An Investigation of Genetic Alterations in Gastric and Colorectal Cancer

by

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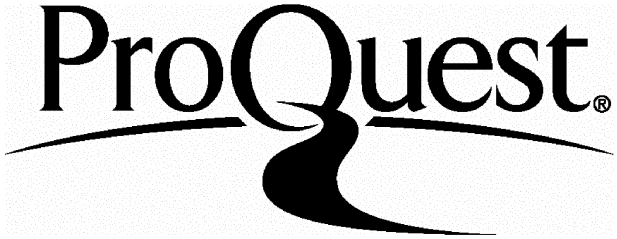

To my parents and my brother

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Abstract

The widely accepted model of colorectal tumourigenesis involves an accumulation of mutations in tumour suppressor genes and oncogenes which include the *APC* (Adenomatous Polyposis Coli) gene, the *p53* gene, a gene on chromosome 18q, and the K-*ras* gene. In addition, replication error (RER) at short repeat sequences is observed in a subset of colorectal tumours and has been associated with a defect in the DNA mismatch repair system. In contrast, the genetic alterations that are most significant in the development and progression of gastric (stomach) cancer are unknown.

In this study, twenty-six gastric carcinoma and corresponding normal tissues were investigated for alterations of the *APC*, *MCC* (Mutated in Colorectal Cancer), *DCC* (Deleted in Colon Carcinoma), *p53*, and *hMSH2* (human *MutS* homologue 2) genes, and for replication error (RER) and loss of heterozygosity (LOH) at twelve microsatellite repeat loci. Some of the genetic alterations were viewed in comparison with colorectal cancer. Forty-three colorectal carcinoma and corresponding normal tissues were investigated for alterations of the *APC* and *hMSH2* genes, and for RER and LOH at at twelve microsatellite repeat loci.

Somatic mutations of the *APC* gene were observed infrequently in gastric carcinomas (4% of tumours). In addition, infrequent LOH was observed at the *APC*, *MCC*, and *DCC* loci, in 8%, 10%, and 5% informative cases respectively. Alterations of the *p53* gene were more frequent: somatic *p53* mutations were detected in 31% of gastric carcinomas while LOH at the *p53* locus was observed in 37.5% of informative cases. RER was detected in 11.5% of gastric carcinomas, at one or more microsatellite repeat loci. Of the 12 microsatellite repeat loci, LOH was most frequently observed in gastric carcinomas at *D22S351* (30% informative cases) suggesting that a tumour suppressor gene on chromosome 22q may be important in gastric tumourigenesis. During mutational analysis of the *hMSH2* gene, an intronic 4 base pair insertion at 31 base pairs upstream of the beginning of exon 13 was detected in both tumour and normal tissue from one gastric carcinoma case. Additionally, a T to C transition polymorphism was detected at the -6 position of the splice acceptor site of *hMSH2* exon 13, and characterized in the 26 gastric carcinoma cases, 43 colorectal carcinoma cases, and 50 unaffected unrelated individuals.

Somatic mutations of the *APC* gene were observed in 37.2% of colorectal carcinomas. In 3 tumours, two independent somatic *APC* mutations were observed. LOH at *APC* was observed in 24.3% of informative colorectal tumours. RER was detected in 14% of colorectal carcinomas, at one or more loci. Of the microsatellite repeat loci, LOH was most frequently observed in colorectal carcinomas at *p53CA* (58.1% informative cases) and at *D18S61* (33.3% informative cases).

This study confirms the importance of alterations of the *APC* gene and RER in colorectal tumourigenesis. The results are not supportive of a common molecular pathogenesis for colorectal and gastric cancer, not even for the intestinal histological type of gastric cancer. Tumour suppressor genes other than those investigated in the present study must play a more important role in gastric tumourigenesis.

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Abbreviations

A	adenine residue.
ACF	aberrant crypt foci.
AMPS	ammonium persulphate.
APC	Adenomatous Polyposis Coli.
bp	base pair.
C	cytosine residue.
cDNA	complementary deoxyribonucleic acid.
cM	centimorgan.
CMC	chemical mismatch cleavage.
dATP	2'-deoxyadenosine 5'-triphosphate.
DCC	Deleted in Colon Cancer.
dCTP	2'-deoxycytidine 5'-triphosphate.
DEPC	diethyl pyrocarbonate.
DGGE	denaturing gradient gel electrophoresis.
dGTP	2'-deoxyguanosine 5'-triphosphate.
DNA	deoxyribonucleic acid.
dNTP	2'-deoxynucleoside 5'-triphosphate.
dTTP	2'-deoxythymidine 5'-triphosphate.
EDTA	ethylenediaminetetraacetic acid.
FAP	Familial adenomatous polyposis.
g	grams.
G	guanine residue.
GTBP	G/T mismatch-binding protein.
hMLH1	human *mutL* homologue 1.
hMSH2	human *mutS* homologue 2.
hMSH3	human *mutS* homologue 3.
hMSH6	human *mutS* homologue 6.
HNPCC	Hereditary nonpolyposis colorectal cancer.
hPMS1	human post-meiotic segregation 1.
hPMS2	human post-meiotic segregation 2.
H. pylori	*Helicobacter pylori.*
IVSP	in vitro synthesized protein.
kb	kilobase pair.
kDa	kilodalton.
l	litre.

LOH	loss of heterozygosity.
M	molar.
Mb	megabase pair.
MCC	Mutated in Colorectal Cancer.
MCR	mutation cluster region.
µg	microgram.
µl	microlitre.
mg	milligram.
ml	millilitre.
mM	millimolar.
MMR	mismatch repair.
ng	nanogram.
PAGE	polyacrylamide gel electrophoresis.
PCR	polymerase chain reaction.
pmol	picomoles.
PTT	protein truncation test.
RER	replication error.
RFLP	restriction fragment length polymorphism.
RNA	ribonucleic acid.
RNase	ribonuclease.
SDS	sodium dodecyl sulphate.
SSCP	single-strand conformation polymorphism.
T	thymine residue.
TGF-β	transforming growth factor-β.
TPR	translocated promoter region.
TEMED	NNN'N' tetramethylethylenediamine.
U	units.
VNTR	variable number of tandem repeats.
v/v	volume for volume.
w/v	weight for volume.

