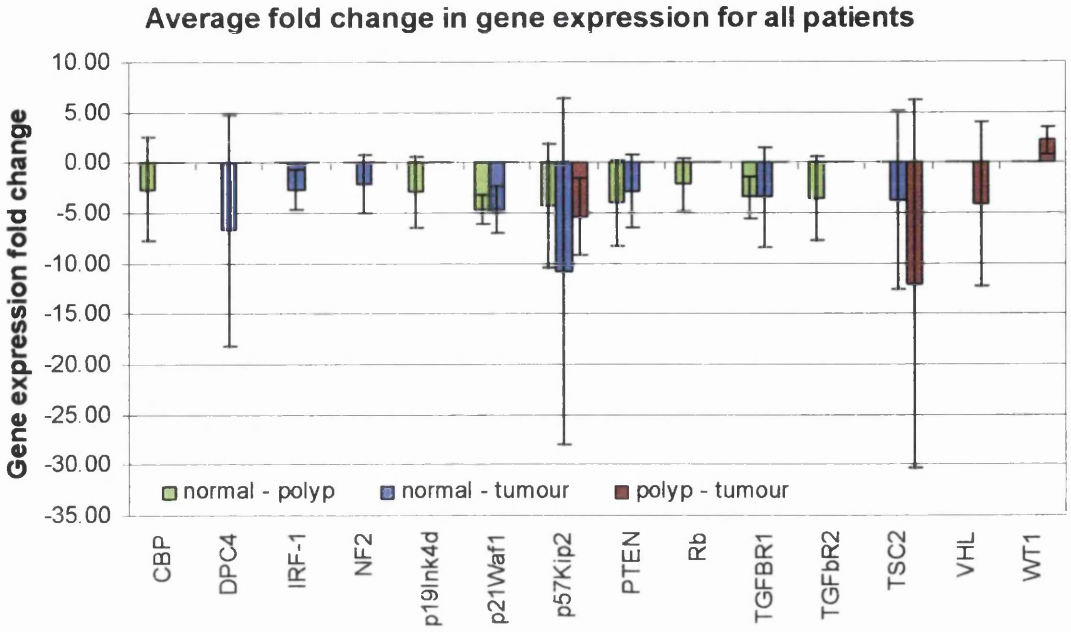


Figure 5.8

This graph shows the average fold changes between the tissue types for the genes where the changes in gene expression are over 2 fold. The error bars for each gene indicate +/- standard deviation between the average results for each patient.

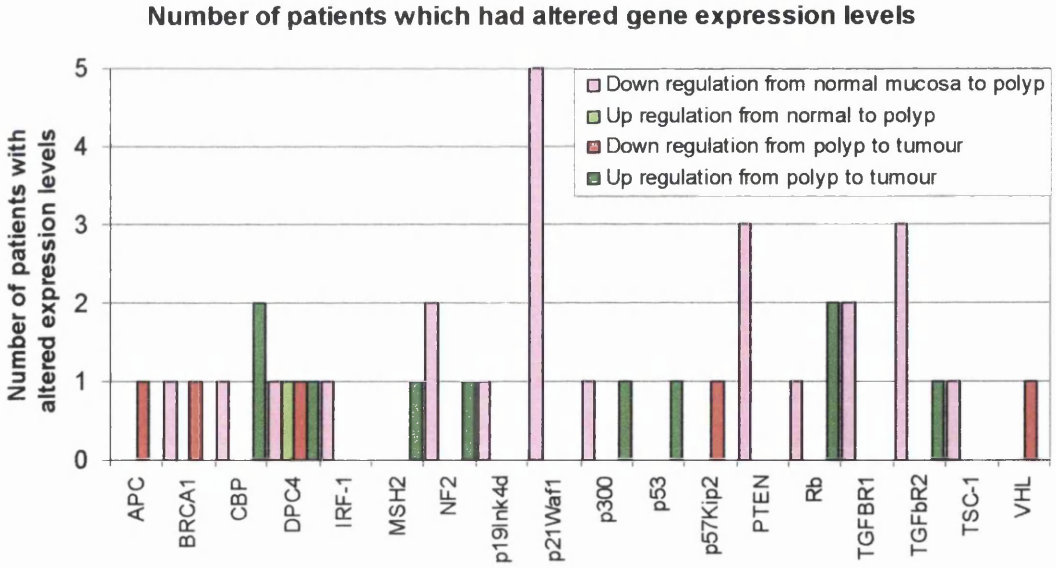


Figure 5.9

This graph shows the number of patients which exhibited alteration in gene expression between the tissue types for the genes where the changes in expression are over 2 fold once the standard deviations have been taken into account.

5.4 Discussion

5.4.1 RNA quality and quantity

The quality of RNA extracted across the samples was uniformly high as determined by spectrophotometry. The use of RNA*later* (Ambion, UK) on ice in the sample collection process is likely to be a contributing factor to this as it has been proven to be an effective preservative of RNA in solid tissue samples preventing the degradation of RNA (Mutter *et al.*, 2004). The logistical aspects of sample collection are another factor responsible for the high quality yields despite small sample sizes. The adjacent locations of the hospital and university laboratories, as well as co-operation between the surgical and pathology department staff enabled the resection and collection of surgical samples to occur within minutes of the sample being removed from the patient.

5.4.2 The control genes and cDNA sequences

5.4.2.1 β-actin

Beta-actin is a housekeeping gene and is therefore constitutively expressed in cells and so acts as both a positive control and also enables arrays to be normalised so the data from one array can be compared with another. There has though been a paper published reporting the expression levels of β-actin in colorectal samples to increase as tissues progress through the process of carcinogenesis. Higher levels of β-actin were detected in adenomas and metastatic polyps than in normal colonic tissue and even higher levels were detected in carcinomas, especially those which were poorly differentiated (Naylor *et al.*, 1992).

5.4.2.2 pUC18

This is a bacterial plasmid included on the array as a negative control as there should be none of this RNA present in a human tissue sample and so blank areas on the array for pUC18 confirm there has been no contamination of the array or sample and is

used in the array analysis to remove background noise from the intensities of the cDNA spots.

5.4.3 Gene expression changes detected in patient one

Patient one was male, 67 years of age at time of operation with Dukes stage C rectal cancer. A photograph of the array images captured by the ChemiDoc apparatus (BioRad, UK) can be seen in Figure 5.2. From this image it is possible to see some of spots on the array are darker than other suggesting these genes are expressed at a high level. However it is not possible to assess the change in gene expression without normalising the data and subtracting background noise. This and further analysis was undertaken and the average fold changes detected can be seen in Table 5.5.

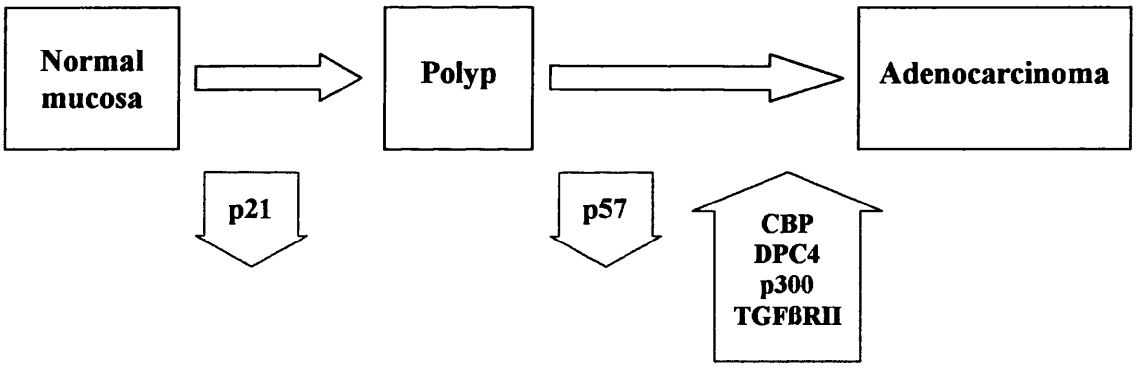


Figure 5.9

This diagram shows the gene changes that were detected in patient one between the stages of cancer progression. The red arrows represent down regulation and the green arrows indicate up regulation of the genes within each arrow. All the genes included had changes of over 2 fold detected.

Table 5.5 lists the gene expression fold changes for all of the genes on the array used for patient one. Where the expression level was so low that the spot intensity was <10% of the intensity of that of β-actin these results have been removed as an accurate reading cannot be obtained below this threshold. For comparisons between

arrays for these genes the fold change value has been replaced by 0.00. Figures 5.3 and 5.9 show the genes which had expression level changes of ±2 fold or greater. These results are classed as significant using the recommendations applied to array analysis where a 2 fold change is the limit of sensitivity of membrane arrays (Quackenbush, 2002). These graphical and pictorial representations of the data also show at which stage in progression of rectal carcinogenesis these gene expression changes occurred. The variation between experiments is represented on Figure 5.3 as error bars of ± the standard deviation (SD).

From normal to rectal polyp, p21 was the only gene down-regulated over 2 fold (-7.01, 1.21 SD). This change was apparent even by simple observation of the array images and as it occurred at such an early stage this may provide an important clinical biomarker. cDNA array studies have found p21 to be 6-fold overexpressed in mucinous colorectal cell lines but not in non-mucinous colorectal cell lines. These increases in expression were not detected in the tissue samples, possibly due to the heterogeneity of tumour tissue (Backert *et al.*, 1999). HNPCC tumours, which are frequently mucinous, have also been reported with overexpressed levels of p21 (Sinicrope *et al.*, 1998). In contrast, a study has reported p21 expression in non-mucinous colorectal carcinoma tissue samples to be down-regulated to around a third of that of normal tissue (Matsushita *et al.*, 1996). The array findings of p21 down regulation for this patients studied in this chapter are in line with these studies as the patient had non-mucinous non-hereditary colorectal cancer.

The differences in gene expression between rectal polyp and rectal adenocarcinoma were predominantly increases in gene expression with the exception of a down regulation of p57 (-4.87). Cyclin-dependant kinase inhibitor 1C or $p57^{Kip2}$, as the gene is also known, is an important inhibitor of cell cycle progression. $p57^{Kip2}$ is inactivated in 10-30% of tumours by hypermethylation of the promoter region and histone demethylation in various human cancers types such as colorectal, gastric, hepatocellular, pancreatic and in cases of myeloid leukemia and is linked to poor prognosis (Kikuchi *et al.*, 2002; Li *et al.*, 2003). $p57^{Kip2}$ is situated in the nucleus of colorectal cancer tissues and a low expression of the gene is associated with a large tumour size and the presence of tumours in female patients (Noura *et al.*, 2001). In

contrast to this current study, recent research of colorectal carcinogenesis has found $p57^{Kip2}$ expression to increase from normal colonic mucosa to adenoma tissue and decrease from adenomas to carcinomas whereas the expression levels remain unchanged when primary carcinomas metastasized to lymph nodes (Li *et al.*, 2003). The expression levels between repeat array experiments of p57 varied to the extent that further experiments would have to be undertaken to draw conclusions for gene expression values for this gene.

The genes which increased in expression from rectal polyp to rectal adenocarcinoma with a fold increase of over 2 were CBP (+2.14), DPC4 (+2.96), p300 (+2.52) and TGF-βRII (+2.64).

CBP and p300 are very similar proteins which have similar roles within the cell.. Human CREB-binding protein is an acetyltransferase enzyme and acetylates proteins, including histones, tagging specifically for transcriptional activation. p300 is also a histone acetyltransferase that functions as a transcriptional regulator for several nuclear proteins (Chan and La Thangue, 2001). p300, along with CBP, controls the stability of the p53 protein by facilitating p53 degradation (Grossman *et al.*, 2003). p300 also functions as an essential coactivator in p53-dependant transactivation of target genes as part of the cells response to DNA damage (Espinosa and Emerson, 2001). Studies in colorectal cell lines have shown that p300 regulates p53-dependent apoptosis after DNA damage and an absence of p300 results in failure to activate p21 transcription which prevents p21-dependant cell cycle arrest to occur, leading to increasing apoptosis in response to DNA damage (Iyer *et al.*, 2004). This experiment suggests that p300 is a key regulator of p53 response and the inhibition of this gene could be used to modulate chemotherapy. These findings highlights the importance of this gene in colorectal cancer and measuring the expression levels of p300 in colorectal cancer patients with wild-type p53 may give insight into how a patient may respond to various chemotherapy agents (Iyer *et al.*, 2004). In this current experiment, patient one showed increases in expression from polyp to carcinoma of CBP and p300 as well as an increase in p21 expression of 2 fold (0.40 SD). This suggests a p21-dependant cell cycle arrest response rather than apoptosis of the tumour cells and this patient may therefore benefit from p300 inhibition to promote cell death within the tumour. As p53 mutations are a late genetic alteration in colorectal carcinogenesis and

there have been no significant changes in p53 expression for most of the patients in this study, the role of CBP, p300 and p21 in the control of cell cycle arrest and apoptosis balance is of great importance.

Human homozygous deletion target in pancreatic carcinoma (DPC4) gene, also known as smad4, had an increased expression level from polyp to tumour. DPC4 is involved in the TGF-ß signaling transduction pathway. This gene is so called as it is inactivated in around 55% of pancreatic cancers and is also inactivated in around 15% of colorectal cancers either by loss of heterozygosity or mutation (McCarthy *et al.*, 2003). The loss of DPC4 expression in colonic adenocarcinomas is reported a late event in the adenoma to carcinoma pathway and shows a weak correlation with metastatic cancer (Maitra *et al.*, 2000; Takaku *et al.*, 1998).

TGF-ßRII, which showed no significant change in expression level from normal mucosa to polyp, showed a significant rise in gene expression from polyp sample to rectal carcinoma with a rise of +2.64 fold (0.35 SD). The TGF-ßRII gene is mutated in many cancers exhibiting MSI and because of this the gene is also termed HNPCC6 as it is frequently mutated in the mismatch repair deficient hereditary disease HNPCC (Shin *et al.*, 2000). TGF-ßRII is inactivated by mutation within an A_{10} tract which disables the receptor and makes the cell resistant to the growth inhibitory effects of TGF-ß (Markowitz *et al.*, 1995). TGF-ßRII mutations are thought to be the earliest mutational event in MSI tumours and have been linked with the progression of adenomas to cancer (Duval *et al.*, 2001; Markowitz *et al.*, 1995; Young *et al.*, 1995; Abdel-Rahman *et al.*, 1999). There have been no expression studies to date of this gene in colorectal cancers other than to define the effect of the inactivating mutation. The increase of TGF-ßRII expression in the progression of the cancer pathway from polyp to carcinoma for this patient may increase the cells sensitivity to the growth inhibitor TGF-ß. This conclusion does assume that the increase in TGF-ßRII expression correlates with an increase in the level of functional TGF-ßRII protein and as this gene is frequently mutated in colorectal cancer this is not a safe assumption to make. It would be of interest to detect the protein levels of TGF-ßRII and TGF-ß before drawing any further conclusions. The MSI status of this patient is unfortunately not known but as the patient had sporadic rectal cancer the tumour is unlikely to be MSI positive as these tumours predominantly occur in the proximal

colon (Thibodeau *et al.*, 1998), but having this confirmed would again be of interest as although the mechanisms behind the mutation of this gene and the alteration of expression levels are very different it would be interesting to see if these occurrences showed any form of correlation. The increase in TGF-ßRII expression seen in this patient occurred at the stage of carcinogenesis at which TGF-ßRII mutations have been reported to be pivotal in tumour progression. It may be that in a subset of colorectal patients TGF-ßRII expression plays a part in tumour progression.

There were also genes that, although showed no significant change in expression between stages, changed significantly in terms of expression from normal mucosa to rectal carcinoma tissue. These genes were p27 (+2.31, 0.26 SD), TSC2 (-5.31, 2.06 SD) and WT1 (-5.61, 2.80 SD). All of these genes had low levels of expression which typically resulted in larger standard deviations between repeats than genes expressed at higher levels. An application of different techniques such as RT-PCR would be an appropriate method of further analysing these genes in order to either confirm these results or to find if these are merely artefacts.

5.4.4 Gene expression changes detected in patient two

Patient 2 also had rectal cancers and samples of normal mucosa, polyp and carcinoma were all taken from this region. The predominant observation for this patient was again a general decrease in gene expression of the majority of genes on the array from normal mucosa to rectal polyp followed by a general increase in expression of these genes between polyp sample and rectal carcinoma. This is a very similar pattern to the results seen from patient 1. Both of these patients were male, in their 60s and the tumours developed in the rectal region of the colorectum. The carcinoma from patient 2 was though less advanced and was categorised as Dukes' stage A whereas patient 1 had a more advanced carcinoma at Dukes stage C. Table 5.6 lists the gene expression fold changes for all of the genes on the array used for patient two. A value of 0.00 is used where spot intensity was very low and an accurate reading cannot be obtained. Figures 5.4 and 5.10 are graphical and pictorial representations of this data which show the genes which had expression level changes of ±2 fold or greater.

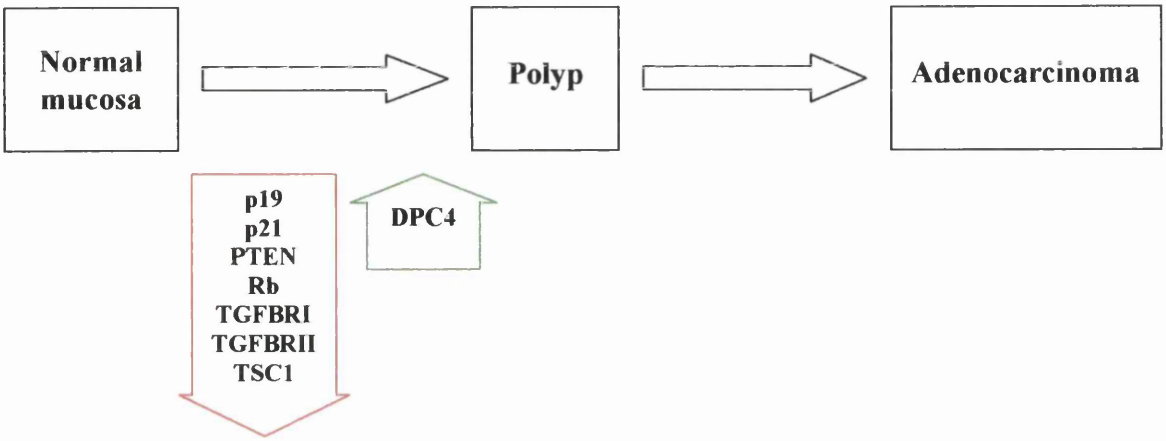


Figure 5.10

This diagram shows the gene changes that were detected in patient two between the stages of cancer progression. The red arrows represent down regulation and the green arrows indicate up regulation of the genes within each arrow. All the genes included had significant changes of over 2 fold detected. There are no genes labelled for the development of a polyp into carcinoma for this patient as there were no significant gene expression changes at this stage.

After taking into account standard deviation, the significant gene expression changes from normal mucosa to polyp were p19 (-8.18, 1.28 SD), p21 (-3.96, 1.84 SD), PTEN (-11.31, 3.42 SD), Rb (-5.97, 0.23 SD), TGF-ßRI (-6.98, 0.14 SD), TGF-ßRII (-10.76, 5.86 SD) and TSC1 (-3.78, 1.20 SD).

The cyclin-dependant kinase inhibitors p19 and p21 were both down-regulated in the polyp sample compared to the normal mucosa. p19 functions to regulate the cell cycle and apoptosis by blocking the degradation of the tumour suppressor p53. p19 expression has been reported to be down-regulated in murine colonic cell lines with truncated β-catenin which prevents apoptosis from occurring (Wagenaar *et al.*, 2001). β-catenin is mutated in many colorectal cancers (Morin *et al.*, 1997) and it would be interesting to know if was functional in this patient. There are currently no published studies of p19 expression in human colorectal cancer tissues but the findings for this patient correspond to those of the study of mouse cell lines.

Phosphatase and tensin homolog (PTEN) gene contains two $(A)_8$ repeats which are susceptible to mutation in MSI tumours (Guanti *et al.*, 2000). Mutations and allelic losses of PTEN can be found in brain tumours and endometrial cancers (Mutter *et al.*,

2000). Germline PTEN mutations can cause Cowden syndrome and Bannayan-Ruvalcaba-Riley syndrome, a symptom of both of these conditions is the development of juvenile polyps in the gastrointestinal tract (Woodford-Richens *et al.*, 2000). Although there are no published studies of PTEN expression in colorectal cancers, PTEN gene expression loss has been detected by immunohistochemistry in primary breast cancers and more advanced prostate carcinomas (Perren *et al.*, 1999; McMenamin *et al.*, 1999). These reports of PTEN down regulation are in agreement with the findings of this current study for this patient.

The retinoblastoma 1 (Rb) encodes a protein which regulates cell cycle progression at the transition from the G_1 to the S phase (Goodrich *et al.*, 1991). Mutation of this gene is rare but when present causes aggressive tumours of the retina. In contrast to this current study which found a down regulation of this gene, Rb levels have been found to be increased in colorectal cancers (Gope *et al.*, 1990). The increased levels have been detected in adenomatous polyps and increase further in carcinoma tissue; these increased levels may protect cells from growth inhibition and apoptosis (Yamamoto *et al.*, 1999).

Transforming growth factor-beta receptor I (TGF-ßRI) is involved in the signal transduction for growth inhibition and was down-regulated by 6.98–fold in the polyp of this patient in comparison to the normal colonic mucosa. Cellular release from the growth control of TGF-ßRI-dependent growth inhibition is an early event in the development of many malignancies (Souchelnytskyi, 2002). Increases in TGF-ßRI expression are correlated to an increased tumourigenicity, invasion and drug resistance in many cancer tissues including colon and gastric as well as several breast and prostate cancer models (Teicher, 2001). The expression level of TGF-ßRI is important when deciding upon a treatment for cancer as leukemia cells resistant to the anti-cancer drug cisplatin have been found to have impaired TGF-ßRI signaling (Stoika *et al.*, 2003).