Chapter 1

Introduction

1. INTRODUCTION

1.1. Pathogenesis of gastric cancer

This thesis has mainly involved the investigation of genetic alterations in gastric cancer viewed in comparison with colorectal cancer. Before the epidemiology of gastric cancer can be considered it is necessary to define the classification systems used for the various histological types.

1.1.1. Classification

The majority of gastric tumours are adenocarcinomas, the remainder being mainly lymphomas and leiomyosarcomas (Watanabe *et al.*, 1990). Numerous classifications have been proposed for gastric carcinoma because of the wide variation in its morphological features. The systems most commonly used are those of Lauren (Lauren, 1965), the Japanese Research Society for Gastric Cancer (1981), the World Health Organisation (WHO) (Watanabe *et al.*, 1990), and Ming (Ming, 1977).

The Lauren classification has wide application particularly in Western countries because it describes two main histopathological types, intestinal and diffuse, that differ in their pathogenesis as will be discussed in further sections. The intestinal type is characterized by cohesive malignant cells that form glandular structures similar to those found in the intestine. The neoplasm typically forms a distinct margin of spread. Diffuse gastric carcinoma on the other hand is characterized by poorly cohesive cells that infiltrate the stomach wall without forming well defined structures. The tumour is poorly demarcated. A minority of gastric carcinomas cannot be classified as one of the two main types. These are known as mixed type or atypical carcinomas and include tumours with both intestinal and diffuse characteristics and others with features of neither.

In the system proposed by the Japanese Research Society for Gastric Cancer, carcinomas are classified as differentiated type (tubular and papillary carcinomas) or as undifferentiated type (mucinous, signet-ring cell, and poorly differentiated carcinomas). These two types are considered to be similar to the intestinal and diffuse subtypes in Lauren's classification. Tumours are also staged by division into early and advanced stages. Early gastric carcinoma is defined as a carcinoma confined to the mucosa or to the submucosa, regardless of the presence or absence of

lymph node metastasis. Advanced carcinoma is defined by invasion into or through the muscularis propria.

The WHO classification is based on standard histological criteria that are employed for the majority of tumours. Gastric adenocarcinomas are typed as papillary, tubular, mucinous, or signet-ring cell types. Tumours are also graded as low grade (well or moderately differentiated carcinomas) or high grade (poorly differentiated carcinomas). Well differentiated carcinomas have a regular glandular structure that often resembles metaplastic intestinal epithelium, while poorly differentiated carcinomas are composed of irregular glands or cell clusters or of single cells, and moderately differentiated carcinomas are intermediate between the two. As gastric adenocarcinomas can be strikingly heterogeneous so that tumours fall into more than one category, the WHO recommend the typing be based on the predominant feature with minor components referred to in the description.

Ming's classification describes two types of gastric carcinoma: expanding and infiltrative. These categories roughly correspond to the intestinal and diffuse subtypes but the advantage of this system over Laurens' is that most if not all cases can be classified as one of the two types.

1.1.2. Anatomy of the stomach

Anatomic subdivision of the stomach is depicted in figure 1.1. It has been observed that intestinal type carcinomas predominantly occur in the distal stomach (antrum and pylorus) while diffuse carcinomas are most often located in the proximal stomach (cardia) (Lehtola, 1978).

1.1.3. Epidemiology

Gastric cancer is estimated to be the second most common cancer in the world, after lung cancer (Parkin *et al.*, 1993). In the U.K. it was the sixth most frequent cancer in men and the seventh in women in 1990 (Cancer Research Campaign Factsheet 24, 1995). Internationally gastric cancer is more prevalent in males than in females. Furthermore, the intestinal histological type occurs more frequently in men and in older age groups whereas the diffuse type is represented equally in both sexes and occurs more often at younger ages (Howson *et al.*, 1986).

The incidence and mortality of gastric cancer vary markedly both between and within countries which suggests that as with most other malignancies environmental

Figure 1.1.

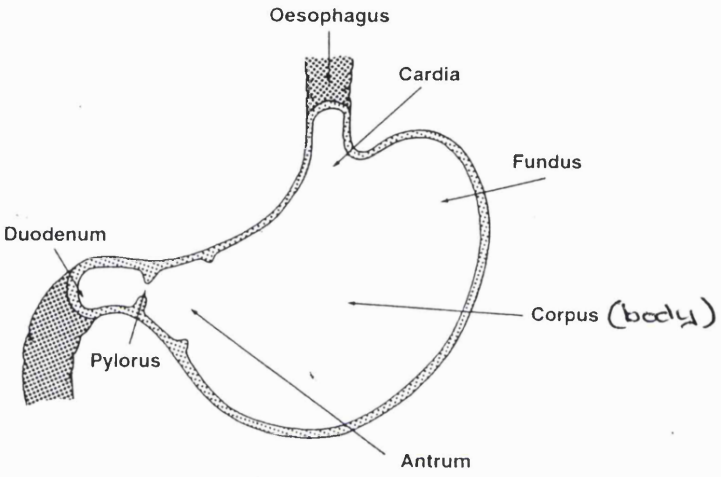


Figure 1.1. Subdivision of the stomach (taken from Cancer Research Campaign Factsheet 24, 1995)

factors are important in gastric carcinogenesis. Risk factors will be discussed in a later section. Incidence rates are highest in Japan where gastric cancer is the most common cancer in both sexes, and high in China, South America, Eastern Europe, Portugal, and Italy (Parkin *et al.*, 1992). In England and Wales the frequency of gastric cancer is high in North Wales in particular, and in Staffordshire and Northern England (Swerdlow and dos Santos Silva, 1993). Analysis of rates by histologic type has revealed that the intestinal form predominates in high risk regions of gastric cancer while the diffuse form is equally prevalent in both high and low risk regions (Munoz *et al.*, 1968; Correa *et al.*, 1973). Studies of migrants from high to low risk areas additionally indicate that environmental exposures are significant early in life. For example Japanese immigrants in Hawaii (Haenszel *et al.*, 1972) and European immigrants in the United States (Haenszel, 1961) display a risk that is more similar to that of their parent nation, but in the offspring rates are displaced towards that of the host nation.

The incidence of gastric cancer has declined throughout the world during this century, at a different time and rate in different countries (Howson *et al.*, 1986), further evidence for an environmental aetiology. The decline has been largely attributed to a decrease in the incidence of intestinal type carcinomas (Munoz and Asvall, 1971; Craanen *et al.*, 1992). In recent years however, the frequency of adenocarcinoma of the cardia (proximal stomach) has risen, and has been accompanied by a decrease in distal gastric tumours (Kampschoer *et al.*, 1989; Powell and McConkey, 1990; Blot *et al.*, 1991). As intestinal type gastric carcinomas are most often distally located the above observation supports evidence of their decline. In addition the incidence of adenocarcinoma of the lower oesophagus has increased. Evidence indicates that these cancers share risk factors with cardia carcinomas that are different from those for distal carcinomas (Powell and McConkey, 1990; Blot *et al.*, 1991).

Epidemiological studies have led investigators to suggest that intestinal and diffuse adenocarcinomas do not have a common pathogenesis. The geographic variation and decreasing frequency of the intestinal type indicates that it is more influenced by environmental factors whereas the diffuse type which has a similar frequency in most populations and is more common in younger people appears to be more closely associated with other as yet unrecognised factors (Howson *et al.*, 1986).

1.1.4. Precursor lesions

A series of precursor lesions have been identified for the intestinal form of gastric carcinoma but diffuse gastric carcinomas lack well defined precancerous changes. The precursors that are most prevalent in patients who develop intestinal type gastric carcinoma are chronic atrophic gastritis and intestinal metaplasia (Munoz and Asvall, 1971; Matsukara *et al.*, 1980; Sipponen *et al.*, 1983). Chronic atrophic gastritis is characterized by a loss of gastric glands. Intestinal metaplasia represents a more advanced degree of chronic atrophic gastritis and is characterized by gradual replacement of the atrophic gastric epithelium by intestinal type epithelium (Correa, 1982). Pathological studies have demonstrated that almost all cases of intestinal type carcinoma, even minute carcinomas of less than 5mm in diameter are surrounded by intestinal metaplasia (Lauren, 1965; Matsukara *et al.*, 1980). The prevalence of intestinal metaplasia in diffuse carcinomas is similar to that in the general population (Correa *et al.*, 1970). Epidemiological studies have also shown that like intestinal type gastric cancer both chronic atrophic gastritis and intestinal metaplasia are more prevalent in regions with a high incidence of gastric cancer than in low incidence regions (Correa *et al.*, 1970; Imai *et al.*, 1971). These observations suggested that intestinal metaplasia and chronic atrophic gastritis are precursors of intestinal and not diffuse gastric cancer. Evidence from pathological and epidemiological studies in populations at high gastric cancer risk have led Correa and co-workers to propose that intestinal type gastric carcinoma is preceded by a prolonged multi-step process that is the result of a series of mutations (Correa, 1988; Correa *et al.*, 1990a) as shown in figure 1.2.

The earliest lesion in the proposed developmental pathway, superficial gastritis, is a mild inflammation which may progress to chronic atrophic gastritis. This lesion usually begins as multiple foci in the distal stomach which eventually coalesce to spread to other areas of the gastric mucosa leading to atrophy of the normal gastric mucosal epithelium. A state of reduced acid production results due to the loss of acid-secreting parietal cells (Correa, 1982). However cell kinetic studies indicate that an enhanced rate of cell renewal as well as high epithelial cell loss is associated with atrophic gastritis (Hart-Hansen et al., 1979). Atrophic gastritis is also associated with a shift of the proliferative zone to the luminal surface from its normal location at the neck of the gastric glands, and it is thought that the hyperproliferative state leads to failure of normal cell maturation and the appearance of undifferentiated cells in the mucosal surface (Deschner *et al.*, 1972; Hart-Hansen *et al.*, 1979).

Figure 1.2.

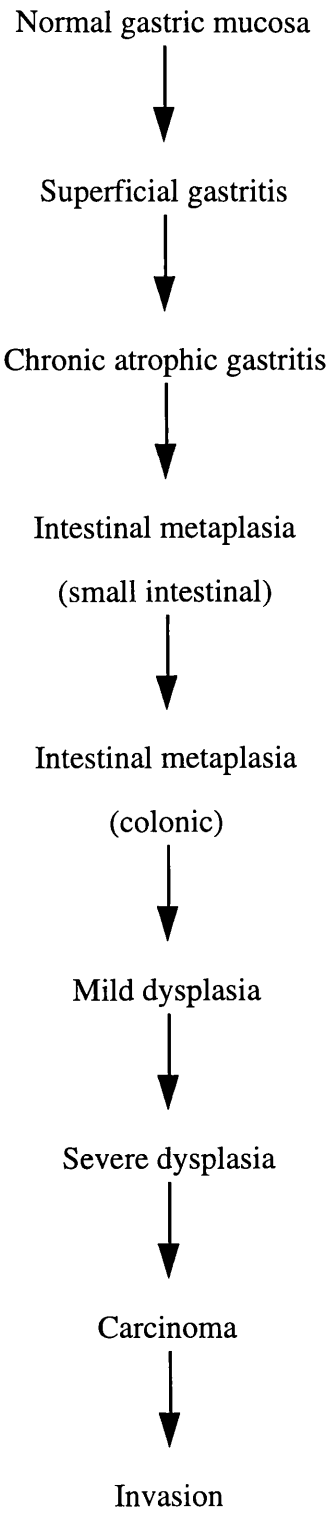


Figure 1.2. Hypothetical pathway of development of intestinal type gastric carcinoma (adapted from Correa, 1988).