Tuberous sclerosis 1 (TSC1) was down-regulated in this patient in the polyp tissue compared to normal colonic mucosa. Mutations in the genes TSC1 and tuberous sclerosis 2 (TSC2) cause the autosomal dominant disease of the same name which is characterized by the widespread development of benign tumours. TSC1 forms a complex with TSC2 which is thought to control cell cycle, endocytosis, cell adhesion

and transcription (Gao and Pan, 2001). The exact role of this complex and how mutations in these genes cause the development of benign tumours is as yet unknown. Biallelic inactivation has not been found in these genes in neurological tumours and so the mechanism behind the role of these TSC genes in tumour development may be through altered gene expression levels rather than mutation (Parry *et al.*, 2000).

There were no significant changes seen from polyp to the rectal tumour. The only significant difference from normal to tumour was a decrease in expression of p21 (-2.72, 0.17 SD) however this is a less striking finding than the initial decrease of this gene at the early stage from normal to polyp (-3.96, SD 1.84).

5.4.5 Gene expression changes detected in patient three

Patient 3 was a 63 year old male with a Dukes stage C carcinoma situated in the sigmoidal region of the colorectum. Samples were taken from normal mucosa, polyp and two samples from the carcinoma, from the peripheral region of the tumour and a further sample from the central region of the tumour. Table 5.7 lists the gene expression fold changes for all of the genes on the array used for patient three. Figures 5.5 and 5.11 are graphical and pictorial representations of this data which show the genes which had expression level changes of ±2 fold or greater.

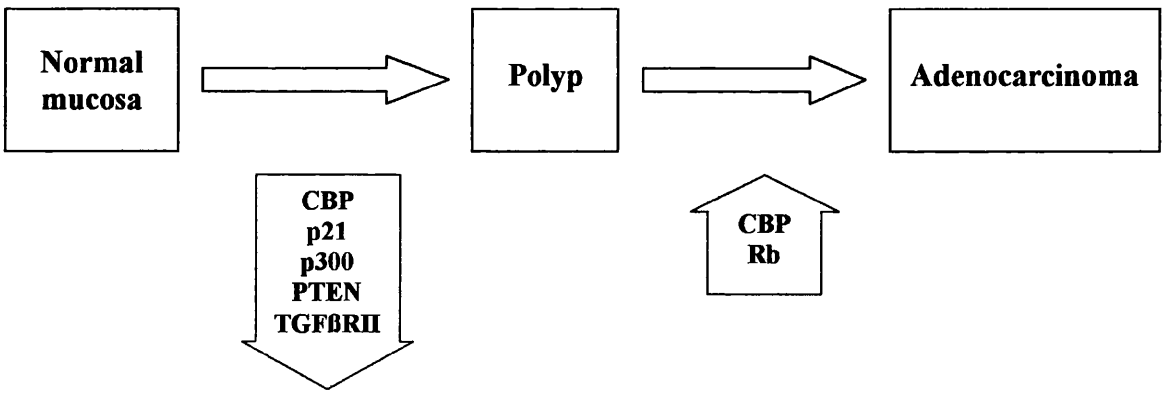


Figure 5.11

This diagram shows the gene changes that were detected in patient three between the stages of cancer progression. The red arrows represent down regulation and the green arrows indicate up regulation of the genes within each arrow. All the genes included had changes of over 2 fold detected.

Again there was a general trend of the genes decreasing in expression level between normal mucosa and sigmoidal polyp and an increase in gene expression from polyp to sigmoidal carcinoma. The changes which were significant after taking into consideration standard deviation were all decreases in expression between the normal mucosa and polyp samples. These genes with significant changes are listed here followed by the fold decreases and standard deviation (SD), CBP (-11.48; SD 0.34), p21 (-4.97; SD 1.50), p300 (-2.54; SD 0.04), PTEN (-2.56; SD 0.01) and TGF-βRII (-2.66; SD 0.03). All of these genes were also found down-regulated in patient one and/or patient two.

As seen in patient one, CBP and p300 were both down-regulated, -11.48 and -2.54 respectively. It is also noteworthy that these genes appear to be regulated together as in patients 2,4 and 5 neither of these genes were down-regulated yet in patients 1 and 3 CBP and p300 were both down-regulated at the normal mucosa to polyp stage of carcinogenesis. This observation is in line with the very similar functions of these proteins as discussed in Section 5.4.3 and this may be a gene expression pattern which is key to certain pathways of carcinogenesis or a factor important in the further development of the polyp to a carcinoma.

The significant changes seen from polyp to the central region of the sigmoidal tumour were CBP (3.73, 1.36 SD) and Rb (2.39, 0.26 SD). Both these genes have been previously discussed. There were no significant changes in gene expression from normal to sigmoidal tumour.

There were unfortunately problems when analysing the peripheral tumour to compare with the expression patterns found in the central tumour sample to assess for clonal differences arisen from the development of the tumour. These problems were a result of arrays being shipped with contamination which was not evident until the end of the experiment and as a result there was only sufficient RNA remaining for one array experiment with the peripheral region of the sigmoidal tumour. From this one experiment slight decreases in expression were seen from the central tumour sample to the peripheral region of the same tumour in the following genes, BRCA1 (-2.36), VHL (-2.36) and WT1 (-2.36). Increases in gene expression were seen in NF2 (2.63),

p19 (2.26) and p21 (2.20). All of these genes were only marginally above the 2 fold threshold regarded as significant and without repeat experiments these initial findings cannot be considered as anything other than observations of the possibility of measurable changes in gene expression within an individual tumour. From this conclusion I raise the suggestion that studies of gene expression in solid tumours would be more useful if more details about the exact sampling technique were provided in publications, especially where small fold changes have been observed. Despite this, the changes observed within the tumour were less pronounced than between the samples of each different stage but further investigation of genetic changes within individual tumours would be worthwhile and provide more insight into the development of the occurrence of clonal growth from which tumours develop.

5.4.6 Gene expression changes detected in patient four

Patient 4 was a 57 year old female with a Dukes stage A carcinoma situated in the rectal region of the colorectum. Samples were taken from normal mucosa, polyp and carcinoma. Table 5.8 lists the gene expression fold changes for all of the genes on the array used for patient four. Figures 5.6 and 5.12 are graphical and pictorial representations of this data which show the genes which had expression level changes of ±2 fold or greater.

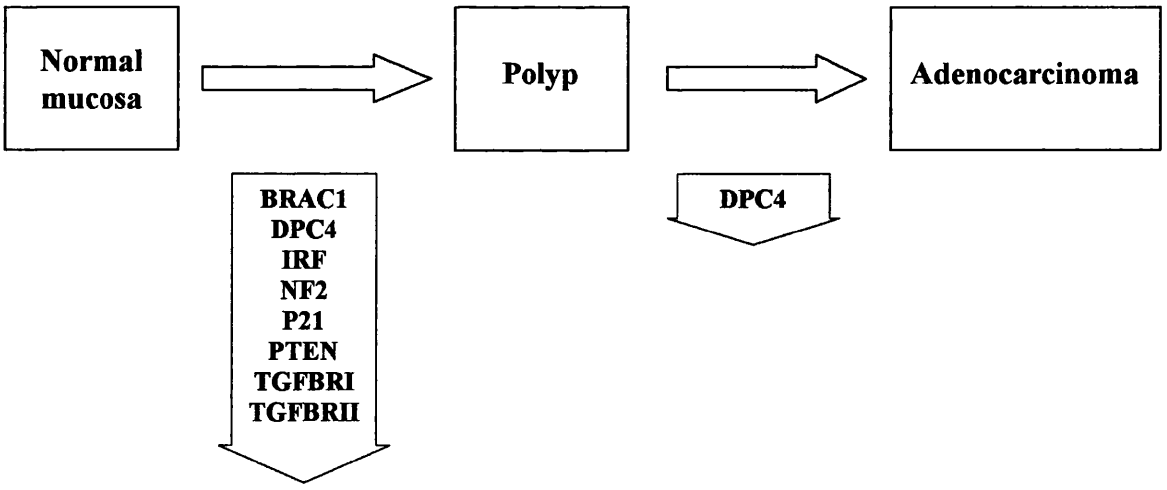


Figure 5.12

This diagram shows the gene changes that were detected in patient four between the stages of cancer progression. The red arrows represent down regulation and the green arrows indicate up regulation of the genes within each arrow. All the genes included had changes of over 2 fold detected.

From the rectal normal mucosa to the rectal polyp the genes with significant changes after taking standard deviation figures into consideration were all decreases in expression and are BRCA1 (-2.48, 0.39 SD), DPC4 (-3.03, 0.76 SD), IRF-1 (-2.53, 0.32 SD), NF2 (-3.17, 0.71 SD), p21 (-3.73, 0.55 SD), PTEN (-3.33, 0.91 SD), TGF-ßRI (-3.01, 0.98 SD) and TGF-ßRII (-2.61, 0.48 SD).

Breast cancer 1, early onset (BRCA1) gene encodes a protein with several functional domains with different roles within the cell. The BRAC1 protein functions as a transcription factor (Monteiro *et al.*, 1996) and also interacts with the repair protein Rad51 (Scully *et al.*, 1997). BRCA1 has been shown to activate the cyclin dependent kinase inhibitor p21 in a p53 independent manner in human colon cancer cell lines in response to DNA damage (Somasundaram *et al.*, 1997). BRAC1 facilitates the cellular response to DNA repair and is required for appropriate cell cycle arrests after ionizing radiation in both the S-phase and the G2 phase of cell cycle. The C terminal region contains the transactivation region of the protein and so germline mutations that result in a truncated protein are in part responsible for the inherited predisposition to ovarian and breast cancers due to loss of function. Loss of heterozygosity of BRCA1 is a molecular alteration present in colorectal carcinomas which is linked with a poor prognosis for overall survival (Garcia *et al.*, 2003). There have been no published studies to date reporting the gene expression levels of BRCA1 in colorectal cancers; it would be very interesting to establish a long term study to find out if there is a connection with decreased gene expression and poor prognosis. Also, as this gene expression decrease has been detected in the progression from normal mucosa to polyp, BRCA1 may be important in determining which polyps continue to develop into carcinomas.

IRF-1 was found down-regulated in patient 4 from normal mucosa to polyp by -2.53 fold (0.32 SD). Interferon regulatory factor 1 (IRF-1) has tumour suppressor activity as the protein collaborates with p53 to induce cell cycle arrest after DNA damage (Tanaka *et al.*, 1996). A raised level of the IRF-1 protein caused by overexpression of this gene induces cell cycle arrest whereas a mutant version of the protein which has been detected in gastric cancer is unable to induce cell-cycle arrest and has a reduced transcriptional activity (Nozawa *et al.*, 1998). A decreased expression level of IRF-1

found in this patient would reduce the capacity of the cells within the polyp to induce cell cycle arrest and therefore may allow mutated cells to divide before repair can occur. This is the first report of IRF-1 expression levels to be reduced in colorectal polyp samples.

Neurofibromin 2 (NF2) mutations causes the rare familial cancer neurofibromatosis 2 which results in brain neoplasms. NF2 mutations have also been detected in multiple tumour types including melanoma and breast carcinoma and therefore NF2 is thought to have a more general role in tumourigenesis than originally surmised (Bianchi *et al.*, 1994). There have been studies of NF2 mutations in colorectal cancer samples, one of which reports the presence of missense mutations in 2/44 (4.5%) (Arakawa *et al.*, 1994) but the majority of these studies have found no NF2 mutations (Sugai *et al.*, 2000; Rustgi *et al.*, 1995). Inactivation of the NF2 gene may therefore be associated with a small minority of colorectal cancers; yet it is possible that NF2 is important in colorectal carcinogenesis with alterations to gene function or expression occurring through mechanisms other than mutation such as promoter hypermethylation. As a down regulation was detected in the polyp of this patient in comparison to normal rectal mucosa, it seems more likely that if NF2 is involved in colorectal carcinogenesis it is through gene regulation rather than mutation.

From the rectal polyp sample to the carcinoma sample the only significant gene expression change was DPC4 (-8.95, 0.07 SD). The pattern of gene expression in this patient was a general decrease in gene expression from mucosa to polyp but, unlike the other four patients, the genes continued to decrease in expression from the polyp to carcinoma sample giving high decreases in expression level when comparing normal mucosa with carcinoma tissue such as DPC4 which decreased by -27.06 fold (6.59 SD).

5.4.7 Gene expression changes detected in patient five

Patient 4 was a 74 year old female with a Dukes stage C carcinoma situated in the rectosigmoidal region of the colorectum. Samples were taken from normal mucosa, polyp and carcinoma. Table 5.9 lists the gene expression fold changes for all of the genes on the array used for patient five. A value of 0.00 is used where spot intensity

was less than the cut off level and as a result an accurate reading cannot be obtained. Figures 5.7 and 5.13 are graphical and pictorial representations of this data which show the genes which had expression level changes of ±2 fold or greater.

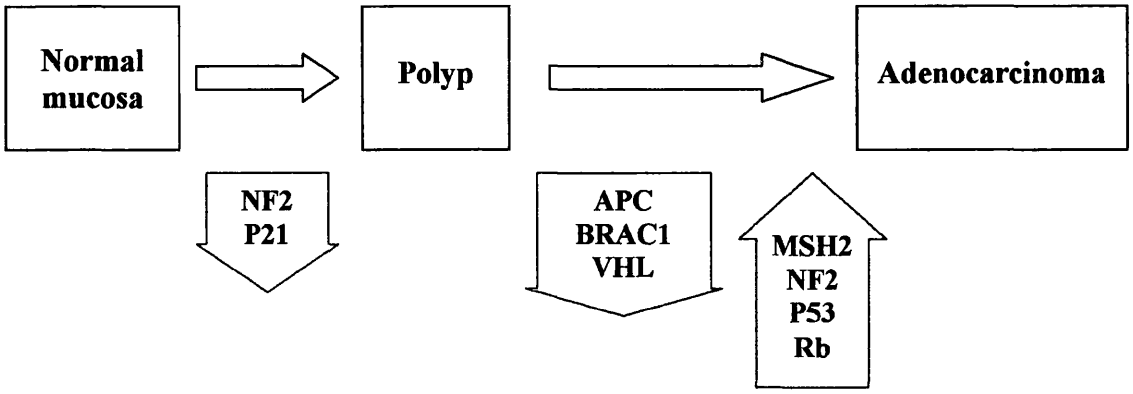


Figure 5.13

This diagram shows the gene changes that were detected in patient five between the stages of cancer progression. The red arrows represent down regulation and the green arrows indicate up regulation of the genes within each arrow. All the genes included had changes of over 2 fold detected.

From the normal mucosa to the rectosigmoidal polyp there were 2 genes with significant changes after taking standard deviation figures into consideration were both decreases in expression and are NF2 (-3.14, 0.01 SD) and p21 (-3.58, 0.26 SD).

From polyp to rectosigmoidal carcinoma there were several genes with significant gene expression changes. Those genes with increases in expression were MSH2 (2.65, 0.03 SD), NF2 (2.94, 0.69 SD), p53 (3.24, 0.25 SD) and Rb (2.74, 0.64 SD). Genes which decreased in expression were APC (-2.90, 0.20 SD), BRCA1 (-3.02, 0.47 SD) and VHL (-15.75, 6.96 SD).

Human mutS homologue 2 (hMSH2) is a mismatch repair gene that is mutated in around 60% of HNPCC cases and is used as a Bethesda marker for MSI (Ward *et al.*, 2001, Loukola *et al.*, 2001). In contrast to the increase in expression level seen here,

immunohistochemical staining of hMSH2 in MSI-H colorectal carcinomas have shown a lack of expression of this MMR gene (Stone *et al.*, 2001). hMSH2 is frequently inactivated through hypermethylation in the majority of sporadic cases (Jass, 2000; Herman *et al.*, 1998; Cunningham *et al.*, 1998).

The tumour suppressor p53 is one of the later genes to become inactivated in the pathway of carcinogenesis outlined by Fearon and Vogelstein (1990). This gene showed no alteration in expression in the polyp tissue compared to normal mucosa in any of the patients and was only found to have significantly altered expression at a later stage in carcinogenesis in patient 5. p53 is a growth regulator of fundamental importance in many cell types and is the gene most often found altered in a wide variety of human cancers. Wild type p53 suppresses cell growth whereas mutants increase cell proliferation. One role of p53 is to mediate G1 arrest in cells following DNA damage with the aim to allow cellular repair to occur before synthesis resumes. Another key role for p53 is the activation of apoptosis inducing genes which can ensure a cell with a large genetic mutation load to undergo apoptosis so that these mutations are not passed on to potential daughter cells. p53 is mutated in 70-80% of sporadic colorectal carcinomas; the mutations are thought to be selected for as they confer resistance to apoptosis rather than due to an increase in mutation rate. This allows tumour cells to survive with an increase in genetic burden as the p53 mutation is relatively late in the adenoma-carcinoma sequence. The presence of mutated p53 in a tumour predicts a reduction in overall survival of that patient (Inieta *et al*, 1998).

The adenomatous polyposis coli (APC) gene was down-regulated in this patient from the polyp sample to the adenoma. APC regulates the processes involved in colonic epithelial cell homeostasis such as cell cycle progression, migration, differentiation and apoptosis. It has been shown to be mutated in approximately 80% of FAP kindred and in sporadic colorectal carcinomas with over 98% of these mutations resulting in the synthesis of a truncated protein (Fearnhead *et al.*, 2001). APC mutations or loss are much less frequent or absent in MSI-H colorectal carcinomas (Iacopetta *et al.*, 1998; Konishi *et al.*, 1996). The APC gene is recognised as one of the first genes disrupted in the adenoma-carcinoma pathway in colorectal cancer and has a major role in preventing uncontrolled proliferation of the epithelium. APC expression increases as the cells progress up the colonic crypt, a pattern which is disrupted by

APC gene mutation (Smith *et al*, 1993). As this current study analysed the overall gene expression in homogenised tissues rather than examining the localised expression of this gene, the scale of alteration of gene expression in this, and the other patients, may be masked. Also this technique cannot detect gene mutations which are the principle way in which this gene is inactivated, rather than through regulation of gene expression.

VHL was decreased in expression by -15.75 fold (6.96 SD) in this patient from polyp to rectosigmoidal carcinoma. Von Hippel-Lindau syndrome is caused by loss or inactivation of the VHL gene which gives the patient a predisposition to retinal angiomas, blastomas of the central nervous system (CNS) and renal cell carcinomas (Latif *et al.*, 1993). VHL acts as a tumour suppressor by down regulation of transcription by binding to transcriptional elongation complexes and as a result suppresses elongation of mRNA and so reduces the transcription of target genes (Duan *et al.*, 1995). Although there are currently no reported gene expression studies of VHL in colorectal carcinomas, decreases in VHL expression has been detected in renal cell carcinoma cell lines using cDNA array analysis (Zatyka *et al.*, 2002). The large significant decrease in expression for VHL in colorectal carcinomas seen in patient 5 suggests that the role of this gene may be broader than originally thought with involvement in cell growth regulation other than CNS and neuronal development and renal carcinogenesis. Despite VHL only registering a significant change in expression for this patient, the gene expression level was altered in several of the patients but due to low expression levels of VHL in all sample types the result was not reproducible leading to large standard deviations. Again, as with other such genes it would be interesting to study the expression of this gene using RT-PCR.

In addition to these genes, p57 (-6.00, 2.09 SD), PTEN (-3.74, 1.48 SD), TGF-ßRI (-9.49, 1.10 SD) and TGF-ßRII (-2.96, 0.70 SD) were all significantly decreased in expression levels from the normal mucosa sample to the rectosigmoidal carcinoma sample.

5.4.8 Gene expression changes detected across all the patients

Table 5.10 lists the gene expression fold changes for all of the genes on the array used for all five patients with minus figures representing a decrease in expression and positive numbers representing gene expression increases. Figure 5.8 is a graphical representation of this data which show the genes which had average expression level changes of ±2 fold or greater. Figure 5.9 shows the number of patients in which the gene expression was altered from normal mucosa to polyp and from polyp to tumour.

A trend seen across all of the patients was a decrease in expression of the majority of genes on the array from normal colorectal mucosa to polyp tissue followed by a general increase in gene expression from polyp to carcinoma sample. This pattern also meant that if just the gene expression changes from normal mucosa to colorectal carcinoma were analysed this gene expression decrease followed by an increase would be masked and there would appear to be very little alteration in gene expression within the process of carcinogenesis.

APC is a tumour suppressor gene which is mutated in patients with FAP and also is one of the earliest genes to be altered in the majority of sporadic colorectal cancer. There was no significant alteration in gene expression of this gene between the polyp and normal mucosa tissues and only a relatively small change (2.90 fold, 0.20 SD) was detected between polyp and carcinoma of patient 5. It is not possible from this study to know whether the APC protein was functional however it stands to reason from the wealth of research of APC mutation in colorectal cancer that it is from this mechanism rather than expression levels that the APC gene is involved in initiating tumour growth in colorectal cancer.

From Figure 5.9 it can be seen that both TGF-βRII and TGF-βRI were down-regulated at the polyp stage in 3/5 (60%) and 2/5 (40%) patients respectively. Both of these receptor genes have been reported to be inactivated early on in colorectal carcinogenesis and this array study is in agreement with these previous findings. TGF-βRII and TGF-βRI have been linked to an improved and reduced prognosis respectively (Duval *et al.*, 2001; Teicher, 2001) yet in this study they were both found

with decreased expression levels in 2/5 (40%) of the patients analysed. It would be very interesting to be able to follow this study up over several years in order to assess the survival and treatment responses of the patients studied in this chapter.

PTEN was also down-regulated in the polyp sample of 3/5 (60%) of the patients. There is currently a lack of expression studies in colorectal cancer for this tumour suppressor despite expression loss being detected in both breast and prostate cancers (Perren *et al.*, 1999; McMenamin *et al.*, 1999). These array results show this gene has a widespread down regulation in this small group of patients and therefore a wider study of this gene would be of interest.

The only gene which was significantly altered in gene expression across all patients was the cyclin dependent kinase inhibitor p21. This gene was found to have a decrease in expression level from normal mucosa to polyp in all patients regardless of polyp location within the colorectum. p21 was the only gene on this array to be consistently down-regulated at this early stage in carcinogenesis.

5.4.9 The role of p21 in colorectal cancer

p21 was the only gene on the array to be significantly down-regulated at the polyp stage in all 5 patients analysed in this study and therefore has the potential for being a biomarker for colorectal cancer. The array findings of p21 down regulation for the patients studied in this chapter are in line with earlier studies of non-mucinous non-hereditary colorectal cancers where p21 was found to be down-regulated to around a third of that of normal tissue (Matsushita *et al.*, 1996).

Progression through the cell cycle is regulated by cyclins and cyclin-dependent kinases (CDKs). High intracellular levels of cyclin kinase inhibitor p21 can induce G1 and G2 arrest and block entry into S phase and M phase respectively by inactivating CDKs or by inhibiting activity of proliferating cell nuclear antigen (PCNA) (Bunz *et al.*, 1998). p21, located on chromosome 6p21, can be transcriptionally induced by the p53 protein and is responsible for the p53-mediated G1 arrest following DNA damage (Waldman *et al.*, 1995). When p21 is disrupted in colorectal cell lines the p53-determined fate of the cell, as a response to DNA damage, favours apoptosis over cell

cycle arrest (Polyak *et al.*, 1996). Monitoring levels of p21 gene expression in patients may provide a predictor for how a patient may respond to chemotherapeutic agents such as 5-fluorouracil which is affected by p53 expression (Violette *et al.*, 2002).

A previous study which found a 6-fold higher level in p21 reported that cell growth was not affected by this increase in expression, possibly due to the overexpressed p21 protein not being functional (Backert *et al.*, 1999). As mutations of p21 are extremely rare (Shiohara *et al.*, 1994), the growth inhibitory effect of p21 may have been prevented by a downstream lesion (Backert *et al.*, 1999). High expression of p21 has also been found to be unable to prevent cell proliferation in human brain tumours (Jung *et al.*, 1995).

To understand whether chromosome number was related to the down regulation of p21 gene expression in colorectal tissue samples from normal mucosa and polyp through to carcinoma samples, FISH was undertaken to assess chromosome number. This is described in chapter 6.

5.4.10 Importance of examining each stage

The design of these experiments allowed comparisons of gene expression to be made not only from normal mucosa to carcinoma but also from normal mucosa to polyp sample and from polyp to carcinoma. This was particularly important as just studying the normal mucosa and the carcinoma can hide gene expression changes which occur at various stages. For example in patient 1, DPC4 showed a decrease in expression level from normal rectal mucosa to rectal polyp however, from polyp to rectal carcinoma this gene was up regulated, giving no overall change in gene expression of DPC4 from normal mucosa to carcinoma. Being able to detect the changes present at each stage may help to explain why some polyps become carcinomas.

5.4.11 Application of this technique for screening purposes

This technique could be used to screen colorectal biopsy tissue samples for alterations in gene expression compared to histologically normal colorectal mucosa from the same patient. This would provide information about the pathways of carcinogenesis

through which the patient's tumour had arisen. This information may be key in allowing the treatment of the cancer to be tailored to the individual patient with the hope of increasing the effectiveness of the chemotherapy provided. This technique may also be useful in determining which polyps will go on to form carcinomas. p21 was down-regulated in all of the polyps tested in this study of colorectal cancer patients. Therefore this gene may be a crucial gene to distinguish between polyps that remain benign and though which become cancerous.

The negative aspects of this technique for use as a screening tool are the expense and time consuming nature of the experiments. If this was taken up as a screening tool then the mass produced nature of the reagents required may well reduce the cost. Several modifications to the protocol or automation of the stages would be needed to reduce the time required for these tests. Also, this technique can only be carried out using tissue samples obtained from a biopsy, which is an invasive procedure. Therefore, whilst important information can be gleaned from such a screen the uptake would be likely to remain relatively low.

5.4.12 Limitations of this study

An observation I have made using these cDNA arrays is that, whilst they provide a rapid and cost effective scan across many genes using minimal RNA quantities, the technique is not as accurate for genes expressed only at low levels as for genes expressed at higher levels. Genes which had very low expression levels gave inconsistent results with large standard deviations between repeat experiments. A technique other than cDNA array analysis would be more appropriate for these genes. RT-PCR would be an excellent method to measure gene expression changes in these low expression level genes, to confirm other results and provide more accurate figures for expression changes. Due to financial and time constraints it was not possible to carry out a follow up study involving RT-PCR analysis however this would be an interesting area of further research. cDNA arrays have been proved to be a valuable scanning tool for the design of further experiments relating to gene expression.

5.4.13 Discussion Summary

cDNA arrays have been shown here to be a useful technique for the rapid screening across many genes for the detection of gene expression changes between various stages within carcinogenesis. The technique was more reliable for the genes expressed at higher levels than those genes with lower levels of expression for which the reproducibility of the experiments was limited. cDNA arrays are best used in conjunction with RT-PCR, this would be an ideal follow up study to the findings in this chapter.

Despite this, the cDNA arrays detected a significant decrease in expression of the cyclin-dependant kinase inhibitor p21 at the polyp stage of tumour development in all 5 patients studied regardless of tumour location, patient sex or age. This gene may therefore be critical in determining progression of a polyp into a carcinoma. p21 expression levels may also indicate which tumours may respond better to certain chemotherapeutic agents and also be an indicator of prognosis in colorectal cancer patients. To try to understand more about the mechanism behind this gene expression decrease of p21, FISH was undertaken of chromosome 6 where the gene is located and these experiments are described in Chapter 6.

Chapter 6

The detection of chromosome 6 and p21 region copy number alterations using fluorescent *in situ* hybridisation

6.1 Introduction

In Chapter 5 I showed that gene expression array analysis detected that p21 expression was down-regulated in polyps from the early stages of colorectal carcinogenesis. The polyps analysed all showed a significant decrease in expression in all 5 of the patients analysed. The purpose of this chapter is to analyse the copy number of this gene and the chromosome on which it is situated in an attempt to establish whether the decrease in p21 expression is an effect of gene dosage due to loss of copy number.

6.1.1 Chromosome instability

Two of the most frequently occurring genetic pathways for colorectal cancer are the pathologically distinct microsatellite instability (MSI) pathway (as discussed in Chapter 4) and the chromosome instability (CIN) pathway which will be discussed here. The CIN pathway is thought to be responsible for around 85% of colorectal cancers (Chung, 2000) and is the pathway which Fearon and Vogelstein (1990) originally described as being the pathway of colorectal carcinogenesis. Since this initial description many more genes have been found to be involved in this pathway and so their model of colorectal carcinogenesis has been considerably elaborated. A diagram of the currently identified genetic changes which occur during the development of cancer through the CIN pathway can be seen in Figure 6.1. CIN increases the rate at which gross chromosomal changes occur during cell division such as chromosome breaks, duplication, rearrangements and deletions (Narayan and Roy, 2003).

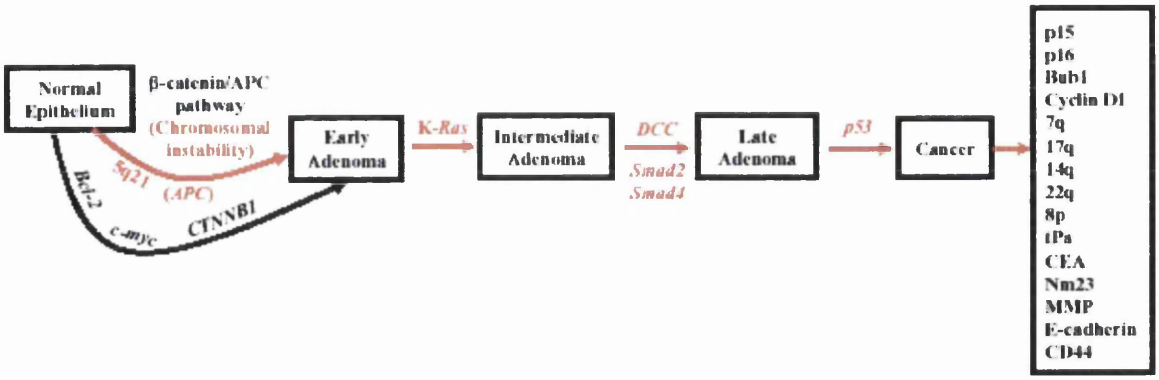


Figure 6.1

Diagram of the chromosome instable (CIN) pathway of colorectal carcinogenesis based on Vogelstein's model. The red arrows indicate the key changes required for cancer progression. Adapted from Narayan and Roy, 2003.

Aneuploidy, which is the loss or gain of one or more chromosomes, can arise from nondisjunction or anaphase lag. Nondisjunction is the failure of paired chromosomes or sister chromatids to disjoin at meiosis I, meiosis II or mitosis. This results in conjoined chromosomes or chromatids migrating to one pole, these will be included in one daughter cell and the other daughter cell will have a loss of genetic material. Chromosomes can also be lost and gained as a result of anaphase lag which is the delayed movement of the chromosome during anaphase (Wolman, 1983). As the process of cell division is extremely complex with a great number of genes playing vital roles at each stage, the mutation, change in expression level or the precise timing of this expression of any one of these genes can result in aneuploidy. One of the key genes involved in this process is the adenomatous polyposis coli (APC) gene which is discussed in Section 6.1.3.

Not all chromosomal abnormalities involve the loss or gain of complete chromosomes. Chromosome breakages can result in the inversion, deletion and translocation of fragments of the arms of chromosomes. Some deletions result in an unstable chromosome; if the deletions remove the telomeres the remaining chromosome is likely to be degraded or if the centromere is deleted the chromosome is likely to be lost at cell division. Deleted regions of chromosomes can go on to form ring chromosomes, if these contain a centromere these may pass through cell division

but acentric ring chromosomes will be lost. Both inversions and translocations can result in the loss of control of the genes in the fragment involved as well as the disruption of genes at the point of insertion. The double strand breaks required for these chromosome abnormalities to occur are often a consequence of oxidative damage or exposure to environmental agents (Gajeka *et al.*, 1999). Double strand breaks are repaired during cell cycle arrest at G2 prior to mitosis but deficiencies in the cells ability to either arrest at this stage or repair the damage can lead to misegregation of the affected chromosome and leads to chromosomal aberrations (Lodish *et al.*, 1995).

6.1.2 Familial adenomatous polyposis (FAP)

Familial adenomatous polyposis (FAP) is an autosomal dominant disease which arises as a result of the germline mutation of the tumour suppressor gene adenomatous polyposis coli (APC) located on chromosome 5p21 (Bodmer *et al.*, 1987; Groden *et al.*, 1991). As can be seen in Figure 6.1, APC is one of the earliest recorded changes in the CIN pathway, the mutation of which causes normal epithelial tissue to form early colorectal adenomas. In FAP patients hundreds to thousands of these adenomas develop during adolescence or early adulthood which, if left untreated leads to colorectal cancer in patients betweens the ages of 30 - 40 (Bodmer, 1996). This disease is further discussed in Chapter 1, Section 1.3.2.

6.1.3 Adenomatous polyposis coli (APC)

APC regulates the processes involved in colonic epithelial cell homeostasis such as cell cycle progression, migration, differentiation and apoptosis and is found to be mutated in around 75% of sporadic colorectal cancers (Leslie *et al.*, 2002; Miyaki *et al.*, 1994). The APC gene product is a large multifunctional protein which interacts with several proteins including β-catenin, end binding protein 1 (EB-1), glycogen synthase kinase (GSK), human homologue of the Drosophila discs large tumour suppressor protein (hDLG) and the mitotic checkpoint kinases Bub-1 and Bub-3 (Leslie *et al.*, 2002; Kaplan *et al.*, 2001). Over 98% of mutations of the gene result in the synthesis of a truncated protein which lacks the ability to degrade β-catenin which then results in increased transcription of many genes including the oncogene c-myc

(He *et al.*, 1998). Wild type APC also binds to and promotes the assembly of microtubules and is involved in chromosome segregation (Smith *et al.*, 1994; Kaplan *et al.*, 2001). APC accumulates at the ends of microtubules embedded in kinetochores and plays a role in stabilising the ends of kinetochore microtubules and assists in their attachment to chromosomes (Kaplan *et al.*, 2001). EB-1 is thought to be involved in connecting APC to microtubules and it has been shown that cells with APC protein lacking this domain display chromosome instability and defective chromosome segregation (Berrueta *et al.*, 1998).

6.1.4 Fluorescent *in situ* hybridisation (FISH)

The development of fluorescent *in situ* hybridisation (FISH) in the mid 1980s was an important technical advancement which enabled the detailed analysis of chromosomal aberrations. Prior to this, histochemical staining methods were used to detect chromosomal aberrations, this technique allowed for the recognition of chromosomal banding patterns making chromosomal losses, gains and structural changes in tumours detectable. A disadvantage of this method was that it required highly skilled researchers and the detection of specific changes was extremely time consuming (Boland *et al.*, 1998).

FISH is a type of chromosome *in situ* hybridisation in which the nucleic acid probe is labelled by incorporation of a fluorophore which fluoresces when exposed to UV irradiation. *In situ* hybridisation was first developed in the 1960s when probes were labelled with radioisotopes, however, to prevent scattering of the signal the isotope needed to be very low energy and so required long developing times of several weeks, which created high levels of background signal (Jin and Lloyd, 1997). These problems were eliminated with the advent of fluorophores which are chemical groups which fluoresce by emitting light of a longer wavelength after being excited with a shorter wavelength. Fluorophores allow a greater resolution than isotopes and are also safer to work with. FISH became an established technique in the early 1990s and various probes have now been developed and are commercially available including centromeric probes, whole chromosome paints and probes for specific loci.

6.1.5 Colorectal cancer analysed by FISH

Since the development of FISH there have been many studies using this technique to analyse chromosome aberrations in colorectal cancer samples from tissue and cell lines. Using cell lines has the advantage of being able to carry out metaphase FISH which provides clearer images and allows the detection of translocations. There is though always the possibility that alterations seen are an artefact of the immortalisation of the cell line as opposed to genetic changes seen in the colorectal tumour from which the cell line is derived. FISH using tissue samples eliminates this problem but it is only possible to carry out interphase FISH which generally only allows the detection of numerical changes of chromosomes and regions. Structural rearrangements such as inversions can be detected by using combinations of probes for different regions but this requires a detailed knowledge of the affected chromosomes and the locations that such rearrangements may occur as well as a series of probes for the region being analysed.

Over 90% of all colorectal cancers show chromosomal aberrations (Muleris *et al.*, 1990). Previous karyotyping and FISH studies of colorectal cancer have detected gains of chromosomes 7, 13 and 20 and loss of chromosome 18 in adenomas and carcinomas and the losses of chromosomes 17 and Y in colorectal carcinomas (Bomme *et al.*, 2001; Herbergs *et al.*, 1996). The most common of these are the loss of chromosome 18 and the gain of chromosome 7, with each aberration occurring in around a quarter of colorectal tumours (Leslie *et al.*, 2002). As well as these alterations in chromosome number, structural rearrangements have also been detected such as del(1p), del(8p), i(13q) and del(17p) in adenomas and in chromosomes 1, 8, 13 and 17 in colorectal carcinomas (Bomme *et al.*, 2001; Di Vinci *et al.*, 1998). Other changes have been reported as being specific to types of colorectal cancer such as chromosome 8p which is commonly lost in CIN+ cell lines and tumours but is gained in MSI+ cancers (Douglas *et al.*, 2004).

6.1.5.1 Chromosome 6 copy number in colorectal cancers

Comparative genomic hybridisation (CGH) is a technique which can be used to compare normal cells against cancer cells to investigate copy number changes across

the whole nuclear genome. CGH has been used to detect the loss of chromosome 6 in colorectal cancer cell lines but this has not been found in primary colorectal carcinomas (Douglas *et al.*, 2004). This suggests that this alteration may be a result of the immortalisation of the colorectal cell lines rather than a chromosome aberration present in colorectal tumours. Other cytogenetic studies of chromosome 6 in colorectal adenomas have reported both the loss and gain of this chromosome with slightly more reported incidences of gains rather than losses currently in the literature (Bardi *et al.*, 1997; Bomme *et al.*, 1996; Bomme *et al.*, 1998). There are also several studies karyotyping colorectal adenocarcinomas where both the gains and losses of chromosome 6 have again been reported, but unlike with the adenomas, there are more reports of losses rather than gains (Reichmann *et al.*, 1981; Bardi *et al.*, 1993; Bardi *et al.*, 1995; Bardi *et al.*, 1997; Parada *et al.*, 1999). These reports are though generally case studies analysing the entire karyotype of only a few patients and so it is difficult to establish any patterns of chromosome 6 losses or gains in colorectal adenoma and adenocarcinoma tissue from these experiments. It is important to realise the limitations of the ability of CGH to detect such changes as >50% of the cells must show the alterations in order to be recognised by this technique (Manoir *et al.*, 1995). Also, the spatial resolution may not be sharp enough to detect some of the smaller chromosomal aberrations. Because of these factors, FISH is recommended as a superior technique to CGH in the determination of chromosomal abnormalities in clinical cancer diagnostics (Liebisch *et al.*, 2003).

6.1.6 p21

In the experiments described in Chapter 5, p21 expression was found to be reduced in the histologically abnormal tissues compared to the levels found in the normal colorectal mucosa of all five patients analysed. Importantly, p21 expression was reduced in polyps, the earliest stage of carcinogenesis studied in these experiments.

6.1.6.1 The role of p21 in colorectal cells

6.1.6.1.1 Cyclin-dependant kinases (CDKs) and CDK inhibitors

Cyclin-dependant kinases (CDKs) regulate the cell cycle by phosphorylation of target substrates involved in DNA replication and mitosis. The G1-S transition of the cell cycle is regulated by the interactions between CDKs, cyclins and CDK inhibitors. CDKs are activated by cyclins, which act as positive regulators and are inhibited by CDK inhibitor proteins such as p15, p16, p21 and p27 (Grana and Reddy, 1995), see Figure 6.2. CDKs activity is also regulated by kinases and phosphatases (Navaratnam *et al*, 1999; Kamb, 1995).

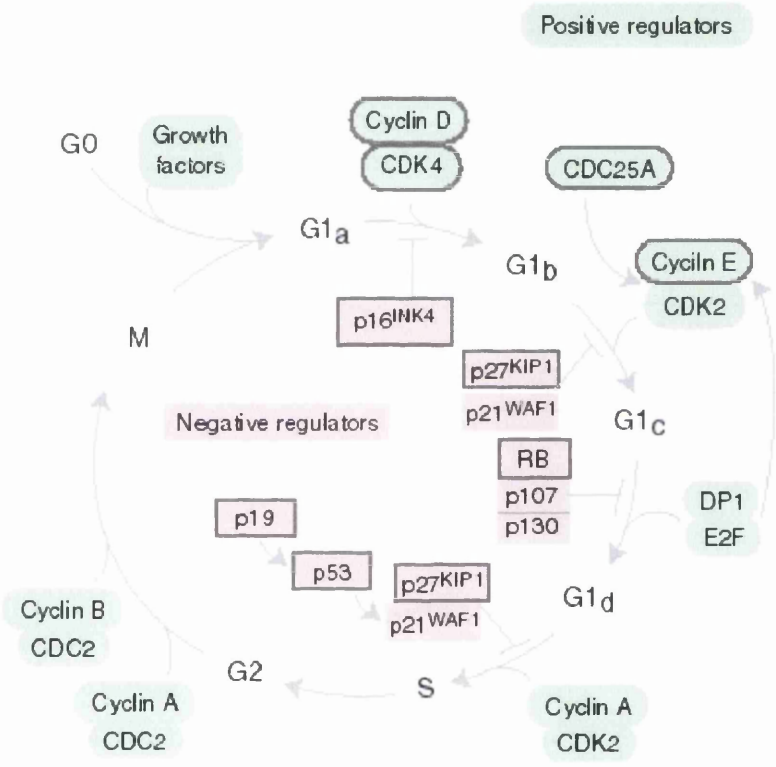


Figure 6.2

This diagram of the cell cycle shows the positive and negative regulators of cell cycle. Proteins bordered in grey have been found to be defective in some cancer cells. From Fred Hutchinson Cancer Research Center www.fhcrc.org

6.1.6.1.2 The Cyclin-dependent kinases inhibitor p21

Cyclin kinase inhibitor p21 transcription is controlled by p53 and can induce G1 arrest and block entry into S phase of the cell cycle by inactivating CDKs or by inhibiting activity of proliferating cell nuclear antigen (PCNA). In normal cells, p21 exists in quaternary complexes with cyclin, CDK, and PCNA (Wang *et al.*, 1994). Transcription of the p21 gene is activated by both p53-dependent and -independent mechanisms. A consequence of a mutation of p53 can be a decreased level of p21 transcription; this in turn leads to CDKs remaining active which results in cumulative DNA damage and premature replication of unchecked DNA (Navaratnam *et al*, 1999; Kamb, 1995). p21 is expressed in terminally differentiating cells of a variety of tissues in a p53-independent manner. Over expression of p21 results in G1 arrest and has been shown to suppress effectively tumour growth in vitro and in vivo (Gartel and Tyner, 2002). Activated APC gene initiated tumour formation is normally suppressed by p21 (Yang *et al.*, 2001(a)). In the colon p21 is expressed as cells exit the proliferative section of the colonic crypt. Loss of expression of p21 and topographical regulation is detected early in colon tumour formation (El-Deiry *et al.*, 1995, Polyak *et al.*, 1996). Decreased p21 expression in colorectal tumours has been reported to be a marker of poor prognosis in patients (Zirbes *et al.*, 2000).