Correa (1988) proposed that these gastric cells are replaced by cells resembing those in the small intestine - small intestinal metaplasia, also known as type I metaplasia or complete metaplasia as the battery of digestive enzymes normally found in the small intestine are present (Stemmerman, 1994). Correa suggested that a gradual transition to metaplastic cells resembling those in the large intestine occurs, which appear less mature and do not show the complete set of aforementioned enzymes. This metaplasia is termed colonic metaplasia or incomplete metaplasia or type III sulphomucin expressing metaplasia (Stemmerman, 1994). It is this type of metaplasia that has been found to frequently accompany small intestinal type gastric carcinomas (Matsukura *et al.*, 1980; Iida and Kasuma, 1982). Correa next suggested that the metaplastic cells can progress to show cellular abnormalities including changes in the size and shape of the cell and nucleus, in the nucleus:cytopasm ratio, and in mitotic activity as well as a distortion of the glandular architecture (dysplasia) (Correa, 1982; 1988). Dysplasias are believed to carry an increased risk of transformation into invasive carcinomas; progression of severe (adenomatous) dysplasia to carcinoma has been reported in up to 80% of gastric cancer patients (Antonioli, 1994). Dysplasia arising in nonmetaplastic glands has been tentatively linked with diffuse type gastric cancer but is more closely associated with the intestinal type (Ming *et al.*, 1984).

Other putative precursors of gastric cancer include pernicious anaemia, a megaloblastic anamia caused by vitamin B-12 deficiency, which is characterized by an immune-mediated atrophic gastritis of the corpus (body) and fundus of the stomach. Patients with pernicious anaemia have a two- to threefold increase in risk for gastric cancer (Brinton *et al.*, 1989; Hsing *et al.*, 1993). Although an association between gastric ulcer and gastric cancer was alleged in the earlier part of this century, follow-up studies demonstrate that malignant transformation of a benign ulcer rarely occurs (none to less than 1% of patients (Lee *et al.*, 1990; Antonioli, 1994). In contrast partial gastrectomy involving resection of the distal stomach, particularly the Billroth II procedure, used for example in the treatment of benign disorders such as peptic ulcer disease, is associated with an increased risk of gastric cancer (Stalsberg and Taksdal, 1971; Caygill *et al.*, 1986). It has been suggested that surgically induced achlorhydria (acid suppression) and damage to the gastric mucosa from bile reflux following the operation may play a role in gastric carcinogenesis (Caygill *et al.*, 1986).

Gastric polyps may be classified into three groups: fundic gland polyps, hyperplastic polyps, and adenomas. Fundic gland polyps are not considered to be premalignant (Marcial *et al.*, 1993), hyperplastic polyps rarely undergo neoplastic

transformation (Daibo *et al.*, 1987), while adenomas have the highest risk of gastric cancer, their malignant potential appearing to be directly related to their size and the degree of dysplasia (Kamiya *et al.*, 1982). Menetrier's disease, characterized by enlarged gastric folds, has also been linked to gastric cancer (Wood *et al.*, 1983) but the rarity of this disease has made it difficult to determine the strength of the association. Finally, the rise in the incidence of adenocarcinoma of the gastric cardia and lower oesophagus is associated with Barrett's oesophagus, a condition in which the squamous epithelium lining the oesophagus becomes replaced with metaplastic epithelium, resulting from gastrooesophageal reflux (Kalish *et al.*, 1984).

1.1.5. Aetiology

Due to the function of the stomach, diet has been extensively investigated as an aetiological factor in gastric cancer. Foods preserved by salting, smoking, or pickling, such as salted or smoked fish and meats and pickled vegetables, are the foods that have most frequently been associated with an increased risk of gastric cancer (Haenszel *et al.*, 1972; Correa *et al.*, 1983; You *et al.*, 1988; Buatti *et al.*, 1989; Ramon *et al.*, 1993). It is believed that the consumption of these preserved products may account for the elevated rates of gastric cancer observed in countries such as Japan and other countries in the Far East, Portugal, Italy, and Columbia. Time trend analyses in Japan have demonstrated that the recent decline in deaths from gastric cancer has been accompanied by a parallel decline in per capita consumption of salted and dried fish (Howson *et al.*, 1986). Excessive dietary salt has been shown to induce gastric atrophy in mice (Kodama *et al.*, 1984) and cause excessive cell replication in rat mucosa, an event known to increase cancer risk because of potentiation of the action of carcinogens and the possibility of increased rate of endogenous mutations (Charnley and Tannenbaum, 1985; Ames and Gold, 1990). Correa (1988) postulated that a high salt diet causes progression to chronic atrophic gastritis in his developmental model of intestinal type gastric carcinoma.

The high risk of gastric cancer associated with preserved foods may also be due to nitrates and nitrites, which have traditionally been used to cure meat, fish, and vegetables. It is thought that potentially carcinogenic N-nitroso compounds, nitrosamides, are produced in the stomach from nitrite and amides derived from food in an acid-catalyzed reaction (Mirvish, 1983). Chronic atrophic gastritis, because of the loss of acid-secreting parietal cells, leads to a higher gastric pH and proliferation of anaerobic bacteria which reduce dietary nitrate to nitrite. The nitrite reacts with other nitrogen-containing compounds to form carcinogenic N-nitroso compounds (Correa, 1992). Correa proposed that these carcinogenic compounds play a role in

the progression from chronic atrophic gastritis to intestinal metaplasia in his model of gastric carcinogenesis (Correa, 1988).