Despite the obvious importance of p21 in regulating colon cell maturation, mice deficient in p21 exhibit no apparent phenotype and develop normally. From this research it appears that the function of p21 under normal circumstances appears to be redundant. However embryonic fibroblasts obtained from these mice are defective in G_1 checkpoint control and the function of p21 has been demonstrated to be necessary for p53-mediated G_1 arrest following irradiation (Deng *et al.*, 1995). Radiation, the short-chain fatty acid butyrate and the nonsteroidal anti-inflammatory drug (NSAID) sulindac all stimulate the cell cycle arrest and apoptosis of colon cancer cells and are all linked to the induction of p21 (Goldberg *et al.*, 1996; Archer *et al.*, 1998; Waldman *et al.*, 1997; Yang *et al.*, 2001(b); Siavoshian *et al.*, 2000).

It is thought that although defective p21 is not itself a cause of tumour formation it has a significant role in the progression of carcinogenesis of colorectal tumours that are initiated by other genetic events, such as mutation of the APC gene (Kinzler and Vogelstein, 1996).

The presence of reduced p21 gene expression levels in the early polyp stages of carcinogenesis, as detected in Chapter 5, indicates that p21 expression levels may be critical in the development of colorectal tumours. The mechanisms underlying this change are not as yet fully understood.

6.1.7 Rationale of the study

Results from Chapter 5 show a reduced level of gene expression of the tumour suppressor gene p21 in early stages of colorectal cancer development, present even in early polyp stages. To follow up these results and to obtain a clearer insight into the mechanisms occurring, FISH was carried out to discover if these findings were a result of variation in copy number of chromosome 6 or of a deletion in the region of the chromosome coding for p21.

6.2 Materials and Methods

6.2.1 Sample collection

Ethical approval was granted for the collection of 63 surgical samples from 13 patients from operations carried out by Mr. Beynon at Singleton Hospital, Swansea as described in section 2.2. Samples were taken of normal mucosa from sites close and distant from the colorectal tumour, the tumour itself where possible from the centre of the tumour as well as the peripheral regions to detect any clonal variation from the tumour. Where this was not possible a single sample was taken from the centre of the tumour. Also, samples were taken from metastatic lymph nodes and polyps where these had developed. Tissues from these sites were also histologically examined by the pathology department at Singleton Hospital, Swansea. These include the same samples as those used in Chapter 5.

6.2.2 Slide preparation

Cytology brushing samples were spun onto slides to give a single layer of cells as described in section 2.2.1 and treated with pepsin to digest cellular proteins as described in section 2.14.1. The slides were also coded at this stage to ensure an unbiased treatment and count of the sample.

6.2.3 FISH with commercially available probe

The commercially available green centromeric probe for chromosome 6 (Abbott) was hybridised to the treated slides; the excess probe was then removed by washing stages as in the protocol described in section 2.14.2. The nuclei were then counterstained using DAPI as described in section 2.14.2.

6.2.4 FISH with probes made from clones

The general procedure for developing the p21 probe using clones is schematically represented in Figure 6.3 and then further described below.

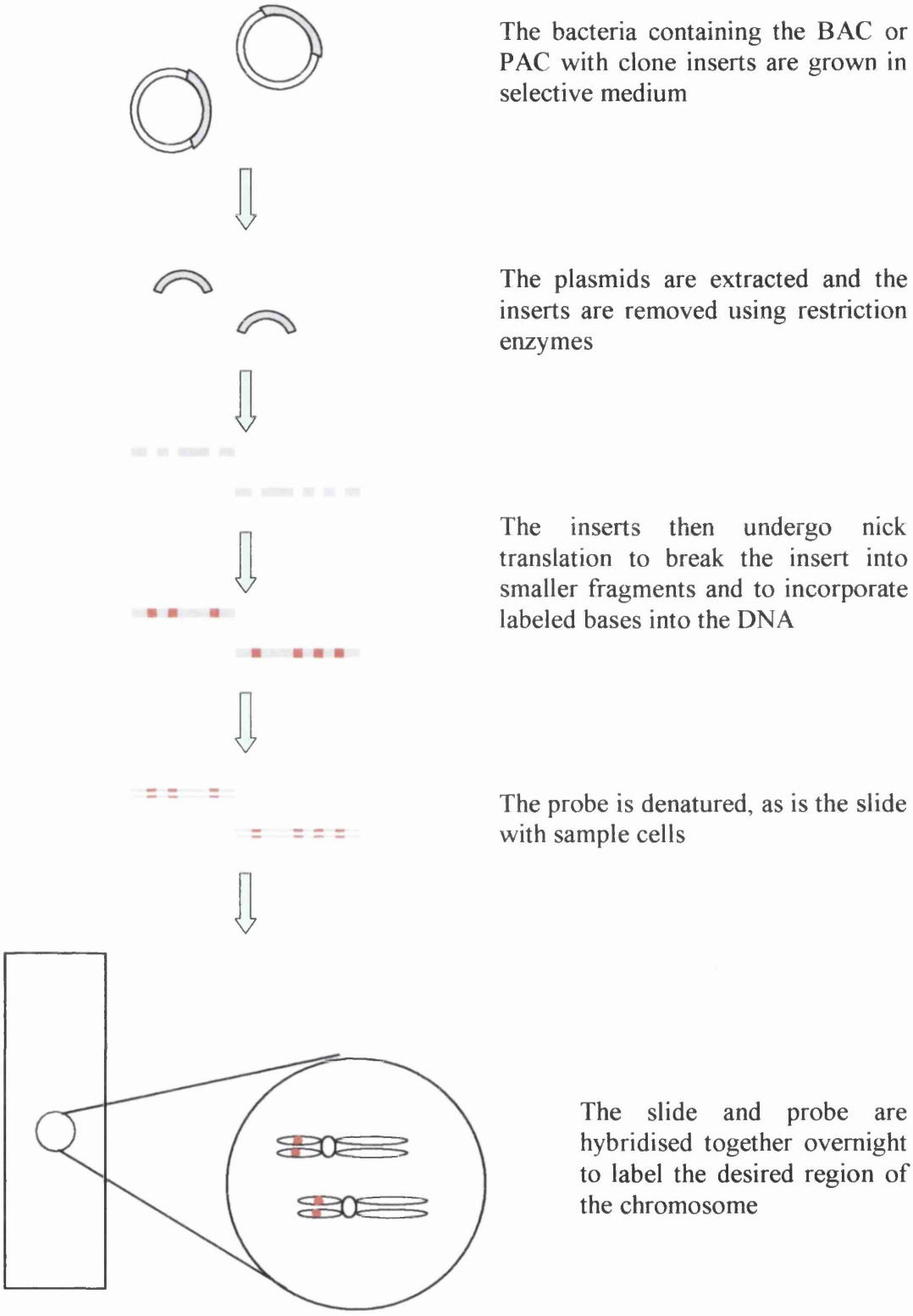


Figure 6.3

Schematic representation of the p21 FISH probe generation process.

The p21 gene is covered by the clone RP3-421A14 but this alone would not give a bright enough signal to score so a clone located just before this and overlapping after the RP3-421A14 clone were also used. These were RP1-108K11 and RP11-52A1, further details including of the length of these clones can be seen in Table 6.1. The clones were purchased from Children's Hospital Oakland Research Institute (CHORI) and cultures of the transformed bacteria where grown as described in section 2.14.4. The plasmids were extracted as described in section 2.14.5 prior to nick translation and purification as in section 2.14.6. The probe was then made by combining the purified nick translation product with human Cot-1 DNA and hybridization mix as described in section 2.14.7 and 2.14.8. The slides and probe were independently denatured and then hybridised overnight following the protocol in section 2.14.9. Excess probe was washed off and the nuclei were then counterstained using DAPI as described in section 2.14.9. A schematic representation of this protocol can be seen in Figure 6.3.

Gene / Clone	Length (kb)	Location	Type of vector	Restriction site
p21	8.6	36754465 – 36763087	-	-
RP-108K11	145.6	36553478 – 36699093	PAC	BamHI
RP3-421A14	195.4	36751257 – 36946619	PAC	BamHI
RP11-52A1	190.2	36788428 – 36978778	BAC	EcoRI

Table 6.1

Table giving the details of p21 and the clones used to make the p21 FISH probe. The information contained in this table is acquired from www.ncbi.nih.gov/genome/guide.

6.2.5 Slide viewing and image capture

Slides were viewed using an epifluorescence microscope and images were captured using charge - coupled device (CCD) camera operated using Mac-Probe 4.1 (Applied Imaging) software as described in section 2.14.3.

6.2.6 Scoring

Slides were scored as described in section 2.14.3 with a minimum of 800 nuclei per sample.

6.2.7 Statistical analysis

To be able to establish whether differences seen were statistically significant, X^2 test was carried out on the actual count data. The resulting p value was then used to determine the level of significance using confidence limits of 95% ($p = <0.05$), 99% ($p= <0.01$) and 99.9% ($p=<0.001$).

6.3 Results

6.3.1 Sample collection

Samples were collected from surgery over a period of 11 months. All the samples were used in this study unless there was information relating to these which would warrant the exclusion from this study of sporadic colorectal case. For example, patient 6 had undergone chemotherapy prior to surgery and so was eliminated from this current study. Other samples were eliminated where there was insufficient tissue to carry out the analysis or there were doubts as to whether the patient had hereditary colorectal cancer which was previously thought to be sporadic. For these reasons the patient numbers are not consecutive but this is not due to any selection process other than that described above.

6.3.2 Chromosome 6 FISH results

A minimum of 800 nuclei were scored from a minimum of 2 slides for each tissue from each patient. Where possible 400 nuclei were scored for each slide but this was not always possible due to overlapping nuclei, very few cells on the slide or bacterial contamination from the colorectal sample. In these cases further slides were scored to give a total nuclei count of 800 for each tissue sample. The full details of these counts are listed in Appendix II. A summary of the average values for these counts for each patient is listed in Table 6.2 below and the overall average data for each tissue type is listed in Table 6.3. Figures 6.4 and 6.5 are photographs of various nuclei observed in this study and are typical of those scored in this experiment.

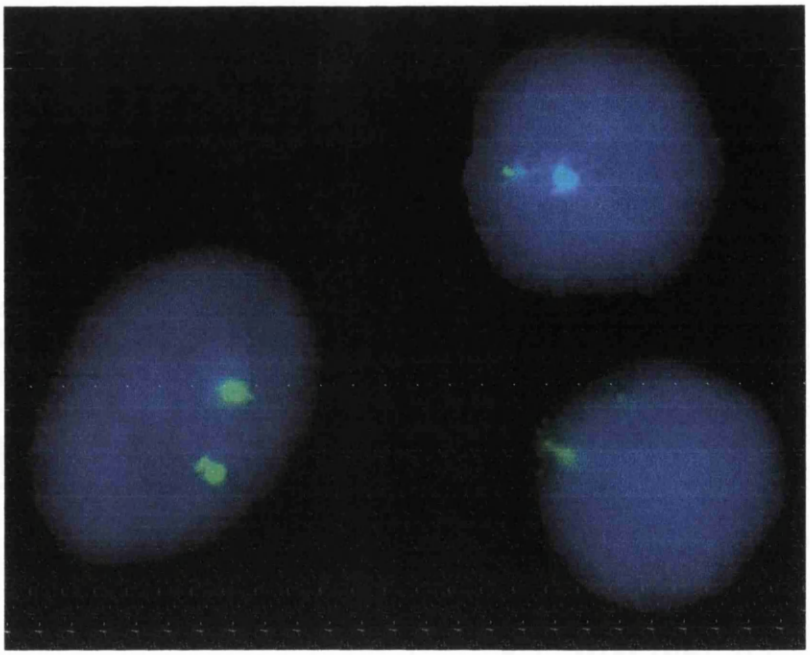


Figure 6.4

Image of several nuclei from the normal adjacent mucosa of patient 1. The nuclei are counterstained blue with DAPI and the centromere of both copies of chromosome 6 in each nucleus are hybridised with the green probe.

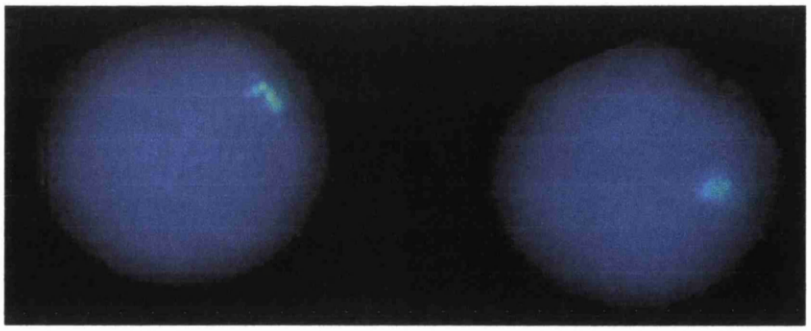
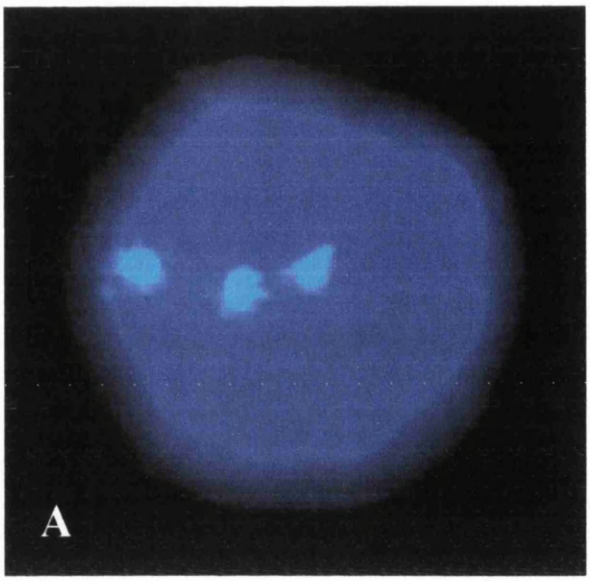


Figure 6.5

This image shows 2 nuclei, counterstained blue with DAPI, from the polyp tissue of patient 4. The green probe has only bound in one location within each nucleus indicating the loss of one copy of chromosome 6 in each.



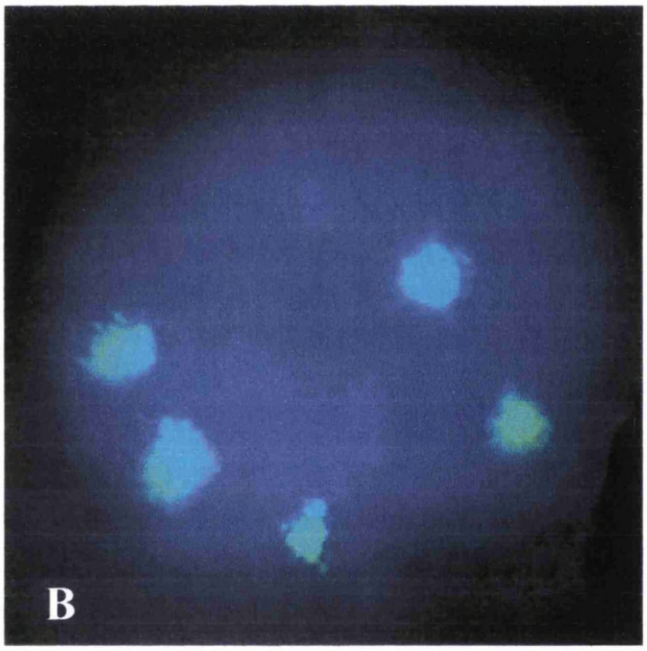


Figure 6.6

These are images of nuclei from the central tumour tissue of patient 11. Again in each picture the nucleus is stained blue with DAPI and the centromeric region of chromosome 6 has hybridised to the green labelled probe. In image A the nucleus has an extra copy of chromosome 6 and in image B the nucleus has 5 copies of chromosome 6.

Patient No.	Patient age	Patient sex	Sample No.	Sample type	Percent of cells			
					2 copies	1 copy	3 copies	4+ copies
1	36	F	1	Central tumour	61.9	7.1	30.4	0.6
			2	Peripheral tumour	51.2	5.6	39.5	3.6
			3	Normal adjacent	78.5	17.2	4.0	0.3
			4	Normal distant	53.1	7.0	39.0	1.0
			5	Lymph node	63.7	5.1	30.2	1.0
2	80	M	6	Tumour	81.5	15.6	2.6	0.4
			7	Normal adjacent	86.9	9.8	3.1	0.1
			8	Normal distant	87.7	9.0	3.3	0.0
4	67	M	12	Tumour	73.3	8.5	17.6	0.7
			13	Normal adjacent	92.1	5.2	2.6	0.1
			14	Normal distant	93.6	4.0	2.5	0.0
			15	Polyp	68.4	20.6	10.8	0.2
5	57	F	16	Tumour	66.2	10.0	21.1	2.7
			17	Normal adjacent	89.6	6.9	3.4	0.1
			18	Normal distant	89.0	7.6	3.1	0.2
			19	Polyp	72.9	24.1	2.9	0.1
7	69	F	23	Central tumour	34.6	9.7	40.5	15.2
			24	Peripheral tumour	52.7	6.9	21.9	18.5
			25	Lymph node	74.2	21.9	3.6	0.4
			26	Normal distant	91.2	5.2	3.3	0.2
			27	Normal adjacent	89.4	7.5	3.0	0.1
8	61	M	28	Central tumour	85.2	14.3	0.4	0.1
			29	Peripheral tumour	44.0	6.2	48.1	1.7
			30	Small polyp	80.7	13.9	5.1	0.4
			31	Large polyp	83.8	13.9	2.3	0.0
			32	Normal distant	92.0	5.3	2.5	0.2
			33	Normal adjacent	90.7	6.3	2.7	0.3
10	63	M	38	Peripheral tumour	73.2	11.5	12.4	2.9
			39	Central tumour	75.7	12.6	7.2	4.5
			40	Normal adjacent	95.1	3.3	1.6	0.0
			41	Normal distant	93.8	3.8	2.3	0.0
			42	Polyp	59.3	16.4	18.8	5.5
11	64	F	43	Normal distant	93.3	6.0	0.7	0.0
			44	Normal adjacent	84.2	6.9	8.2	0.8
			45	Peripheral tumour	79.1	13.1	6.3	1.5
			46	Central tumour	48.2	16.9	22.0	12.9
			47	Lymph node	78.1	19.6	2.2	0.1
13	64	M	52	Central tumour	88.0	10.0	1.6	0.4
			53	Peripheral tumour	90.1	8.9	0.9	0.1
			54	Normal adjacent	96.2	2.5	1.4	0.0
			55	Normal distant	96.8	1.8	1.4	0.0
			56	Polyp	89.9	6.4	3.7	0.0

Table 6.2 continued overleaf

Patient No.	Patient age	Patient sex	Sample No.	Sample type	Percent of cells			
					2 copies	1 copy	3 copies	4+ copies
15	85	F	61	Peripheral tumour	82.8	13.8	3.4	0.0
			62	Central tumour	84.5	14.5	0.9	0.1
			63	Normal adjacent	89.4	6.5	3.9	0.1
			64	Polyp	79.5	11.4	7.8	1.3
			65	Large polyp	80.2	11.1	7.8	1.0
			66	Normal distant	93.6	4.9	1.5	0.0
16	73	F	67	Peripheral tumour	75.1	19.6	4.5	0.7
			68	Normal adjacent	92.1	6.2	1.7	0.0
			69	Normal distant	96.6	2.6	0.9	0.0
			70	Central tumour	75.2	19.3	4.5	0.9
17	58	M	71	Normal distant	95.2	4.0	0.7	0.1
			72	Polyp	78.9	20.2	0.8	0.1
			73	Normal adjacent	91.6	7.3	1.1	0.0
			74	Peripheral tumour	82.2	13.8	3.6	0.4
			75	Central tumour	79.5	7.9	10.2	2.4
			76	Lymph node	26.5	11.6	22.6	39.3
20	74	F	85	Normal distant	90.5	7.0	2.2	0.2
			86	Normal adjacent	90.5	4.4	4.6	0.5
			87	Peripheral tumour	83.2	8.7	7.8	0.2
			88	Central tumour	74.4	24.1	1.3	0.3
			89	Far polyp	84.3	13.5	2.2	0.0

Table 6.2

Table showing the average percent of cells with the normal number of chromosome 6, only 1 copy, 3 copies and 4 and more copies for each tissue sample from each patient.

Sample type	Number of samples	2 copies	1 copy	3 copies	4+ copies
Normal distant	13	89.7 ± 11.3	5.2 ± 2.1	4.9 ± 10.3	0.1 ± 0.3
Normal adjacent	13	89.7 ± 4.6	6.9 ± 3.6	3.2 ± 1.9	0.2 ± 0.2
Polyp	10	81.2 ± 2.3	14.0 ± 3.3	4.3 ± 3.0	0.5 ± 0.6
Peripheral tumour	10	75.5 ± 12.3	11.8 ± 3.9	11.7 ± 13.7	1.0 ± 1.0
Central tumour	13	70.7 ± 17.4	13.6 ± 5.4	11.9 ± 14.2	3.7 ± 5.6
Lymph node	4	60.6 ± 23.5	14.6 ± 7.7	14.7 ± 13.9	10.2 ± 19.4

Table 6.3

Table showing the mean percent of cells with the normal number of chromosome 6, only 1 copy, 3 copies and 4 and more copies for each tissue type. Figures following the mean values are ± standard deviation found between the patients for each tissue type.

Sample type	Normal distant	Normal adjacent	Polyp	Peripheral tumour	Central tumour	Lymph node
Normal distant	-	N.S.D	***	***	***	***
Normal adjacent	N.S.D	-	***	***	***	***
Polyp	***	***	-	**	**	***
Peripheral tumour	***	***	**	-	N.S.D	*
Central tumour	***	***	**	N.S.D	-	*
Lymph node	***	***	***	*	*	-

Table 6.4

Table showing the statistical significance of the levels of aneuploidy found in the sample types using the chi-squared test. N.S.D. represents no significant difference, * , ** and *** indicates a significant difference at confidence limits of 95%, 99% and 99.9% respectively.