A number of case-control studies have reported a positive association of gastric cancer risk with dietary protein intake, particularly from animal sources, which could provide secondary and tertiary amines for gastric nitrosation (Jedrychowski *et al.*, 1986; Buiatti *et al.*, 1990). Furthermore, several foods have been found to contain potential mutagen precursors (nitrosoindoles) which are converted to mutagens after nitrite treatment. These foods include fava beans (Yang *et al.*, 1984) which have been associate with the increased gastric cancer risk in Columbia (Correa *et al.*, 1983), soy sauce (Wakabayashi *et al.*, 1983) and chinese cabbage (Wakabayashi *et al.*, 1985) which are both commonly eaten in Japan. However despite the mutagenicity of N-nitroso compounds epidemiologic data on gastric cancer risk and dietary nitrate and nitrite, from drinking water and vegetable sources as well as preserved foods, have been inconsistent (Palli, 1994).

A high consumption of fresh fruit, especially citrus fruit, and vegetables has been consistently reported to be a strong protective factor against gastric cancer (Haenszel *et al.*, 1972; Buatti *et al.*, 1989; Hansson *et al.*, 1993; Ramon *et al.*, 1993) and also chronic atrophic gastritis (Fontham *et al.*, 1986). The mechanism of protection is unclear although their high content of micronutrients such as vitamin C, vitamin E, and β-carotene is thought to play a role. Indeed these micronutrients have consistently been associated with a decreased risk of gastric cancer (Hansson *et al.*, 1994; Buiatti *et al.*, 1990; Hirohata and Kono, 1997). Vitamins C and E have been shown to inhibit the formation of N-nitroso compounds from nitrite in the stomachs of experimental animals (Mirvish, 1983), and like β-carotene may be protective because of their anti-oxidative effect (Krinsky and Deneke, 1982; Bieri *et al.*, 1983). Low blood levels of β-carotene have been found in patients with gastric dysplasia but not in patients with less severe lesions of the gastric mucosa (Haenszel *et al.*, 1985) leading Correa (1988) to suggest that β-carotene may have a protective effect in the late stages of gastric carcinogenesis.

With respect to an association of gastric cancer risk with starchy food, case-control studies have yielded conflicting results although a positive association has often been reported with whole grain cereals which may physically irritate the stomach and hence promote gastric cancer (Palli, 1984). Gastric cancer risk is positively associated with the age at which food began to be stored in a refrigerator (Buiatti *et al.*, 1989; Coggon *et al.*, 1989; Hansson *et al.*, 1993). Refrigeration has increased the availability of fruits and vegetables, reduced the need for salting or

similar methods of food preservation, and may prevent contamination of food by bacteria and fungi capable of activating various procarcinogens thus it has been proposed their widespread use may account in part for the decline in the incidence of gastric cancer during this century (Coggon *et al.*, 1989). Despite the fact that intestinal type gastric cancer is considered to be more related to environmental factors than the diffuse type (Howson *et al.*, 1986) no difference has been reported between the two histological types in dietary risk factors (Buiatti *et al.*, 1991; Boeing *et al.*, 1991).

A role for tobacco and alcohol in gastric cancer has been studied. Gonzalez and Agudo (1994) reviewed 37 epidemiological studies investigating an association between tobacco smoking and alcohol consumption and gastric cancer and found them to give inconsistent and conflicting results. They concluded that overall the studies suggest that tobacco smoking and alcohol are not causally related to gastric cancer but a weak association could not be ruled out and that these factors may play a role in tumour of the gastric cardia, the incidence of which is rising as previously described.

Infection with the bacterium *Helicobacter pylori* (*H. pylori*) is now regarded as an important risk factor for gastric cancer. *H. pylori* is a common infection usually acquired early in childhood. In developing countries up to 90% of the population are affected while in developed countries the prevalence is lower (approximately 50% of the population) (Munoz and Pisani, 1994). Prospective epidemiological studies using serological techniques have reported that individuals with *H. pylori* infection have a three- to sixfold higher risk of gastric cancer than those without infection (Nomura *et al.*, 1991; Parsonnet *et al.*, 1991; Forman *et al.*, 1991). A number of studies have shown a strong geographic correlation between the incidence of gastric cancer and the prevalence of *H. pylori* infection (Forman *et al.*, 1990; Correa *et al.*, 1990b); The Eurogast Study Group, 1993). However it should be noted that the rate of gastric cancer is low in some regions of the world where *H. pylori* infection is highly prevalent, for example in some African countries where it is found in 50-85% of the population (Munoz and Pisani, 1994). The incidence of *H. pylori* infection is decreasing in developed countries (Parsonnet, 1996) as is the incidence of gastric cancer which may suggest further evidence that the two disorders are related. In addition, *H. pylori* infection has been associated with low socio-economic status, poor housing and overcrowding (Mendall *et al.*, 1992) which are also risk factors positively associated with gastric cancer (Wynder *et al.*, 1963; Barker *et al.*, 1990).

It is now accepted that *H. pylori* infection is the main cause of a type of non-atrophic gastritis known as superficial or chronic gastritis (Appelman, 1994). Correa has proposed that *H. pylori* infection may play a role in the earliest stage of his developmental pathway for intestinal type gastric cancer (Correa, 1992). In a follow-up study of patients with *H. pylori* infection, progression of this superficial gastritis to chronic atrophic gastritis, a precursor of intestinal type gastric carcinoma, has been reported (O'Connor *et al.*, 1996). However while some studies report that *H. pylori* infection is more prevalent in patients with the intestinal type gastric carcinoma than in those with diffuse type carcinoma (Tatsuta *et al.*, 1995; Endo *et al.*, 1995) others report no difference between the two histological types (Wee *et al.*, 1992; Sipponen *et al.*, 1992). Nevertheless some studies have reported that *H. pylori* infection is less frequent in patients with cancer in the gastric cardia than in the distal stomach (Talley *et al.*, 1991; Nomura *et al.*, 1991) which is where intestinal type carcinomas are most often located (Lehtola, 1978).