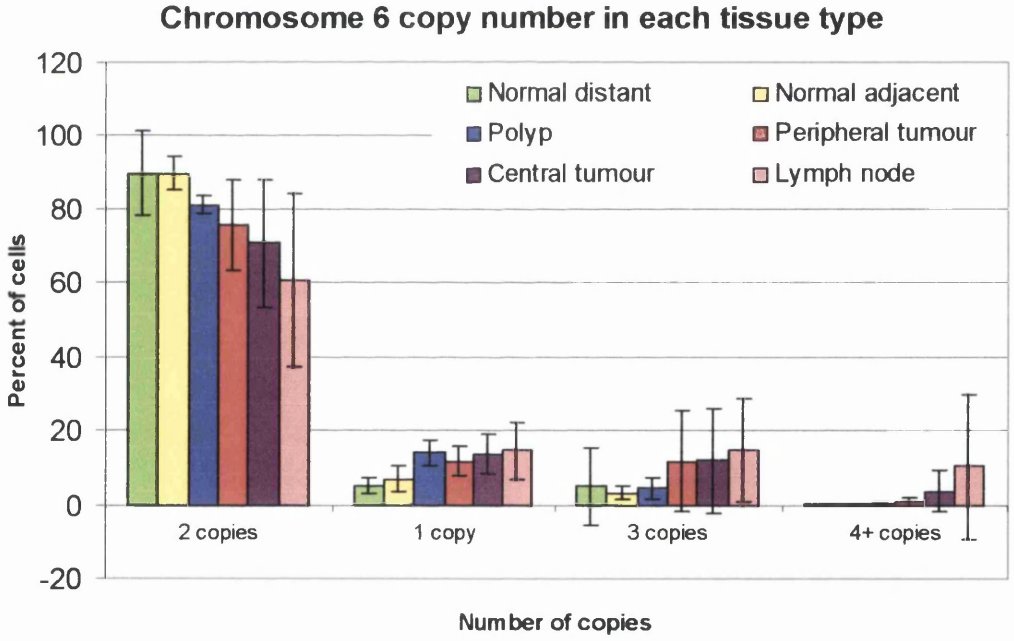


Figure 6.7

This is a graphical representation of the data in Table 6.3. The error bars show +/- 1 standard deviation.

6.3.3 p21 probe results

6.3.3.1 p21 probe optimisation

Optimisation of the protocol developed by Doak (2004) was needed in order to create a probe which bound only in the appropriate region with a strong signal and with minimal background noise. A sufficiently large region of the chromosome had to be covered by the probe so that there was a strong enough signal to be scored on interphase FISH slides where the chromosomes are not condensed as they are at metaphase. This was achieved by using a combination of 3 clones obtained from Children's Hospital Oakland Research Institute (CHORI). During the nick translation process an incubation of 2 hours was found to sufficiently incorporate the labelled bases and also give fragments size of between 200-800bp which has been found to be the optimal size for hybridisation. Sufficient human Cot-1 DNA which is enriched with repetitive DNA sequences was needed to eliminate background noise and the quantity needed for this again required optimisation, adding 15µl to 600ng of labelled probe gave the clearest results. 5 µl of the finished probe was applied per cytodot on each slide. The probe was successfully optimised on normal metaphase cells and a photograph of the optimised probe hybridised to a normal metaphase spread can be seen in Figure 6.8. This photograph clearly shows the probe bound to the correct region of both copies of chromosome 6 and, importantly, not bound to any other regions.

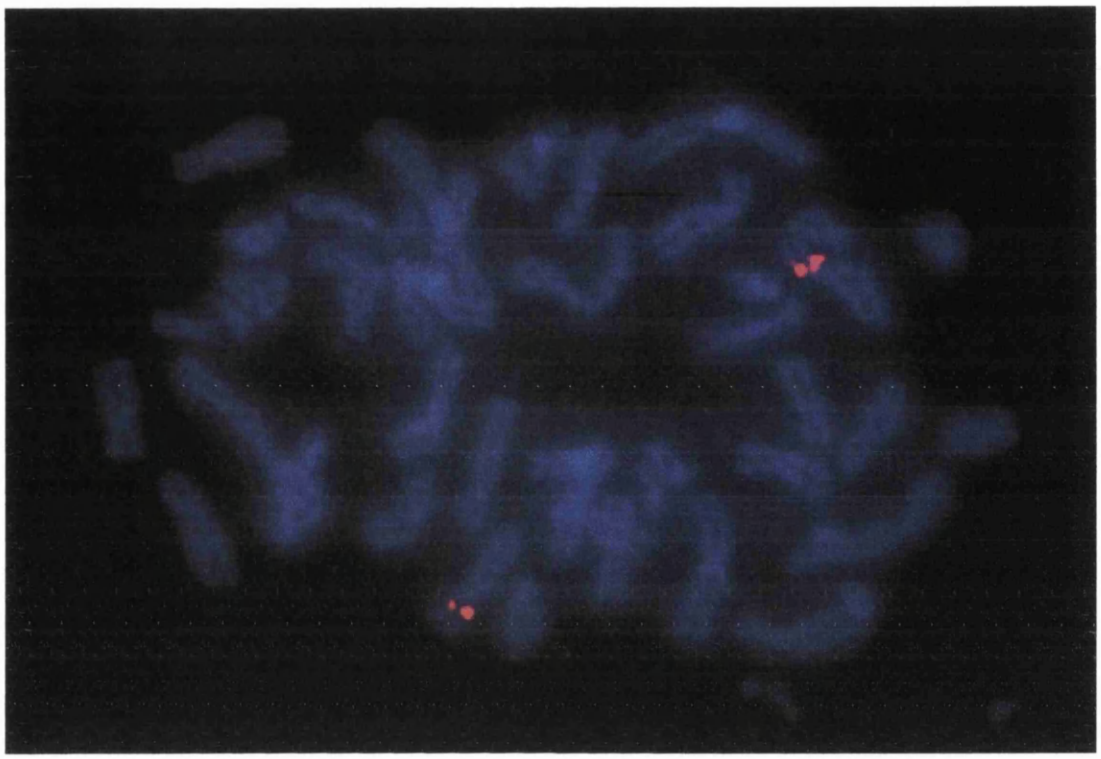


Figure 6.8

This image shows the chromosomes from a metaphase cell stained blue with DAPI and red probe bound to the appropriate region of chromosome 6 on both sister chromatids for both chromosome 6 copies.

Fortunately, no further optimisation was required when using the probe on interphase colorectal cells. Figure 6.9 shows the probe hybridised to chromosomes within the nuclei of an interphase sample of colorectal normal mucosa.

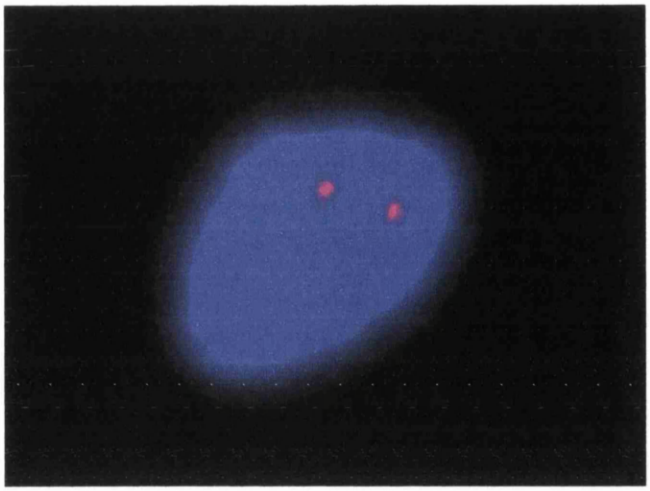


Figure 6.9

An image of a nucleus from a histologically normal colorectal mucosa sample hybridised with the p21 probe. The nucleus is blue as a result of DAPI staining and the red probe indicates the presence of 2 copies of the p21 region.

6.3.3.2 p21 probe results

Due to time constraints it was unfortunately not possible to use this probe with all of the samples used for the chromosome 6 FISH probe. FISH analyses of the patient samples examined by array analysis in Chapter 5 were however analysed using the probe for the p21 region. The results for these experiments are in Table 6.5.

Patient No.	Patient age	Patient sex	Sample No.	Sample type	Chromosome 6 - Percent of cells				p21 probe - Percent of cells			
					2 copies	1 copy	3 copies	4+ copies	2 copies	1 copy	3 copies	4+ copies
4	67	M	12	Tumour	73.3	8.5	17.6	0.7	68.4	7.7	20.6	3.3
			13	Normal adjacent	92.1	5.2	2.6	0.1	94.0	4.1	1.9	0.0
			14	Normal distant	93.6	4.0	2.5	0.0	93.2	5.2	1.7	0.0
			15	Polyp	68.4	20.6	10.8	0.2	63.6	26.9	8.8	0.7
8	61	M	28	Central tumour	85.2	14.3	0.4	0.1	85.2	14.2	0.4	0.2
			29	Peripheral tumour	44.0	6.2	48.1	1.7	38.5	6.4	51.4	3.8
			30	Small polyp	80.7	13.9	5.1	0.4	81.1	16.9	1.9	0.1
			31	Large polyp	83.8	13.9	2.3	0.0	83.6	14.6	1.2	0.6
			32	Normal distant	92.0	5.3	2.5	0.2	90.4	6.7	2.4	0.5
			33	Normal adjacent	90.7	6.3	2.7	0.3	89.3	7.0	3.4	0.2
10	63	M	38	Peripheral tumour	73.2	11.5	12.4	2.9	73.3	13.4	12.2	1.1
			39	Central tumour	75.7	12.6	7.2	4.5	72.8	14.9	9.8	2.6
			40	Normal adjacent	95.1	3.3	1.6	0.0	94.3	3.7	1.8	0.1
			41	Normal distant	93.8	3.8	2.3	0.0	93.3	4.2	2.2	0.2
			42	Polyp	59.3	16.4	18.8	5.5	62.3	11.7	20.3	5.6
5	57	F	16	Tumour	66.2	10.0	21.1	2.7	57.8*	7.8	31.6***	2.7
			17	Normal adjacent	89.6	6.9	3.4	0.1	87.9	8.6	3.2	0.2
			18	Normal distant	89.0	7.6	3.1	0.2	87.0	8.9	4.0	0.1
			19	Polyp	72.9	24.1	2.9	0.1	63.5**	33.4**	2.5	0.6
20	74	F	85	Normal distant	90.5	7.0	2.2	0.2	89.6	7.2	2.6	0.6
			86	Normal adjacent	90.5	4.4	4.6	0.5	88.3	5.5	5.1	1.0
			87	Peripheral tumour	83.2	8.7	7.8	0.2	81.7	9.0	8.7	0.6
			88	Central tumour	74.4	24.1	1.3	0.3	70.7	14.2***	12.6***	2.6
			89	Far polyp	84.3	13.5	2.2	0.0	81.8	15.1	2.0	1.0

Table 6.5

FISH results for chromosome 6 and p21 probes for the 5 patients analysed in chapter 5. The asterisks signify statistical significance, * represents a 95% confidence limit, ** a 99% limit and *** for 99.9%.

Sample type	No. of Samples	2 copies	1 copy	3 copies	4+ copies
Normal distant	5	90.9 ± 2.9	6.3 ± 2.0	2.5 ± 0.9	0.3 ± 0.3
Normal adjacent	5	90.6 ± 2.9	5.9 ± 1.9	3.2 ± 1.3	0.3 ± 0.4
Polyp	6	72.7 ± 10.4	19.8 ± 8.4	6.1 ± 7.5	1.5 ± 2.1
Peripheral tumour	3	64.5 ± 22.9	9.6 ± 3.6	24.1 ± 23.7	1.8 ± 1.7
Central tumour	5	71.0 ± 9.8	11.7 ± 3.6	15.0 ± 11.8	2.3 ± 1.2

Table 6.6

Table showing the mean percentage of cells with the normal number of copies of the p21 region, only 1 copy, 3 copies and 4 and more copies for each tissue type. Figures following the mean values are ± standard deviation found between the patients for each tissue type.

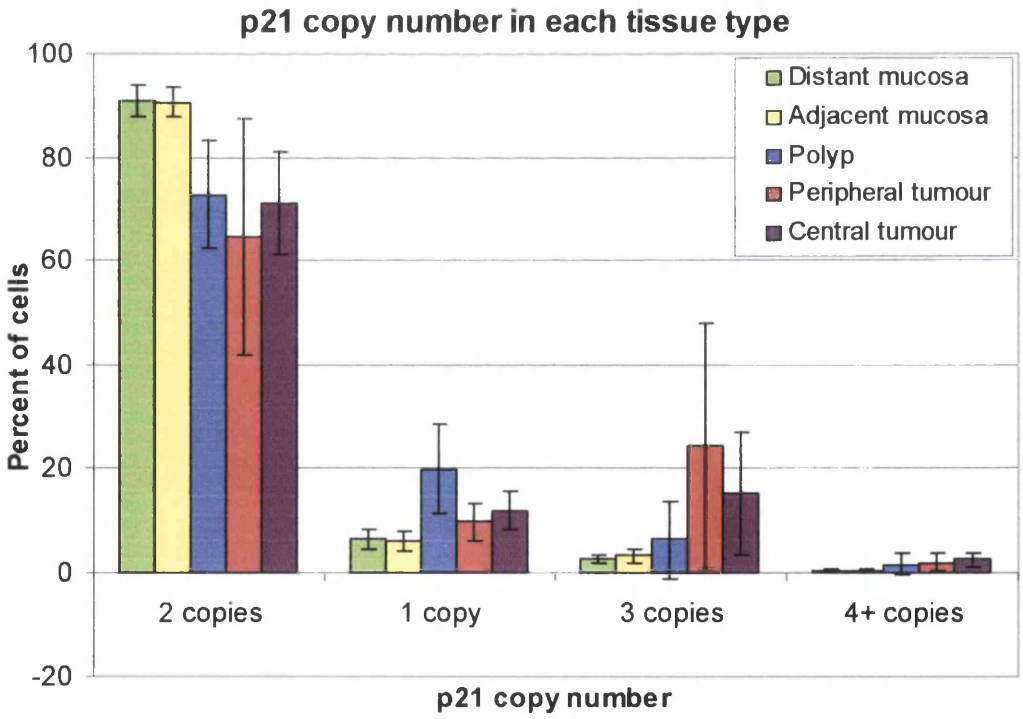


Figure 6.10

This is a graphical representation of the data in Table 6.7. The error bars show +/- 1 standard deviation.

6.4 Discussion

6.4.1 Application of p21 probe

Centromeric FISH probes, as their name indicates, bind to the centromeric region of the chromosome and although these are useful for detecting chromosome number within samples, it is not possible to know whether the entire chromosome is present or for example, if there have been deletions from the arms of the chromosome. For this reason it was necessary to develop the FISH probe for the region of chromosome 6 that the p21 gene is situated. Once optimised, the probe for p21 was straightforward to hybridise and clear to visualise, allowing for easy scoring. There was no statistically significant (using chi-squared test with 95% confidence limit) variation between repeat experiments for the same tissue giving indicating the experiments are highly reproducible. This technique can also be used to make probes for other regions of the genome where clones are available.

Using a combination of both the chromosome 6 centromeric and the p21 region probes not only provides information as to whether there is a change in chromosome 6 number but also, if the counts for each probe are unequal, indicates either the deletion of at least this proportion of the chromosome 6 p arm or a translocation of this region onto another chromosome.

6.4.2 Levels of aneuploidy present

6.4.2.1 Chromosome 6

As the tissue samples progressed through the pathway of carcinogenesis the levels of aneuploidy increased in respect of chromosome 6 copy number as can be seen in Table 6.3 and represented in graphical form in Figure 6.7. The statistical significance between the proportions of cells with 2 copies of chromosome 6 for each sample type can be seen in Table 6.4. There was no significant difference in the number of copies of chromosome 6 between the histologically normal mucosa located close to and distant from the carcinoma with both these sample types having 2 copies of the chromosome in a mean of 89.7% of cells. This shows that there is no localised effect

upon the surrounding histologically normal colorectal mucosa. This information is especially useful for experiments where it is not possible to collect a distal mucosal sample. The remaining cells with an abnormal number of chromosome 6 copies had an approximately even number of gains and losses.

The proportion of cells with a normal copy number of chromosome 6 was significantly lower in the 10 polyp samples than in the normal mucosa samples. Interestingly there was less variation between the polyp samples than in the normal mucosa samples. These results show that already at this early stage in carcinogenesis the cells are losing their chromosomal stability. Patients 6 and 15 both had 2 polyps and in both of these patients the polyps showed a high degree of similarity in terms of chromosome 6 aneuploidy and there was no significant difference between the paired polyps regardless of size. The abnormal cells found in the 10 polyp samples consist of a higher proportion of chromosome losses than gains with 74.5% of these abnormal cells exhibiting chromosome 6 monosomy. This is in contrast to the proportion of colorectal cancer adenoma cases reported which predominantly describe the gain of chromosome 6 (Bardi *et al.*, 1997; Bardi *et al.*, 1993; Bomme *et al.*, 1998), but these generally have limited patient numbers and studies describing chromosome 6 losses have also been published (Bomme *et al.*, 1996; Muleris *et al.*, 1994). Unlike most of these previous studies, this current experiment used FISH analysis of a large number of individual cells from several patients and was a focused study analysing chromosome 6 directly; this allowed the detection of changes which occurred in only a small proportion of cells.

There was a relatively large variation between the polyp and carcinoma tissue between the patients with both the carcinoma samples from the centre of the tumour and the peripheral regions having a higher level of chromosome 6 aneuploidy than the polyp tissue. There was no statistically significant difference overall between the levels of aneuploidy in the 2 different tumour samples with 70.7% of central tumour and 75.5% of peripheral tumour samples having a normal copy number of chromosome 6. Unlike the findings for the polyp samples, the aneuploid cells in the tumour samples showed an even number of chromosome 6 losses and gains. Interestingly, of the 10 patients from which both central tumour and peripheral

tumour samples were taken, there were 5 patients (see Table 6.7) which showed significant differences between these regions of the same colorectal tumour.

Patient No.	Central tumour % cells diploid for chromosome 6	Peripheral tumour % cells diploid for chromosome 6	Confidence interval
1	61.9	51.2	99%
7	34.6	52.7	99.9%
8	85.2	44.0	99.9%
11	48.2	79.1	99.9%
20	74.4	83.2	95%

Table 6.7

Table showing the confidence intervals for the statistically significant differences using chi-squared test seen in chromosome 6 copy number between the central and peripheral regions of the same colorectal tumour.

Patient 8 had half the proportion of cells with a normal chromosome 6 copy number in the peripheral tumour sample than that taken from the centre of the tumour. The abnormal cells also showed different abnormalities with the peripheral tumour having 48% of the cells showing chromosome 6 gains and the aneuploid cells from the centre of the tumour being predominantly monoploid for this chromosome. Almost the opposite observation was made of patient 11 with only 48% of the central tumour cells exhibiting the normal number of chromosome 6 compared with 79% of the peripheral tumour cells. This important observation shows the large scale variation that can be found within a single tumour and highlights the likelihood that the variation between studies, patients and even between tumours from the same patient could be simply due to the variation that exists within cells sampled from a tumour. The variations did not show a pattern correlating to the location from within the tumour from which the sample was taken. This makes it more difficult to remove this variation from studies even by applying strict protocols detailing the sampling site from each tumour. A great deal of care therefore needs to be taken when reporting FISH findings from solid tumours.

The metastatic lymph nodes showed the greatest level of aneuploidy of all of the samples, with only 60.6% of cells having 2 copies of chromosome 6. The alterations in copy number not only occurred at a greater frequency than in the other tissue samples but also consisted predominantly of chromosome gains with 24.9% of the metastatic lymph node cells having gains of chromosome 6 with 10.2% having 4 or more copies. This indicates the level of chromosome instability in metastatic tissues to be greater than found in the primary carcinomas. There was also the greatest variation between these samples from different patients with a large standard deviation. It does need to be highlighted that there was only 4 metastatic lymph nodes analysed in this study and further samples would need to be examined before any sound conclusions can be drawn from this data.

6.4.2.2 Aneuploidy levels detected in the p21 region

Due to the time required for the optimisation of the probe for p21 region, it was not possible to apply this probe to all 63 samples from the 13 patients analysed using the probe for chromosome 6. The samples from the 5 patients that were analysed in Chapter 5 for the gene expression study were all analysed along with any additional tissue samples that were not analysed by the arrays. Unfortunately, this cohort of samples did not include any metastatic lymph node tissue. These patients are listed in Table 6.5 along with the FISH results for both the chromosome 6 and p21 region probe. Where there is a statistically significant difference between the copy numbers of these probes in the same tissue of each patient these are marked with a number of asterisks depending upon the degree of significance.

The number of cells with a normal number of copies of the p21 region decreased as the samples progressed through the pathway of carcinogenesis just as was observed for the chromosome 6 centromeric probe. For 4 of the 5 patients there was no significant difference between the copy numbers of the chromosome 6 centromeric probe and that of the p21 region probe. This suggests that, as there was no significant increase or decrease in p21 region copy number in comparison to chromosome 6, there were no significant levels of translocation or deletion of the p21 region in any of the samples of these patients and the changes in p21 copy number were a result of the

loss or gain of the whole of chromosome 6. A review of DNA copy number losses in human neoplasms found that, although 6q losses are some of the most frequently reported aberrations, 6p losses are relatively rare (Knuutila *et al.*, 1999). This is in agreement with the findings in this current study which found no significant decrease in the copy number of this 6p region in comparison to that of the number of chromosome 6 copies in most of the patients analysed.

Patient 5, despite showing no significant difference between the chromosome 6 and p21 region copy numbers in the normal mucosa samples, did show differences between copy numbers of each probe in the tumour and polyp samples. In both of these tissue samples the percent of cells with a normal complement of the p21 region was approximately 9% lower than seen for chromosome 6. This suggests that in addition to whole chromosome 6 aberrations there was also a high level of 6p structural alterations. The polyp sample showed an increase in p21 region losses in comparison to chromosome 6 copy number indicating that 6p deletions had occurred. The tumour sample however showed a comparative gain of this region suggesting that this proportion of chromosome 6 had translocated onto another chromosome. There are reports of chromosomal abnormalities occurring at band 6p21 in many types of cancer, such as renal and ovarian cancer (Argani *et al.*, 2001; Wake *et al.*, 1980) and these aberrations are especially common in leukemia (Stock and Dennis, 1999; La Starza *et al.*, 2002). It is not possible from this study to establish onto which chromosome this region of chromosome 6 has translocated nor the size of the fragment either translocated or, in the case of the polyp, deleted. Chromosome painting techniques would provide some of these answers as well as giving greater insight into the overall levels of aneuploidy present and having the advantage of being able to detect balanced chromosomal abnormalities, which is not possible in this current study. The decrease in expression of p21 as detected in Chapter 5 was greater for the polyp sample than for the tumour sample and this correlation therefore may indicate that copy number is at least in part an explanation for this. This is further discussed in the final discussion in Chapter 7.

Using the type of interphase FISH applied in this study it is not possible to analyse very small regions of the genome. In order for the p21 FISH probe to be visible for scoring in interphase cells the probe needed to cover a much larger region than the

8.6kb which make up the p21 gene. A total of 373.1kb was covered by the 3 clones used to create the p21 region probe. Therefore these experiments would not be able to detect if a small fragment of the chromosome covered by the probe was deleted. For example, if the 8.6kb which code for p21 were deleted there would still be a strong signal from the remaining probe as such a small proportion of the overall 373kb probe would be unlikely to affecting the binding of the remaining probe. These smaller scale deletions can be detected using molecular genetic techniques such as PCR and it would be interesting to analyse p21 at this level of detail in future studies.

6.4.3 Other genes covered by the p21 region FISH probe

As described above, the p21 region FISH probe had to cover a larger region than just the p21 gene itself so that it could be visualised. The clones used to create the probe spanned over 373kb and were located on chromosome 6 positions 36553478 – 36699093 and 36751257 – 36978778. In addition to p21, the probe covered the chromosome 6 locations for the genes splicing factor, arginine/serine rich 3 (SFRS3), located at position 36670138-36679187, serine/threonine kinase 38 (STK38), position 36569654-36623225 and a gene for a hypothetical protein located at 36773606-3680938.

SFRS3 is involved in the tissue-specific differential splicing by mediating the protein-protein interaction within the intron during spliceosome assembly (Jumaa *et al.*, 1997). There are currently no published experiments examining the role of this gene in the development of any form of cancer. STK38 regulates cell division and morphology. Despite the role of this gene in cell cycle control there is, as yet, only one study published which has analysed this gene in cancerous tissues. This study of human extramedullary plasmacytomas found a downregulation of STK38 by 1.8 fold (Hedvat *et al.*, 2003). There is no explanation for this downregulation offered but it would be interesting to see if this correlates with the downregulation observed for p21 as it is possible that these decreases in expression are the result of a common cause, such as deletion of this region of chromosome 6p21.3.

6.4.4 Other cancer development candidate genes on chromosome 6

Although tumor necrosis factor-alpha (TNFα) is located outside of the p21 region FISH probe, at position 31651k-31654k, it does map to position 6p21.3 and therefore any large scale chromosome aberrations which involve p21 may also involve the TNFα gene. TNFα is a cytokine involved in the inflammatory process and has cytotoxic and anti-tumour activity (Jong *et al.*, 2002). TNFα has been examined in colorectal cancers where an allelic imbalance between normal and tumour DNA was reported (Honchel *et al.*, 1996). Since this study, further research has established that various alleles of this gene provide decreased risks of colorectal cancer but that another allele increases the risk (Jong *et al.*, 2002). Raised serum levels of TNFα have been described in colorectal cancer patients with metastatic disease (Ardizzoia *et al.*, 1992) again indicating this gene has a potential role in colorectal cancer development. It is therefore important not to assume that any changes to tissues with gains or losses of chromosome 6p21 are entirely as a result of change of copy number of the gene p21.

Other candidate tumour suppressor genes on chromosome 6 are glutamate receptor-ionotropic-kinase 2 and angiopoietin-1 as these are located within a region which has shown high levels of loss of heterozygosity (LOH) in ulcerative colitis-associated colorectal cancers (Ezaki *et al.*, 2003). If these genes are involved in ulcerative colitis-associated colorectal cancer development it seems unlikely that these relate also to sporadic cases as LOH for this region was not detected in these latter carcinomas.

The study of other forms of cancer may bring to light other genes on this chromosome which are also important in the development of colorectal cancer. Retinoblastoma is a type of paediatric eye tumour initiated by the inactivation of the gene of the same name. These tumours have been frequently found to have gains of chromosome 6p22 and the increased gene expression of E2F3 and DEK correspond to these DNA gains (Grasemann *et al.*, 2005). Allelic loss of regions of 6q have been reported in breast cancer patients, with the genes ESR and IGF2R mapping to this region and therefore having the potential to be involved in cancer development (Roy *et al.*, 2001). It is

possible that these genes may also play a role in the development of other cancers, including colorectal carcinogenesis.

6.4.5 Alternative pathways of colorectal carcinogenesis

Although the mean values of copy number for both chromosome 6 and p21 provide information regarding the general trends seen in these experiments across the tissue types examined, the means also mask some of the findings of this study. There was a large variation between a number of the patients in some tissue types. As described earlier there was little variation in chromosome 6 and p21 region copy number in the normal mucosa and also the polyp samples but between the tumour samples and also the metastatic lymph node samples there was a much larger variation. For example, the tumour from patient 7 had only 34.6% of the tumour cells with 2 copies of chromosome 6 and gains in 55.7% of its cells, whereas the tumour from patient 2 had 81.5% diploid tumour cells and chromosome 6 gains in only 3% of its cells (see Table 6.2). Patient 13 also had low levels of aneuploidy in all of the samples from normal mucosa through to tumour samples, this may indicate that this tumour has arisen from a pathway other than the chromosomal instability pathway, perhaps from the microsatellite instability pathway.

6.4.6 The application of these FISH experiments for patient screening

Significant changes were detected for both the chromosome 6 and the p21 region probes between normal mucosa and polyp tissue and between poly and carcinoma. This shows the potential of using this technique to analyse the various colorectal tissue samples from patients. By finding out the extent of the chromosome aberrations for each patient it may be possible to establish an accurate prognosis and be able to make informed decisions as to which treatment may be the most appropriate for each individual case. Unfortunately, as with the array experiments, the downside to these experiments as a screening technique is the requirement of tissue samples obtained from biopsy. This is therefore unlikely to appeal to any of the population that currently does not take up the existing colonoscopy screening programme.

6.4.7 Discussion Summary

These experiments have shown that it is relatively simple and cost effective to produce FISH probes for chromosome regions that are not as yet commercially available provided that clones are available.

The results also show that as expected the proportion of cells with abnormal chromosome 6 and p21 copy numbers increased with progression of carcinogenesis. Comparisons of the copy numbers of chromosome 6 centromeric probe and that of the p21 region probe indicated there was no significant difference between the copy numbers of these regions. This indicates that the changes in p21 region copy number are a result of loss and gain of the whole of chromosome 6 rather than deletions and translocations of just the p21 region.

One of the more striking observations made from this study was the level of statistically significant variation in the FISH results for chromosome 6 copy number from samples taken from different regions of the same tumour. This highlights the caution required when reporting FISH data from colorectal tumours.

Chapter 7
General Discussion

7.1 General background to the hypotheses behind this thesis

Despite the advances made in medicine over the last few decades 70% of patients with colorectal cancer will die from this disease (Mella *et al.*, 1997). This is often the result of patients presenting with late stages of colorectal cancer as the 5 year survival of patients with Dukes' stage A cancer is 95% whereas once the cancer has metastasised the survival drops to only 3% (Rhodes, 2000). Current screening techniques such as colonoscopy are risky procedures and the uptake in screening the over 50s is still relatively low, thought to be because of the invasive nature of the procedure. Colonoscopy is also an expensive procedure considered borderline in its cost effectiveness as a screening tool (Brenner and Rennert, 2005). Other screening tests such as the faecal occult blood test (FOBT) although are non-invasive are also not ideal as these produce large numbers of false positive results which can cause alarm and unnecessary colonoscopies. Also, more dangerously, FOBTs also produce false negative results as not all colorectal cancers bleed (Rennert, 2003). Faecal based DNA tests are emerging as a potential new colorectal cancer screening method. PCR based studies have been undertaken to examine faecal samples for DNA containing mutations in the adenomatous polyposis coli (APC), p53 and K-ras genes (Osborn and Ahlquist, 2005). These, and similar, studies have had a sensitivity of around 52% for adenocarcinomas and 15% - 40% for adenomas, with a specificity of 95% (Osborn and Ahlquist, 2005; Imperiale *et al.*, 2005). These results show a greater sensitivity than FOBTs yet maintain a higher specificity. These studies show the potential of these faecal DNA based screening methods yet increasing the sensitivity is obviously a goal. These tests are also very expensive and therefore are currently not viable for population-based screening (Song *et al.*, 2004). To reduce these costs it would be ideal to find smaller regions of DNA containing hotspots which are mutated in a larger proportion of both colorectal adenomas and adenocarcinomas. If such a non-invasive test for colorectal cancer could be developed then this could be a very

influential factor in reducing the mortality of colorectal cancer as patients may be able to be detected earlier where the chances of survival are much greater. Such a test can only result from a better understanding of early stages of disease.

There is currently limited understanding of what makes some polyps progress to malignancy whilst others remain benign. 25% of the western population over 50 years of age and 50% of over 75 year olds will develop colorectal polyps (Greenwald *et al.*, 1995). Around 10% of these over 1cm will become malignant within 10 years (Stryker *et al.*, 1987). As yet it is not known which of these polyps do progress and therefore it would be useful to know which of these polyps were likely to develop into cancers and would require more follow up treatment than those which were likely to remain benign.

7.2 Overall objectives of thesis

The experiments described in this thesis were designed to explore the various genetic pathways of colorectal cancer development by studying surgically removed tissue from normal colonic mucosa and intermediate stages of carcinogenesis as well as cancerous tissue and, where possible, metastatic lymph nodes from each patient. These experiments examined the occurrence of mutations in the mitochondrial genome, how these related to the microsatellite status of the nuclear genome, the alterations in gene expression and larger scale chromosomal alterations examined using fluorescent *in situ* hybridisation (FISH). The emphasis of these experiments was to find mutations, gene expression changes or chromosomal aberrations which occur early in the development of colorectal cancer that may allow for the detection of these tumours whilst the chance of patient survival is still relatively high.

7.3 Summary of the findings of the experiments

The experiments described in this thesis all used the largest number of patients samples that could be obtained over a three year period and totalled 128 tissue samples from 37 patients. As these samples were collected on a chronological rather than selective basis there were some tissue types, such as metastatic lymph nodes, that have a lower number of samples than would be ideal. The sample numbers from this

study are still frequently greater than many previously published studies within the same field and the results provide an excellent stimulus for further research areas and larger studies.

The experiments in this thesis are summarised here and the main findings feature in Figure 7.1 which also indicates the timing these changes occur within the normal to adenoma to carcinoma progression. These changes may not though all occur within the same pathway. For example, neither patient with nuclear microsatellite instability also had mitochondrial DNA mutations and therefore these may be changes that occur in separate genetic pathways of colorectal carcinogenesis.

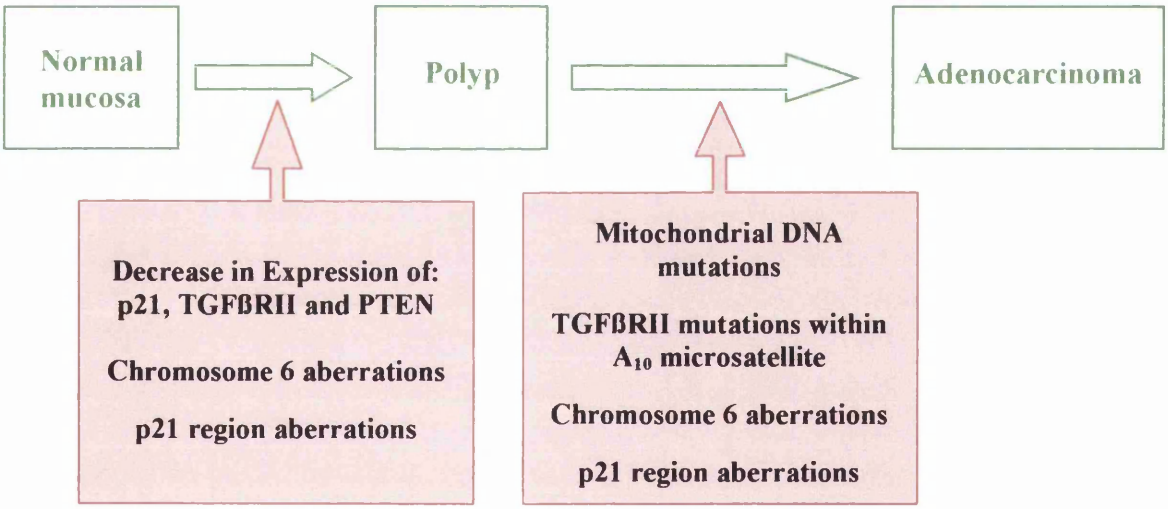


Figure 7.1

This diagram summarises the genetic changes found at each stage in the experiments described in this thesis. The changes detected in this thesis are in the red boxes and indicate the stages of colorectal carcinogenesis (as seen in the green boxes) at which these were identified. The full names for the genes abbreviated above are transforming growth factor, beta receptor II (TGFßRII), phosphatase and tensin homolog (PTEN) and p21 is the cyclin-dependent kinase inhibitor 1A.

7.3.1 Summary of the mitochondrial DNA study

Previous studies of the mitochondrial genome found mitochondrial DNA (mtDNA) mutations present in the lung lavage fluid, urine and saliva of patients with lung, bladder and head and neck tumours respectively (Fliss *et al.*, 2000). As there are more copies of mtDNA per cell than the nuclear genome, these mtDNA mutations could be more readily detected in the paired bodily fluids than nuclear mutations. MtDNA mutations therefore provide potential for finding a mtDNA mutation screen for colorectal cancer from the faeces of affected patients. In Chapter 3, I describe how a 379 base pair section of the displacement loop (D-loop) of the mitochondrial genome was amplified and analysed for mutations in 65 samples from 24 patients with sporadic colorectal cancer. The D-loop is so called at it contains the origin of replication for both strands of the mitochondrial genome and when replication occurs the strands become displaced and effectively single-stranded during this process. This helps to contribute to a higher mutation rate in this region than for the rest of the mtDNA and is the reason this region of the mitochondrial genome was selected for this study.

Tumour specific mtDNA mutations occurred in 9/24 (37.5%) of patients. Recently published studies found very similar levels of mutation with 10/25 (40%) and 142/365 (38.9%) of colorectal samples containing mutations within the D-loop (Lee *et al.*, 2005; Lièvre *et al.*, 2005). In this current study, no mutations were present in any of the normal, adenoma and hyperplastic polyp samples suggesting that these mitochondrial mutations occur at a late stage of colorectal cancer progression. This was also indicated as in one patient a mutation was found in metastatic adenocarcinoma tissue but not in matched adenocarcinoma tissue. As these mutations occur at a later stage they may be therefore useful in differentiating between polyps which remain begin and those which become cancerous. It is increasingly likely that a panel of markers will be required to detect colorectal cancers and this could include early biomarkers which may detect all polyps and later markers which may detect polyps which are likely to become cancerous. D-loop mutations have been found at high levels in a wide range of human cancers including cervical, endometrial, ovarian, breast, Oesophageal, bladder, lung and head and neck tumours (Wang *et al.*, 2005; Kumimoto *et al.*, 2004; Fliss *et al.*, 2000). This shows the potential of this region as a

biomarker for cancer detection. Importantly, in a study of 365 colorectal patients, D-loop mutations have been found to correlate with poor prognosis and a factor of resistance to fluorouracil-based adjuvant chemotherapy (Lièvre *et al.*, 2005). This may be due to this drug interfering with the mitochondrial function and requiring a functional respiratory chain to be efficient. It is becoming increasing likely that mtDNA determines the cellular response to some cancer therapeutic agents (Costantini *et al.*, 2000; Singh *et al.*, 1999). These factors all add weight to the importance of mtDNA mutation detection in cancer patients. It would be interesting to be able to follow up the patients examined in this thesis with regard to assessing their response to any chemotherapy undertaken as well as assessing if there is any correlation with prognosis in this sample group.

The results from this thesis are also important in the context of elucidating more about the different mechanisms in the carcinogenesis of colorectal tumours in different locations within the colon. Of the samples analysed, mtDNA mutations were found in 6/12 (50%) rectal tumour samples and both of the sigmoid tumours but none of the transverse or caecal tumour samples. This could be a result of different mechanisms causing cancer in the proximal and distal regions of the colon. Dietary factors may play a larger role in colorectal cancer development of the distal regions of the colorectum where the faeces are more concentrated and the exposure time to the mucosa is longer. This appears to correlate as diet is thought to be responsible for 80% of colorectal cancers (Bingham, 2000) and around 75% of cancers occur in the distal regions of the colon (Mella *et al.*, 1997). From this, it is likely that the mtDNA mutations observed were a result of exposure to mutagenic chemicals within the faeces.

20% of the mutations detected were transversion mutations, characteristic of the result of damage due to reactive oxygen species (ROS) which can damage guanine bases and cause these to mispair with deoxyadenosine (Grollman and Moriya, 1993). This is described in more detail in Chapter 3, section 3.1.1.4 and in Figure 3.4. Levels of oxidative damage are higher in the mitochondrion than in the remainder of the cell as this is the site of oxidative phosphorylation (OXPHOS) (Richter *et al.*, 1988). It is thought that the ROS lipid peroxidases produced as a result of OXPHOS preferentially damage the D-loop of the mitochondrial genome due to this region

containing the membrane attachment site and is therefore closest to the location of oxidative phosphorylation which occurs on the inner mitochondrial membrane (Hruszkewycz, 1978). The frequency of transversion mutations in the portion of D-loop studied was lower in this study than for other regions of the mitochondrial genome (Lewis *et al.*, 2002) but this was a study of smokers and so these were likely to have higher levels of oxidative damage. Other studies of various cancer types have found lower levels with between 8-10% of the mutations found were transversion mutations (Polyak *et al.*, 1998; Kumimoto *et al.*, 2004; Fliss *et al.*, 2000).

4/9 (44%) of the patients with mutations had either deletion or insertion mutations within a C-tract within the D-loop. This finding was particularly interesting as around 12% of sporadic colorectal cancers show microsatellite instability (MSI) in the nuclear genome (Ionov *et al.*, 1993). I then designed further experiments to establish whether there was a connection between nuclear MSI (nMSI) and mitochondrial MSI (mtMSI), described in Chapter 4 and below in Section 7.3.3. Due to the mtDNA mutations being predominantly in microsatellites with the mtDNA, these results also may add insight into the mismatch repair mechanisms of the mitochondrial genome.

7.3.2 Nuclear microsatellite instability discussion summary

To try to establish any connection between mtMSI and nMSI I have assessed all the samples analysed in Chapter 3 for nMSI, this research is the content of Chapter 4. The well documented nMSI markers located within the genes BAX, transforming growth factor-β receptor II (TGFβRII), human *Saccharomyces cerevisiae* mutS homolog 3 (hMSH3) and insulin-like growth factor II receptor gene (IGFIIR) were examined (Souza *et al.*, 1996; Rampino *et al.*, 1997; Ikeda *et al.*, 1998; Molenaar *et al.*, 1998). None of the samples showed MSI in the BAX, IGFIIR or hMSH3 microsatellites. 2/24 patients had mutations within the A10 microsatellite in the TGFßRII gene. This gene is mutated in between 90% - 100% of MSI colorectal cancers (Parsons *et al.*, 1995; Souza *et al.*, 1996) and has not been reported to be mutated in any microsatellite stable colorectal cancers. This result was therefore interpreted that these 2 patients were MSI. Although it is possible that there were false negative results this is unlikely as TGFßRII alone is a highly sensitive marker of MSI (Parsons *et al.*, 1995; Souza *et al.*, 1996) and in combination with the BAX, IGFIIR or hMSH3

markers it would be very unlikely that any MSI positive tumours were not detected as such. The overall level of MSI in this study is 2/24 (8.3%), this level correlates well with previous studies. The level is at the lower end of previously recorded MSI levels in sporadic colorectal cancer. This may be due to the high proportion of patients within the sample group with distal colorectal cancers; as MSI tumours tend to be located in the proximal regions of the colorectum. In this study proximal tumours were shown to more frequently contain microsatellite mutations with 1/6 (16.7%) being MSI as opposed to 1/18 (5.6%) of the distal tumours. This is though, based on only 2 MSI+ samples and therefore can only be considered an observation warranting further investigation rather than a result in itself.