Mechanisms by which *H. pylori* increases the risk of gastric cancer are being investigated: *H. pylori* infection has been found to increase the rate of proliferation of gastric epithelial cells (Lynch *et al.*, 1995), decrease gastric secretion of ascorbic acid (Banerjee *et al.*, 1994), and result in infiltration of white blood cells in the gastric mucosa which synthesize oxygen radicals with mutagenic potential (Drake *et al.*, 1995). As gastric carcinoma develops in only a small proportion of individuals infected with *H. pylori* this suggests that cofactors, environmental or genetic, are required. Finally it is interesting to note that a type of gastric lymphoma, low-grade gastric mucosa-associated lymphoid tissue (MALT) lymphoma has also been linked to infection with *H. pylori* (Wotherspoon *et al.*, 1993). Antibiotic therapy has been used to eradicate *H. pylori* infection: in some patients with low-grade primary gastric MALT lymphoma and *H. pylori* infection, complete regression of the lymphoma after eradication of the *H. pylori* infection with antibiotic treatment has been observed (Wotherspoon *et al.*, 1993; Bayerdorffer *et al.*, 1995). Perhaps the use of antibiotics has contributed to the decline in the incidence of gastric carcinoma through incidental eradication of *H. pylori* (Simini, 1996).

1.1.6. Diagnosis and Treatment

At the early stage of disease, gastric carcinoma typically produces no symptoms thus the majority of tumours are not detected until they are metastatic. Symptoms of advanced disease include vomiting, abdominal pain, and weight loss (Fuchs and Mayer, 1995; Cancer Research Campaign Factsheet 24, 1995). Surgery is the main form of treatment for gastric cancer, however in the U. K. late diagnosis

means that the majority (80%) of patients present too late for curative resection (Ellis and Cunningham, 1994). Prognosis is poor with approximately 10% of patients alive 5 years after diagnosis (Cancer Research Campaign Factsheet 24, 1995). Tumours located in the proximal stomach have a worse prognosis than those in the distal stomach. This could be due to the technical difficulty of resecting proximal tumours or because proximal tumours are more often of the more aggressive diffuse histological type (Cancer Research Campaign Factsheet 24, 1995; Fuchs and Mayer, 1995). Diffuse gastric carcinomas have been reported to have a poorer prognosis than intestinal type tumours (Lauren, 1965; Craanen *et al.*, 1992). In Japan, because of the high incidence of gastric cancer, annual screening by radiograph or endoscopy has been recommended for persons over 50 years of age. The Japanese also use a more aggressive surgical procedure involving extended resection of lymph nodes, thus Japan has a higher proportion of patients with early stage disease and much better survival rates than in the West (Maruyama *et al.*, 1987).

1.1.7. Heredity and gastric cancer

Less is known about the role of hereditary factors in the pathogenesis of gastric cancer than about environmental factors. Nevertheless several lines of evidence support a contribution of genetic factors. Several families with an accumulation of gastric cancers have been described in the literature although the mode of inheritance has not been established (Macklin, 1960; Woolf and Isaacson, 1961). Hereditary Nonpolyposis Colorectal Cancer (HNPCC) is an autosomal dominant disorder characterized by early onset of colorectal cancer, the occurrence of multiple tumours, and high frequencies of extra-colonic malignancies (Lynch *et al.*, 1993). Gastric cancer has been shown to be part of the HNPCC tumour spectrum and there is evidence that it is the second most common extra-colonic neoplasm associated with HNPCC (Vasen *et al.*, 1990; Watson and Lynch, 1993). An increased risk of gastric cancer is also associated with the cancer predisposition syndromes characterized by intestinal polyposis including Familial Adenomatous Polyposis (Jagelman *et al.*, 1988) and Peutz-Jeghers syndrome (Giardiello *et al.*, 1987).

Case-control studies have indicated that first degree relatives of patients with gastric cancer have a two- to threefold increase in risk of developing the disease (Zanghieri *et al.*, 1990; La Vecchia *et al.*, 1992; Nagase *et al.*, 1996). Moreover, it has been shown that the majority of patients with this familial occurrence of gastric cancer have the diffuse histological type of this tumour (Lehtola, 1978; Mecklin *et al.*, 1988; Zanghieri *et al.*, 1990). Also, the elevated risk appears to be highest in first degree relatives of gastric cancer patients with an early age of onset than in those with

later onset, again suggesting an association with diffuse gastric carcinoma as this type occurs more frequently in younger individuals (Howson *et al.*, 1986). Further support for a genetic influence is provided by reports that blood group A is more prevalent among gastric cancer patients than controls and again is primarily associated with diffuse type lesions (Wynder *et al.*, 1963; Correa *et al.*, 1973; Mecklin *et al.*, 1988).

The above observations have led to the generally accepted view that genetic factors are more linked to diffuse type carcinomas than to the intestinal type. In a further study a large kindred appeared to develop foveolar hyperplastic polyps and later diffuse gastric carcinoma, suggesting that hyperplastic polyps may be a precursor for this tumour type (Carneiro *et al.*, 1993a). However in another report seven individuals in two generations of one family developed either *Helicobacter pylori*-positive, chronic atrophic gastritis, intestinal metaplasia, or dysplasia at an early age (less than 30 years) while two others developed early onset gastric cancer of the intestinal type (under 40 years) (Scott *et al.*, 1990). The authors concluded that the results may suggest a dominantly inherited predisposition to the metaplasia/ dysplasia/carcinoma sequence described by Correa (1988). Pernicious anaemia, also recognized as a precursor of gastric carcinoma, shows a distinct familial tendency, and an autosomal dominant type of inheritance has been suggested for the atrophic gastritis associated with pernicious anaemia (Varis *et al.*, 1980).