7.3.3 Is there a correlation between mtMSI and nMSI?

None of the 4 patients with mtMSI had nMSI mutations and although there were only 2 patients showing nMSI this data suggests there is no link between mtMSI and nMSI and these occur through different pathways. The 2 patients with nuclear MSI had no mtMSI. The tumour locations of the mtMSI+ samples were all distal but 93% of nMSI+ sporadic tumours occur in the proximal colon (Thibodeau *et al.*, 1998; Ward *et al.*, 2001). This indicates that the nuclear mismatch repair mechanism may be separate to the repair system responsible for maintaining these repeat sequences in the mitochondrial genome. However, the mismatch repair (MMR) protein human *Saccharomyces cerevisiae* mutS homolog 1 (hMSH1) has been found to be involved in the maintenance of the mitochondrial genome stability (Mookerjee *et al.*, 2005). This may indicate that the MMR system in the nuclear and mitochondrial genomes have at least some common proteins.

7.3.4 Array Discussion summary

The development of array technology has made it possible to examine the levels of gene expression of many genes in one experiment. This technique was appropriate for this thesis as many of the polyp samples were extremely small and this approach can cover many genes at once using minimal quantities of RNA. Although arrays are available which cover several thousand genes, these are very expensive and require specialist equipment and expertise to extract the data from these experiments (Cheung

et al., 1999). I therefore chose to use a membrane cDNA array known as the GEArray human cancer/tumour suppressor array from SuperArray (USA) which focused on 23 tumour suppressor genes. These arrays were a useful technique for the rapid screening of gene expression changes between various stages within carcinogenesis. The array results were more consistent for the genes expressed at higher levels than those expressed at lower levels. It was difficult to obtain reproducible results for the genes expressed at low levels as the background noise was often higher than the readings for these genes. Despite this, cDNA arrays are ideal preliminary scanning tools especially where tissue samples are small, yielding only low quantities of RNA.

The cDNA arrays detected a significant decrease in the expression of the tumour suppressor genes transforming growth factor, beta receptor II (TGFßRII) and transforming growth factor, beta receptor I (TGFßRI) in 60% and 40% respectively of the patients analysed. Phosphatase and tensin homolog (PTEN) was also significantly down-regulated in the polyp samples of 60% of the patients studied. Down-regulation of PTEN gene expression is thought to promote colorectal cancer progression by activating nuclear factor-KB and β-catenin causing deregulated growth and resistance to apoptosis (Agarwal *et al.*, 2005).

The most notable detected change in expression was of the cyclin-dependant kinase inhibitor 1A known as p21 which was decreased at the polyp stage of tumour development in all 5 patients studied regardless of tumour location, patient sex or age. This gene may therefore be critical in determining progression of a polyp into a carcinoma. p21 expression levels may also indicate which tumours may respond better to certain chemotherapeutic agents and also be an indicator of prognosis in colorectal cancer patients. It has been reported that decreased p21 expression is linked with poor prognosis (Zirbes *et al.*, 2000). As this gene was down-regulated in all the patients analysed, either all 5 patients had a poor prognosis or perhaps there is a scale where those patients with a larger fold-decrease in p21 expression have a poorer prognosis than those with only a small decrease in p21 expression. It would be interesting to analyse more patients to see if this study is truly representative of a wider population. To try to understand more about the mechanism behind this gene expression decrease of p21, FISH was undertaken and these experiments are described in Chapter 6.

7.3.5 Fluorescent *in situ* hybridisation discussion summary

To further explore the gene expression findings of Chapter 5, where the kinase inhibitor p21 was found down-regulated in the polyp tissue of all 5 patients examined, FISH analysis was undertaken to determine if these gene expression changes were the result of gross chromosomal alterations. A probe for the centromeric region of chromosome 6, on which the p21 gene is located, was used to detect numerical aberrations and a probe specifically for the p21 region was developed to detect aberrations at a higher resolution. It was simple and cost effective to produce FISH probes for chromosome regions that are not as yet commercially available by labeling clones for the chromosome regions. As expected, due to the hostile microenvironment within a tumour and the accumulation of genetic damage, the FISH results also show that the proportion of cells with abnormal chromosome 6 and p21 copy numbers increased with progression of carcinogenesis.

As well as varying from patient to patient, the proportion of cells with abnormal copy numbers of both chromosome 6 and the p21 region varied significantly even within the same tumour of 50% of the samples studied. This lack of uniformity within solid tumours highlights variability and therefore the need for multiple sampling within individual tumours. These findings of such variation within a single tumour may result in different areas of a single cancerous growth responding differently to treatments such as chemotherapy. It would be interesting to further investigate this aspect of the results, possibly by establishing cell lines from different areas from within a single tumour and exposing these to various chemotherapeutic agents to establish any differences in reaction to these chemicals.

1 of the 5 patients analysed using the p21 region probe had a significant difference between the chromosome 6 and p21 region number. This patient showed deletions of the p21 region in the polyp tissue and an increase in the carcinoma tissue, both in relation to the number of copies of chromosome 6. This shows that deletions and rearrangements of chromosome arms as well as gross changes in chromosome number occurred in this patient.

The results of this FISH study have shown some interesting findings and this data provides an incentive to expand this study to also examine different regions of some of the larger polyps as well as the continuing to examine these regional differences found within carcinomas. It would also be interesting to examine other chromosomes to gauge the overall level of aneuploidy in the cells and how this compares to the levels of chromosome 6 alterations. More metastatic lymph nodes need to be analysed using FISH for chromosome 6 and also for the p21 region probe.

7.3.6 Correlation between Fluorescent *in situ* hybridisation results and gene expression data

Even at the polyp stage there was a significant reduction in the number of cells with normal copy numbers of both chromosome 6 and the p21 region. This may be a contributing factor to the reduction of p21 gene expression observed in Chapter 5, especially in polyps where those cells that did not have the normal number of chromosome 6 and p21 region mainly had losses (74.5% of aneuploid cells) rather than gains (25.5%).

In the tumour samples analysed, from both central and peripheral regions, the aneuploid cells had a fairly even proportion of losses and gains. Although from a smaller sample number of 4 lymph nodes, the trend observed in the aneuploid cells from these metastatic samples was a majority of gains of chromosome 6 with 63.0% showing gains and of these 41.0% had gains of at least 2 additional copies of chromosome 6. Unfortunately no lymph nodes were examined for gene expression alterations in Chapter 5 due to low RNA yields but it would be interesting to see if this overall increase in chromosome 6 copy number correlates with an increase in p21 expression.

By comparing the percentage of cells in each sample with chromosome 6 and p21 region losses with the fold decrease it appears that there is little correlation between these figures. For example, patient 20 (array 5) has 13.5% of polyp cells and 24.1% of tumour cells with 1 copy of chromosome 6. If the reduction in p21 gene expression was thought to be a result of these chromosome aberrations, then it would be expected that the fold decrease in expression would be greater for the tumour than the polyp.

This was not the case with both these tissue samples exhibiting extremely similar p21 expression levels with fold decreases in comparison to normal colonic mucosa of -3.58 for the polyp and -3.38 for the tumour. Whilst patient 4 (array 1) had the highest percentage of cells with 1 copy of chromosome 6 in the polyp sample along with the greatest fold-decrease in expression, the reverse was true for patient 10 (array 3). This lack of correlation indicates that the mechanism behind the decreases in p21 expression seen is not a result of chromosomal aberrations for this chromosome or region.

Other possible mechanisms which could have caused the decrease in p21 expression observed in Chapter 5 include mutation or hypermethylation of the gene or promoter region or to any other the genes involved within this complex pathway. For example, a reduction in expression of functional p53 could cause a reduction in p21 expression; however p53 is reported to be a late occurring change in colorectal cancer.

It would be particularly interesting to study metastatic lymph nodes using the p21 region probe as one previous study of secondary liver tumours has found chromosome 6p gains to be more common in colorectal cancer metastases than in primary colorectal carcinomas (Parada *et al.*, 1999). It is therefore thought that there are genes of importance in the metastatic process of colorectal cancer in this region. The tumour suppressor gene p21 has been reported to be involved early in the development of colorectal cancer and so it is likely that there are further genes which have later roles in this pathway which are also situated on chromosome 6p.

To further this study it would be interesting to analyse all the samples using the p21 probe for the patients that were examined with the chromosome 6 probe. This would allow a more accurate assessment of whether translocations or deletions of the p21 region did play a part in the mechanism behind the decrease in p21 expression levels observed in Chapter 5. This would also determine whether the correlation between chromosome 6 centromeric and p21 region aberration levels is representative of the whole sample group.

7.4 Discussion of techniques applied in this thesis

A wide range of techniques were applied throughout these experiments covering many aspects of genetics from mutation research to cytogenetics. Mutations were detected by single-stranded conformation polymorphism (SSCP), fragment analysis and sequencing. Whilst sequencing gave more information about the mutations detected by both SSCP and fragment analysis, both of these latter techniques were proven to be both sensitive and accurate in detecting these mutations and could be used for rapid cost effective screening where sequencing all samples is not a viable option. Fragment analysis gave neither false positive or negative results and was simple to carry out. SSCP also gave no false positive results but one of the mutations was not detected using this technique. SSCP does have the advantage that it requires no expensive or specialist laboratory equipment or consumables. Sequencing not only confirmed the results of these techniques but also provided specific information regarding the nature of the mutations and therefore enables the elucidation of the mechanism behind these mutations. For example, from sequencing data it was possible to determine that the mutations detected in the mitochondrial genome were predominantly insertions or deletions in repeat base sequences as a result of unrepaired strand slippage.

cDNA arrays were extremely useful for scanning across many genes to detect gene expression alterations between different tissue samples. As these experiments often relied on small quantities of RNA due to the small size of the tissue sample, particularly for polyps, it was useful to be able to detect the changes in 23 tumour suppressor genes in one experiment. These arrays were also useful to use in smaller laboratories which do not have specialist glass chip array readers or the bioinformatics experience required to mine the vast amount of data produced from the arrays which have several hundred genes. Despite these benefits of smaller cDNA arrays, there was a problem with contaminated arrays being supplied by the manufacturer. Also, for genes which were only expressed at low levels, the results were very variable as the background noise levels were too high to obtain significant results and therefore this was not a suitable technique for analysing these genes. Nevertheless, cDNA membrane arrays are an ideal means of screening across large numbers of genes at once so that it can be established which genes are expressed at these levels and may

require further analysis by methods such as reverse transcription PCR and quantative real time PCR (Q-RT-PCR). cDNA arrays also allow observations to be made to the patterns of expression seen in groups of genes of similar function; for example, a pattern was observed in the patients studied that the expression of these genes was generally down-regulated from normal mucosa to polyp followed by a general pattern of up-regulation from polyp to adenocarcinoma.

Fluorescent *in-situ* hybridisation (FISH) analysis was used to follow up the results from the gene expression studies. FISH allowed the analysis of individual cells from each tissue sample. This was carried out using a commercially available centromeric probe to determine chromosome 6 copy number. To discover whether there were any chromosomal alterations at a micro level, I developed a probe for the p21 region. The probe bound exclusively to the p21 region and was clear and easy to score. This was a straight forward and cost effective technique which can be used to make FISH probes for any region for which clones are available.

By taking samples of mucosa close to and distant from the tumour it was possible in all of these experiments to assess for any differences that may be localised to around the tumour site. There were no significant differences found between these 2 normal mucosa samples and this result can be used as justification for using only one normal mucosa sample in future experiments. In contrast, there were large significant differences found in the FISH study between the central and peripheral tumour samples from within a single neoplasm. This highlights the need for multiple sampling sites within a tumour and may explain the differences seen not only between various studies and patients but also from tumours from the same patient and various biopsies of the same tumour.

7.5 Future work

One of the most time consuming aspects of the research described in this thesis was the collection and processing of surgical samples. Over several months I built up a large collection of colorectal tissues, RNA, DNA and cell brushings which can be used for future experiments and therefore vastly reduce the time required for further studies.

As well as continuing to build on the sample numbers, especially of polyps, metastatic lymph nodes and secondary tumours, for all of the studies described, it would be interesting, where necessary, to confirm some of the results already observed using additional techniques. For example, Q-RT-PCR could be used to confirm the gene expression changes seen in Chapter 5. This could give accurate measures of gene expression change not just for those genes found significantly down-regulated in this study, such as p21 and PTEN, but also for those genes such as Wilms tumor 1 (WT1), that had large fold differences but were not significant due to the low initial levels of gene expression. Also, it would be interesting to use immunohistochemistry to confirm the MSI status of those patients found with TGFßRII microsatellite mutations by staining for the mismatch repair genes hMSH2 and hMLH1.

Given more time it would be possible to follow up these patients to see if any of the findings in the experiments described in this thesis correlate with patient prognosis and survival. It would be particularly stimulating to continue the research in to the mitochondrial genome. Recently, a correlation of D-loop mutations with poor prognosis and resistance to fluorouracil chemotherapeutic drugs has been found, (Lèivre *et al.*, 2005), and it would be interesting to establish whether this is also true for the patients studied in this thesis. Mutations in the coding region of the mtDNA have recently been found to promote cancer by preventing apoptosis (Shidara *et al.*, 2005). It would be interesting to see if the patients with D-loop mutations also had these mutations in the coding regions where mutations have a clear cancer causative affect.

It would also be interesting to know more about the lifestyles of these patients for factors such as diet, exercise levels, whether they smoke and if so how much as well as alcohol consumption. Although these are all well documented risk factors for colorectal cancer it would be interesting to see if some factors affect particular pathways more than others. This would need a large sample size due to the number of variables within each person's lifestyle but I believe this may provide more information behind the mechanisms of each individual pathway.

7.6 Final Conclusions

- Mitochondrial DNA (mtDNA) mutations have been found to be frequent in colorectal tumours and occur during a late stage of carcinogenesis

- Transforming growth factor, beta receptor II (TGFßRII) is an excellent marker for nuclear microsatellite instability and levels of sporadic mismatch repair deficient tumours within this examined South Wales population are in line with those reported in previous studies for the rest of the Western World.

- Neither patient with nuclear microsatellite instability had mtDNA mutations. This may indicate mtDNA has an independent repair mechanism however further studies are needed to confirm this.

- Phosphatase and tensin homolog (PTEN) and TGFßRII are down-regulated at the polyp stage in 3/5 patients analysed suggesting an early role in the development of at least a subset of colorectal cancers

- The cyclin-dependent kinase inhibitor p21 was down-regulated in the polyps of all the patients analysed, this gene may be key to the early stage development of the majority, if not all, of colorectal cancers

- Fluorescent *in situ* hybridisation (FISH) probes can be relatively easily developed at little cost for regions of the genome available from clone libraries

- Chromosome 6 aberrations were detected in increasing frequency in colorectal tissue samples with increasingly developed tumours.

- Chromosome 6 and p21 region aberrations are unlikely to be responsible for the down-regulation observed of the tumour suppressor p21

- The large variation within a single tumour observed especially from the FISH studies show how essential it is that research and diagnostic tests are based on either large portions of a tumour or from multiple smaller biopsies.

Appendix I

Fragment analysis data table

Patient number	Sample Number	Age	Sex	Sample type	TGF-ßRII		BAX		hMSH3		IGFIIR	
1	4	64	F	Rectal Polyp	84.89	84.98	92.19	92.28	130.99	130.92	108.84	108.91
	5			Adjacent mucosa	84.87	85.06	92.08	91.97	130.96	130.92	108.89	108.82
2	9	80	M	Rectal tumour	**85.92**	**85.83**	92.11	92.05	130.92	131.07	108.85	108.89
	10			Distant mucosa	84.89	85.06	92.15	92.03	130.92	131.00	108.90	108.94
3	11	79	F	Lymph node	84.87	84.95	92.12	92.11	130.91	131.03	109.00	108.92
	13			Rectal tumour	84.92	84.89	92.09	92.10	131.04	130.93	108.77	108.93
	14			Distant mucosa	84.90	84.98	92.18	92.09	130.99	130.99	108.84	108.85
4	16	64	M	Rectal tumour	84.86	84.95	92.10	92.06	131.07	131.02	108.87	108.97
	17			Adjacent mucosa	84.93	85.00	92.14	92.06	131.00	131.03	108.80	109.00
5	19	64	F	Distant mucosa	84.91	85.08	92.14	92.13	131.03	131.02	108.90	108.82
	20			Sigmoid polyp	84.88	84.84	92.12	92.12	131.04	130.94	108.88	108.82
6	24	71	F	Distant mucosa	84.87	85.14	92.13	92.09	130.93	131.00	108.88	108.86
	25			Rectal tumour	84.90	84.96	92.18	92.14	130.99	130.97	108.91	109.02
7	28	78	M	Distant mucosa	84.91	84.92	92.09	92.21	131.05	130.88	109.01	108.89
	29			Rectal tumour	84.89	85.09	92.11	92.04	131.07	130.92	108.94	108.97
8	30	64	M	Rectal polyp	84.87	85.12	92.10	92.08	131.03	130.90	108.89	108.95
	31			Liver biopsy	84.82	85.05	92.13	92.23	131.02	130.98	108.93	108.82
	33			Distant mucosa	84.83	84.81	92.16	92.05	130.94	131.03	108.91	108.82
	34			Rectal tumour	85.01	84.83	92.10	92.19	130.98	130.95	108.79	108.80
	35			Adjacent mucosa	84.87	84.85	**94.37** | 92.14 | 92.16		130.90	130.92	108.80	108.77
9	36	70	F	Sigmoid tumour	84.89	85.03	92.12	92.17	130.93	130.92	108.84	108.80
	37			Distant mucosa	84.86	84.84	92.11	92.22	131.00	130.91	108.76	108.90
	38			Sigmoid polyp	84.84	85.01	92.18	92.09	130.97	130.92	108.92	108.83
	39			Adjacent mucosa	84.87	84.88	92.19	92.30	130.90	130.94	108.75	108.78
10	40	59	F	Adjacent mucosa	84.89	84.61	92.16	92.13	130.93	130.93	108.79	108.89
	42			Transverse polyp	84.80	84.84	92.23	92.20	130.92	130.92	108.81	108.77
	43			Transverse tumour	84.90	84.79	92.12	92.16	130.97	130.95	108.84	108.82
11	44	67	M	Distant mucosa	84.85	84.89	91.98	92.13	130.90	130.99	108.85	108.81
	45			Rectal tumour	84.86	84.92	92.22	92.08	130.96	130.95	108.87	108.80

Table A1 continued overleaf

Patient number	Sample Number	Age	Sex	Sample type	TGF-ßRII		BAX		hMSH3		IGFIIR	
12	46	63	F	Rectal tumour	84.95	85.00	92.16	92.24	130.95	131.00	108.83	109.00
	47			Distant mucosa	84.91	84.96	92.13	92.14	131.03	130.93	108.90	108.81
13	50	73	M	Sigmoid polyp	84.76	84.84	92.16	92.33	130.95	130.95	108.85	108.86
	51			Adjacent mucosa	84.81	84.92	91.99	92.20	130.90	130.93	108.91	108.73
14	53	68	M	Distant mucosa	84.83	84.86	92.14	92.15	130.92	130.89	108.86	108.94
	54			Lymph node	84.87	84.94	92.15	92.16	130.98	131.00	108.82	108.68
	55			Rectal tumour	85.03	84.97	92.16	92.15	130.92	130.89	108.88	108.87
	56			Liver biopsy	84.84	85.04	92.14	92.20	130.94	130.97	109.00	108.99
15	57	85	M	Transverse tumour	85.00	84.92	92.13	92.19	130.93	131.03	108.90	108.93
	58			Distant mucosa	84.88	84.93	92.08	92.14	130.93	130.87	108.80	108.79
	60			Caecal polyp	84.61	84.91	92.11	92.10	130.92	130.91	108.84	108.76
16	68	68	F	Lymph node	84.89	84.95	92.10	92.26	130.99	131.02	108.80	108.81
	69			Distant mucosa	84.84	85.09	92.15	92.14	130.95	131.13	108.86	108.85
	70			Rectal tumour	84.89	84.90	92.17	92.12	130.92	130.98	108.78	108.87
	71			Adjacent mucosa	84.92	84.89	92.16	92.15	131.00	131.05	108.81	108.83
17	72	65	F	Distant mucosa	84.87	85.12	92.18	92.21	130.93	130.96	108.95	108.88
	73			Sigmoid polyp	85.04	85.06	92.12	92.15	130.92	130.99	108.96	109.96
	74			Adjacent mucosa	84.96	84.97	92.12	92.14	130.97	130.96	108.89	108.84
18	76	65	F	Distant mucosa	84.83	85.07	92.09	92.16	130.93	130.96	108.92	108.85
	77			Caecal tumour	84.89	84.98	92.04	92.12	130.97	131.00	108.94	108.88
	78			Caecal polyp	84.88	84.91	92.19	92.10	130.93	131.01	108.91	108.90
19	81	73	M	Distant mucosa	84.97	84.93	92.15	92.07	130.89	130.93	108.90	108.87
	82			Lymph node	85.03	84.92	92.13	92.15	131.00	130.91	108.89	108.80
	83			Transverse tumour	85.06	85.01	92.11	92.15	130.89	130.91	108.97	108.86
20	87	64	F	Rectal tumour	84.90	85.00	92.09	92.15	130.89	131.12	108.92	108.93
	88			Adjacent mucosa	84.94	84.98	92.10	92.24	130.97	131.00	109.01	108.94

Table A1 continued overleaf

Patient number	Sample Number	Age	Sex	Sample type	TGF- ßRII		BAX		hMSH3		IGFIIR	
21	99	83	F	Transverse tumour	84.89	85.05	92.13	92.22	130.99	131.00	108.97	108.91
	100			Adjacent mucosa	84.88	84.98	92.20	92.11	130.96	130.95	108.97	108.95
22	102	58	M	Sigmoid tumour	84.93	85.00	92.11	92.16	130.88	130.98	108.96	109.06
	104			Distant mucosa	84.94	84.92	92.18	92.14	130.91	131.03	109.01	109.02
	105			Lymph node	84.87	85.02	92.17	92.26	130.94	130.88	108.95	109.00
23	122	70	F	Rectal tumour	85.05	84.90	92.16	92.19	131.08	131.06	108.96	108.99
	124			Distant mucosa	84.84	84.97	92.14	92.17	131.06	130.92	109.00	108.92
24	96	58	M	Caecal tumour	**83.98**	**83.93**	92.25	92.21	130.96	130.94	108.89	108.89
	97			Adjacent mucosa	84.87	84.96	92.15	92.18	130.91	130.96	108.86	108.94
	98			Distant mucosa	84.79	84.85	92.19	92.11	131.04	130.99	108.97	108.78

Table A1

This table contains the fragment analysis data. For each sample tissue from each patients fragment analysis was undertaken as described in Chapter 4, for the microsatellite within the genes transforming growth factor β receptor II (TGF-ßRII), Bax, human *Saccharomyces cerevisiae* MutS homolog 3 (hMSH3) and insulin-like growth factor II receptor gene (IGFIIR). The figures are the length of the PCR fragment in base pairs. Figures in bold are those fragment analysis results which were not the wild type length. These were further examined in Chapter 4, section 4.3.1 and 4.3.2.