Carneiro *et al.* (1993b) demonstrated that more than 95% of first degree relatives of patients with intestinal or diffuse type gastric cancer had both *Helicobacter pylori* infection and gastritis, a significantly higher number than in a matched group of dyspeptic patients. However the relatives of patients with intestinal type gastric carcinoma showed a higher prevalence of chronic atrophic gastritis, intestinal metaplasia, and dysplasia than the relatives of patients with diffuse type gastric carcinoma. This study , while in agreement with the hypothesis that different pathways exist for the development of intestinal and diffuse gastric carcinomas, also supported views that common environmental and genetic factors may equally contribute to some apparently familial gastric cancers (Woolf and Isaacson, 1961; Lehtola, 1978).

1.2. Pathogenesis of colorectal cancer

1.2.1. Classification

Dukes devised a classification system in the 1930s that is still widely used, in which colorectal carcinomas are subdivided into three main categories (Dukes, 1932).

In Dukes stage A, the tumour has extended to the muscularis propria layer of the bowel wall. In stage B tumours infiltrate beyond the muscularis propria but do not spread to the lymph nodes. Dukes stage C indicates lymph node metastases, with stage C1 indicating local node involvement and stage C2, local and distant nodal involvement.

The TNM (Tumour Node Metastasis) system like Dukes's classification is based on the extent of spread. Stages I, II, and III correspond to Dukes's A, B, and C stages (Hermanek and Sobin, 1987). The histopathological classification of the WHO is also frequently used, adenocarcinomas are graded as being well, moderately, or poorly differentiated. Most colorectal carcinomas (approximately 85%) are adenocarcinomas (Morson and Sobin, 1987). More recently Jass *et al.*, (1987) have described a new classification to provide indication of clinical outcome. Four groups with differing prognosis are described, patients in group I having an excellent prognosis and patients in group IV having poorest prognosis. The survival figures of new prognostic groups I, II, III, and IV were found to be similar to Dukes's A, B, C1, and C2 stages respectively, but the new prognostic classification allowed twice as many patients to be placed into categories.

1.2.2. Anatomy of the colorectum

The large bowel may be divided into nine regions (figure 1.3). Tumours in the caecum, ascending colon, hepatic flexure, and transverse colon are classified as right-sided, and tumours in the splenic flexure, descending colon, sigmoid colon, and rectum are classified as left-sided. Tumours occur more frequently in the left than the right side of the colorectum, those in the sigmoid colon, rectosigmoid junction, and rectum accounting for almost 70% of cases (Cancer Research Campaign Factsheet 18, 1993). The colorectum wall is composed of essentially five layers: the mucosa which forms the luminal surface, submucosa, muscularis propria, subserosa, and serosa. The mucosa itself consists of an epithelium with invagenous crypts termed the crypts of Lieberkuhn, the lamina propria, and the muscularis mucosae which forms the border to the submucosa layer (Hermanek *et al.*, 1983).

1.2.3. Epidemiology

In Westernized countries, colorectal cancer is the second most common malignancy in both sexes. Incidence rates are high in the USA, northern and western Europe, and low in Africa and Asia (Parkin *et al.*, 1993). In the U.K. there are over 28,000 new cases each year and over 19,000 deaths due to this tumour. The

Figure 1.3.