Appendix II

Fluorescent *in situ* hybridisation data table

Patient number	Sample no and tissue type	Total counted	Normal	1 copy	3 copies	4+ copies	% normal	% 1 copy	% 3 copies	% 4+ copies
1	1 - central tumour	427	267	31	127	2	62.53	7.26	29.74	0.47
	1 - central tumour	406	249	28	126	3	61.33	6.90	31.03	0.74
	2 - peripheral tumour	413	206	25	171	11	49.88	6.05	41.40	2.66
	2 - peripheral tumour	401	211	21	151	18	52.62	5.24	37.66	4.49
	3 - adjacent mucosa	401	312	71	17	1	77.81	17.71	4.24	0.25
	3 - adjacent mucosa	390	309	65	15	1	79.23	16.67	3.85	0.26
	4 - distant mucosa	401	212	32	153	4	52.87	7.98	38.15	1.00
	4 - distant mucosa	432	230	26	172	4	53.24	6.02	39.81	0.93
	5 - Lymph node	419	274	23	118	4	65.39	5.49	28.16	0.95
	5 - Lymph node	403	250	19	130	4	62.03	4.71	32.26	0.99
2	6 - Tumour	404	323	68	11	2	79.95	16.83	2.72	0.50
	6 - Tumour	420	349	60	10	1	83.10	14.29	2.38	0.24
	7- adjacent mucosa	177	152	18	7	0	85.88	10.17	3.95	0.00
	7- adjacent mucosa	401	360	33	7	1	89.78	8.23	1.75	0.25
	7- adjacent mucosa	108	92	12	4	0	85.19	11.11	3.70	0.00
	8 - distant mucosa	115	97	14	4	0	84.35	12.17	3.48	0.00
	8 - distant mucosa	216	186	23	7	0	86.11	10.65	3.24	0.00
	8 - distant mucosa	424	393	18	13	0	92.69	4.25	3.07	0.00
4	12 - Tumour	68	51	5	12	0	75.00	7.35	17.65	0.00
	12 - Tumour	439	310	42	78	9	70.62	9.57	17.77	2.05
	12 - Tumour	116	86	10	20	0	74.14	8.62	17.24	0.00
	13 - adjacent mucosa	182	169	7	6	0	92.86	3.85	3.30	0.00
	13 - adjacent mucosa	264	240	16	7	1	90.91	6.06	2.65	0.38

Table A2 continues overleaf

Patient number	Sample no and tissue type	Total counted	Normal	1 copy	3 copies	4+ copies	% normal	% 1 copy	% 3 copies	% 4+ copies
4	13 - adjacent mucosa	411	380	24	7	0	92.46	5.84	1.70	0.00
	14 - distant mucosa	401	373	17	11	0	93.02	4.24	2.74	0.00
	14 - distant mucosa	408	384	15	9	0	94.12	3.68	2.21	0.00
	15 - Polyp	147	99	36	12	0	67.35	24.49	8.16	0.00
	15 - Polyp	111	74	20	17	0	66.67	18.02	15.32	0.00
	15 - Polyp	170	121	33	15	1	71.18	19.41	8.82	0.59
5	16 - Tumour	409	267	39	91	12	65.28	9.54	22.25	2.93
	16 - Tumour	402	270	42	80	10	67.16	10.45	19.90	2.49
	17 - adjacent mucosa	403	360	27	16	0	89.33	6.70	3.97	0.00
	17 - adjacent mucosa	428	385	30	12	1	89.95	7.01	2.80	0.23
	18 - distant mucosa	400	352	33	14	1	88.00	8.25	3.50	0.25
	18 - distant mucosa	404	364	28	11	1	90.10	6.93	2.72	0.25
	19 - polyp	400	293	92	14	1	73.25	23.00	3.50	0.25
	19 - polyp	441	320	111	10	0	72.56	25.17	2.27	0.00
7	23 - central tumour	414	148	36	169	61	35.75	8.70	40.82	14.73
	23 - central tumour	398	133	43	160	62	33.42	10.80	40.20	15.58
	24 - Peripheral tumour	409	220	26	88	75	53.79	6.36	21.52	18.34
	24 - Peripheral tumour	403	208	30	90	75	51.61	7.44	22.33	18.61
	25 - lymph node	411	304	91	16	0	73.97	22.14	3.89	0.00
	25 - lymph node	402	299	87	13	3	74.38	21.64	3.23	0.75
	26 - distant mucosa	401	365	22	13	1	91.02	5.49	3.24	0.25
	26 - distant mucosa	420	384	21	14	1	91.43	5.00	3.33	0.24
	27 - adjacent mucosa	408	366	29	12	1	89.71	7.11	2.94	0.25
	27 - adjacent mucosa	397	354	31	12	0	89.17	7.81	3.02	0.00

Table A2 continues overleaf

Patient number	Sample no and tissue type	Total counted	Normal	1 copy	3 copies	4+ copies	% normal	% 1 copy	% 3 copies	% 4+ copies
8	28 - central tumour	400	342	56	2	0	85.50	14.00	0.50	0.00
	28 - central tumour	424	360	62	1	1	84.91	14.62	0.24	0.24
	29 - Peripheral tumour	404	166	18	210	10	41.09	4.46	51.98	2.48
	29 - Peripheral tumour	425	199	34	188	4	46.82	8.00	44.24	0.94
	30 - Small polyp	402	312	70	20	0	77.61	17.41	4.98	0.00
	30 - Small polyp	406	340	42	21	3	83.74	10.34	5.17	0.74
	31 - large polyp	400	335	56	9	0	83.75	14.00	2.25	0.00
	31 - large polyp	413	346	57	10	0	83.78	13.80	2.42	0.00
	32 - distant mucosa	222	204	12	5	1	91.89	5.41	2.25	0.45
	32 - distant mucosa	404	366	24	14	0	90.59	5.94	3.47	0.00
	32 - distant mucosa	107	100	5	2	0	93.46	4.67	1.87	0.00
	33 - adjacent mucosa	117	106	7	3	1	90.60	5.98	2.56	0.85
	33 - adjacent mucosa	409	374	26	9	0	91.44	6.36	2.20	0.00
	33 - adjacent mucosa	296	267	19	10	0	90.20	6.42	3.38	0.00
10	38 - Peripheral tumour	439	321	53	52	13	73.12	12.07	11.85	2.96
	38 - Peripheral tumour	424	311	46	55	12	73.35	10.85	12.97	2.83
	39 - central tumour	408	310	53	26	19	75.98	12.99	6.37	4.66
	39 - central tumour	400	302	49	32	17	75.50	12.25	8.00	4.25
	40 - distant mucosa	403	379	16	8	0	94.04	3.97	1.99	0.00
	40 - distant mucosa	417	401	11	5	0	96.16	2.64	1.20	0.00
	41 - Normal distant	400	373	17	10	0	93.25	4.25	2.50	0.00
	41 - Normal distant	412	389	14	9	0	94.42	3.40	2.18	0.00
	42 - Polyp	417	238	70	82	27	57.07	16.79	19.66	6.47
	42 - Polyp	406	250	65	73	18	61.58	16.01	17.98	4.43

Table A2 continues overleaf

Patient number	Sample no and tissue type	Total counted	Normal	1 copy	3 copies	4+ copies	% normal	% 1 copy	% 3 copies	% 4+ copies
11	43 - distant mucosa	400	369	27	4	0	92.25	6.75	1.00	0.00
	43 - distant mucosa	405	382	21	2	0	94.32	5.19	0.49	0.00
	44 - adjacent mucosa	207	177	14	16	0	85.51	6.76	7.73	0.00
	44 - adjacent mucosa	428	354	31	37	6	82.71	7.24	8.64	1.40
	44 - adjacent mucosa	318	268	21	26	3	84.28	6.60	8.18	0.94
	45 - Peripheral tumour	407	321	57	22	7	78.87	14.00	5.41	1.72
	45 - Peripheral tumour	401	318	49	29	5	79.30	12.22	7.23	1.25
	46 - central tumour	422	203	69	91	59	48.10	16.35	21.56	13.98
	46 - central tumour	373	180	65	84	44	48.26	17.43	22.52	11.80
	47 - lymph node	420	333	79	8	0	79.29	18.81	1.90	0.00
	47 - lymph node	389	299	79	10	1	76.86	20.31	2.57	0.26
13	52 - central tumour	423	367	47	8	1	86.76	11.11	1.89	0.24
	52 - central tumour	381	340	34	5	2	89.24	8.92	1.31	0.52
	53 - peripheral tumour	401	364	32	4	1	90.77	7.98	1.00	0.25
	53 - peripheral tumour	416	372	41	3	0	89.42	9.86	0.72	0.00
	54 - adjacent mucosa	405	389	11	5	0	96.05	2.72	1.23	0.00
	54 - adjacent mucosa	405	390	9	6	0	96.30	2.22	1.48	0.00
	55 - distant mucosa	418	402	9	7	0	96.17	2.15	1.67	0.00
	55 - distant mucosa	417	406	6	5	0	97.36	1.44	1.20	0.00
	56 - Polyp	415	378	21	16	0	91.08	5.06	3.86	0.00
	56 - Polyp	451	400	35	16	0	88.69	7.76	3.55	0.00
15	61 - Peripheral tumour	400	334	52	14	0	83.50	13.00	3.50	0.00
	61 - Peripheral tumour	447	367	65	15	0	82.10	14.54	3.36	0.00

Table A2 continues overleaf

Patient number	Sample no and tissue type	Total counted	Normal	1 copy	3 copies	4+ copies	% normal	% 1 copy	% 3 copies	% 4+ copies
15	62 - central tumour	406	347	55	4	0	85.47	13.55	0.99	0.00
	62 - central tumour	401	335	62	3	1	83.54	15.46	0.75	0.25
	63 - adjacent mucosa	401	359	26	15	1	89.53	6.48	3.74	0.25
	63 - adjacent mucosa	132	116	9	7	0	87.88	6.82	5.30	0.00
	63 - adjacent mucosa	286	260	18	8	0	90.91	6.29	2.80	0.00
	64 - Polyp	400	323	42	30	5	80.75	10.50	7.50	1.25
	64 - Polyp	476	372	59	39	6	78.15	12.39	8.19	1.26
	65 - Large polyp	407	326	42	35	4	80.10	10.32	8.60	0.98
	65 - Large polyp	415	333	49	29	4	80.24	11.81	6.99	0.96
	66 - distant mucosa	400	371	22	7	0	92.75	5.50	1.75	0.00
	66 - distant mucosa	422	399	18	5	0	94.55	4.27	1.18	0.00
16	67 - peripheral tumour	415	313	79	20	3	75.42	19.04	4.82	0.72
	67 - peripheral tumour	400	299	81	17	3	74.75	20.25	4.25	0.75
	68 - normal adjacent	401	369	24	8	0	92.02	5.99	2.00	0.00
	68 - normal adjacent	417	384	27	6	0	92.09	6.47	1.44	0.00
	69 - normal distant	401	388	9	4	0	96.76	2.24	1.00	0.00
	69 - normal distant	415	400	12	3	0	96.39	2.89	0.72	0.00
	70 - central tumour	426	325	78	19	4	76.29	18.31	4.46	0.94
	70 - central tumour	422	313	86	19	4	74.17	20.38	4.50	0.95
17	71 - distant mucosa	402	383	17	1	1	95.27	4.23	0.25	0.25
	71 - distant mucosa	407	387	15	5	0	95.09	3.69	1.23	0.00
	72 - Polyp	105	84	19	2	0	80.00	18.10	1.90	0.00
	72 - Polyp	134	105	29	0	0	78.36	21.64	0.00	0.00
	72 - Polyp	181	148	32	1	0	81.77	17.68	0.55	0.00

Table A2 continues overleaf

Patient number	Sample no and tissue type	Total counted	Normal	1 copy	3 copies	4+ copies	% normal	% 1 copy	% 3 copies	% 4+ copies
17	72 - Polyp	0	0	0	0	0	0.00	0.00	0.00	0.00
	72 - Polyp	121	95	25	1	0	78.51	20.66	0.83	0.00
	72 - Polyp	207	162	42	3	0	78.26	20.29	1.45	0.00
	72 - Polyp	128	98	29	0	1	76.56	22.66	0.00	0.78
	73 – adjacent mucosa	79	72	7	0	0	91.14	8.86	0.00	0.00
	73 – adjacent mucosa	138	129	7	2	0	93.48	5.07	1.45	0.00
	73 – adjacent mucosa	310	281	20	9	0	90.65	6.45	2.90	0.00
	73 – adjacent mucosa	193	173	18	2	0	89.64	9.33	1.04	0.00
	73 – adjacent mucosa	101	94	7	0	0	93.07	6.93	0.00	0.00
	74 - Peripheral tumour	404	329	57	17	1	81.44	14.11	4.21	0.25
	74 - Peripheral tumour	401	333	54	12	2	83.04	13.47	2.99	0.50
	75 - central tumour	258	197	24	26	11	76.36	9.30	10.08	4.26
	75 - central tumour	193	151	18	22	2	78.24	9.33	11.40	1.04
	75 - central tumour	442	371	23	40	8	83.94	5.20	9.05	1.81
	76 - Lymph node	0	0	0	0	0	0.00	0.00	0.00	0.00
	76 - Lymph node	255	54	31	61	109	21.18	12.16	23.92	42.75
	76 - Lymph node	317	99	36	70	112	31.23	11.36	22.08	35.33
	76 - Lymph node	276	75	31	60	110	27.17	11.23	21.74	39.86
20	85 - distant mucosa	260	226	25	8	1	86.92	9.62	3.08	0.38
	85 - distant mucosa	242	217	21	4	0	89.67	8.68	1.65	0.00
	85 - distant mucosa	302	287	8	6	1	95.03	2.65	1.99	0.33
	86 - adjacent mucosa	100	86	5	8	1	86.00	5.00	8.00	1.00
	86 - adjacent mucosa	169	151	7	11	0	89.35	4.14	6.51	0.00
	86 - adjacent mucosa	249	231	11	7	0	92.77	4.42	2.81	0.00

Table A2 continues overleaf

Patient number	Sample no and tissue type	Total counted	Normal	1 copy	3 copies	4+ copies	% normal	% 1 copy	% 3 copies	% 4+ copies
20	86 - adjacent mucosa	105	98	4	2	1	93.33	3.81	1.90	0.95
	86 - adjacent mucosa	205	187	9	8	1	91.22	4.39	3.90	0.49
	87 - peripheral tumour	152	127	15	10	0	83.55	9.87	6.58	0.00
	87 - peripheral tumour	76	62	8	6	0	81.58	10.53	7.89	0.00
	87 - peripheral tumour	172	148	11	13	0	86.05	6.40	7.56	0.00
	87 - peripheral tumour	412	337	33	38	4	81.80	8.01	9.22	0.97
	87 - peripheral tumour	0	0	0	0	0	0.00	0.00	0.00	0.00
	88 - central tumour	479	362	112	5	0	75.57	23.38	1.04	0.00
	88 - central tumour	400	293	99	6	2	73.25	24.75	1.50	0.50
	89 - Far polyp	245	208	34	3	0	84.90	13.88	1.22	0.00
	89 - Far polyp	163	132	27	4	0	80.98	16.56	2.45	0.00
	89 - Far polyp	402	350	40	12	0	87.06	9.95	2.99	0.00

Table A2

This table contains the fluorescent *in situ* hybridisation data for the chromosome 6 probe. These results are analysed in Chapter 6.

Patient number	Sample no and tissue type	Total counted	Normal	1 copy	3 copies	4+ copies	% normal	% 1 copy	% 3 copies	% 4+ copies
4	12 - Tumour	214	146	18	42	8	68.22	8.41	19.63	3.74
	12 - Tumour	327	223	24	71	9	68.20	7.34	21.71	2.75
	12 - Tumour	117	81	9	24	3	69.23	7.69	20.51	2.56
	12 - Tumour	146	99	11	30	6	67.81	7.53	20.55	4.11
	13 - adjacent mucosa	401	374	20	7	0	93.27	4.99	1.75	0.00
	13 - adjacent mucosa	400	379	13	8	0	94.75	3.25	2.00	0.00
	14 - distant mucosa	291	271	16	4	0	93.13	5.50	1.37	0.00
	14 - distant mucosa	240	222	13	5	0	92.50	5.42	2.08	0.00
	14 - distant mucosa	396	372	18	6	0	93.94	4.55	1.52	0.00
	15 - Polyp	426	264	122	38	2	61.97	28.64	8.92	0.47
	15 - Polyp	171	112	44	14	1	65.50	25.73	8.19	0.58
	15 - Polyp	290	184	76	27	3	63.45	26.21	9.31	1.03
5	16 - Tumour	404	235	34	124	11	58.17	8.42	30.69	2.72
	16 - Tumour	405	233	29	132	11	57.53	7.16	32.59	2.72
	17 - adjacent mucosa	409	361	34	13	1	88.26	8.31	3.18	0.24
	17 - adjacent mucosa	428	375	38	14	1	87.62	8.88	3.27	0.23
	18 - distant mucosa	408	356	36	16	0	87.25	8.82	3.92	0.00
	18 - distant mucosa	400	347	36	16	1	86.75	9.00	4.00	0.25
	19 - polyp	404	253	137	11	3	62.62	33.91	2.72	0.74
	19 - polyp	405	261	133	9	2	64.44	32.84	2.22	0.49

Table A3 continues overleaf

Patient number	Sample no and tissue type	Total counted	Normal	1 copy	3 copies	4+ copies	% normal	% 1 copy	% 3 copies	% 4+ copies
8	28 - central tumour	404	345	56	2	1	85.40	13.86	0.50	0.25
	28 - central tumour	400	340	58	1	1	85.00	14.50	0.25	0.25
	29 - Peripheral tumour	400	149	24	211	16	37.25	6.00	52.75	4.00
	29 - Peripheral tumour	400	159	27	200	14	39.75	6.75	50.00	3.50
	30 - Small polyp	402	320	75	7	0	79.60	18.66	1.74	0.00
	30 - Small polyp	401	331	61	8	1	82.54	15.21	2.00	0.25
	31 - large polyp	409	340	62	6	1	83.13	15.16	1.47	0.24
	31 - large polyp	400	336	56	4	4	84.00	14.00	1.00	1.00
	32 - distant mucosa	418	376	28	11	3	89.95	6.70	2.63	0.72
	32 - distant mucosa	406	369	27	9	1	90.89	6.65	2.22	0.25
	33 - adjacent mucosa	399	358	28	12	1	89.72	7.02	3.01	0.25
	33 - adjacent mucosa	237	211	17	8	1	89.03	7.17	3.38	0.42
	33 - adjacent mucosa	204	182	14	8	0	89.22	6.86	3.92	0.00
10	38 - Peripheral tumour	400	294	56	46	4	73.50	14.00	11.50	1.00
	38 - Peripheral tumour	405	296	52	52	5	73.09	12.84	12.84	1.23
	39 - central tumour	408	300	61	36	11	73.53	14.95	8.82	2.70
	39 - central tumour	400	288	59	43	10	72.00	14.75	10.75	2.50
	40 - distant mucosa	400	379	14	7	0	94.75	3.50	1.75	0.00
	40 - distant mucosa	413	388	16	8	1	93.95	3.87	1.94	0.24
	41 - Normal distant	408	381	16	10	1	93.38	3.92	2.45	0.25
	41 - Normal distant	404	377	18	8	1	93.32	4.46	1.98	0.25
	42 - Polyp	400	251	45	79	25	62.75	11.25	19.75	6.25
	42 - Polyp	401	248	49	84	20	61.85	12.22	20.95	4.99

Table A3 continues overleaf

Patient number	Sample no and tissue type	Total counted	Normal	1 copy	3 copies	4+ copies	% normal	% 1 copy	% 3 copies	% 4+ copies
20	85 - distant mucosa	411	370	28	11	2	90.02	6.81	2.68	0.49
	85 - distant mucosa	400	357	30	10	3	89.25	7.50	2.50	0.75
	86 - adjacent mucosa	404	356	21	22	5	88.12	5.20	5.45	1.24
	86 - adjacent mucosa	316	278	19	16	3	87.97	6.01	5.06	0.95
	86 - adjacent mucosa	223	198	12	11	2	88.79	5.38	4.93	0.90
	87 - peripheral tumour	205	166	20	18	1	80.98	9.76	8.78	0.49
	87 - peripheral tumour	298	242	26	28	2	81.21	8.72	9.40	0.67
	87 - peripheral tumour	402	333	34	32	3	82.84	8.46	7.96	0.75
	88 - central tumour	402	288	56	48	10	71.64	13.93	11.94	2.49
	88 - central tumour	409	285	59	54	11	69.68	14.43	13.20	2.69
	89 - Far polyp	255	207	39	6	3	81.18	15.29	2.35	1.18
	89 - Far polyp	301	246	47	6	2	81.73	15.61	1.99	0.66
	89 - Far polyp	402	332	58	7	5	82.59	14.43	1.74	1.24

Table A3

This table contains the fluorescent in situ hybridisation data for the p21 region probe. These results are further discussed in Chapter 6

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