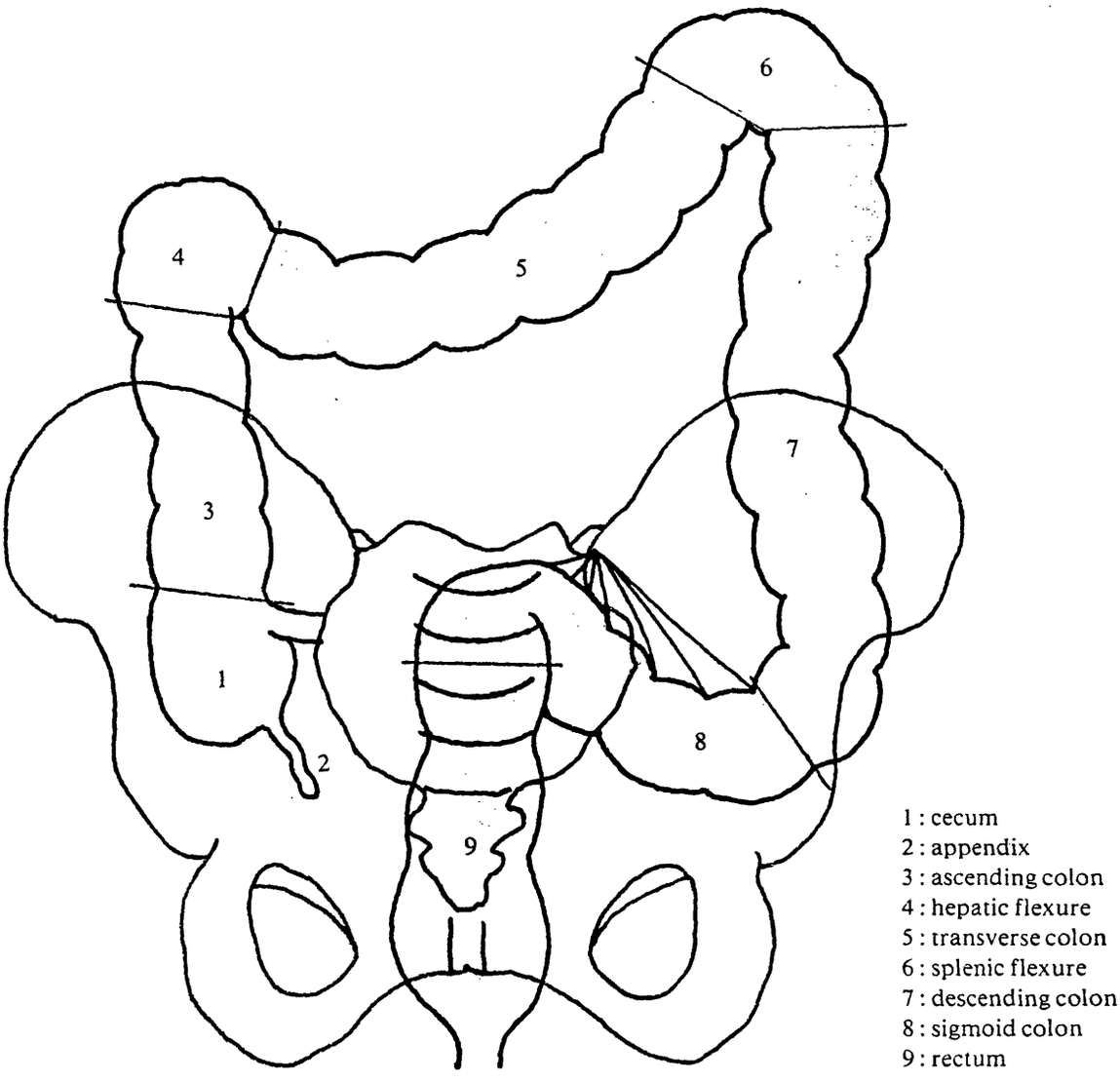


Figure 1.3. Subdivision of the colorectum (taken from Hermanek *et al.*, 1983)

incidence of colon cancer is higher in Scotland than in England and Wales, while that of rectal cancer is similar in all regions (Cancer Research Campaign Factsheet 18, 1993).

The geographic variation in incidence indicates that environmental factors play a major role in the genesis of colorectal cancer. This suggestion is supported by studies of migrants from low to high risk areas, who take on the incidence of the host country within a single generation (Haenszel and Kurihara, 1968). There have been significant changes in colorectal cancer risk in some populations, for example incidence rates have increased strikingly in Japan during this century to approach those in Western countries (Parkin *et al.*, 1992), providing further evidence for the importance of environmental factors.

1.2.4. The Adenoma - Carcinoma Sequence

It is generally accepted that the vast majority of colorectal cancers arise from benign tumours, termed adenomas (Bussey, 1975; Hermanek *et al.*, 1983). Cell kinetic studies involving in situ DNA labelling of colonic epithelial cells have demonstrated that the proliferative zone, which is confined to the lower two-thirds of the crypts of Lieberkuhn in normal colorectal mucosa, is shifted to the surface of the crypts in adenomas and in adjacent mucosa (Deschner and Lipkin, 1975). Further evidence suggests that widespread hyperproliferation of the colorectal mucosa precedes the development of colorectal carcinoma (Ponz de Leon *et al.*, 1988).

Histologically adenomas are divided into tubular, villous and tubulo-villous adenomas. Tubular adenomas (also known as adenomatous polyps) are the most common type, and are composed of branching tubules embedded in the lamina propria. Villous adenomas are composed of finger-like processes of lamina propria covered by epithelium reaching down to the muscularis mucosae, and tubulo-villous adenomas have a histology intermediate between tubular and villous (Bussey, 1975; Muto *et al.*, 1975). Tubular and villous adenomas are not separate entities but represent two extremes of a histological spectrum of tumours that are essentially part of the same neoplastic process (Bussey, 1975).

A gradual transition from benign to malignant tumour is suggested, associated with increasing size of adenoma and grade of dysplasia (Morson, 1974; Muto *et al.*, 1975; Fearon and Vogelstein, 1990). Villous adenomas have the highest malignancy rate. The histological distinction between an adenoma with severe dysplasia and an

adenocarcinoma is infiltration through the muscularis mucosae into the submucosa (Morson, 1974; Hermanek *et al.*, 1983).

More recent studies suggest that microscopic epithelial lesions called dysplastic aberrant crypt foci (ACF) are the precursors of adenomas, and therefore the earliest identifiable precursors of colorectal neoplasia (Pretlow *et al.*, 1991; Roncucci *et al.*, 1991; Jen *et al.*, 1994b). ACF are composed of clusters of abnormally large dark staining slightly elevated mucosal crypts.

1.2.5. Aetiology

The high incidence of colorectal cancer in industrialized nations is considered by some to be related to a 'Western' diet, with high fat and meat consumption and low fibre intake (Wynder and Shigematsu, 1967; Burkitt, 1971), but the evidence is not conclusive. For example while some case-control studies support a strong positive association between total dietary fat intake and colorectal cancer risk, others report no significant association (Willett, 1989). The type of fat (eg. animal fat) rather than the total amount may be the important factor. Mechanisms for how fat is involved in colorectal carcinogenesis have been proposed: high levels of dietary fat result in an increased concentration of bile acids and cholesterol in the gut and modify the activity of gut bacteria. The bacteria convert these substances into secondary bile acids and cholesterol metabolites, which are thought to act as tumour promoters (Hill *et al.*, 1971; Reddy, 1981). Secondary bile acids enhance epithelial cell proliferation in the large bowel (DeRubertis *et al.*, 1984).

Burkitt (1971) suggested how fibre may protect against bowel cancer by hypothesizing that a higher dietary fibre intake increases the stool bulk and speed of transit, diluting potential carcinogens and reducing their contact with the colonic mucosa. In addition, fibre and resistant starch are substrates for anaerobic fermentation by flora in the bowel which results in the production of short chain fatty acids such as butyrate (Cummings and Bingham, 1987). Sodium butyrate has been shown to inhibit the proliferation of colorectal tumour cells (Augeron and Laboisse, 1984). Fermentation of fibre lowers colonic pH and it has been proposed that this inhibits the conversion of primary to secondary bile acids (Thornton, 1981). Several, but not all, case-control studies report that a reduction in colorectal cancer risk is associated with intake of fibre, mainly from fruit and vegetables (Willett, 1989; Bingham, 1990). However these foods are the main dietary source of certain micronutrients that may have a protective role in colorectal cancer, such as β-carotene and vitamin C (La Vecchia *et al.*, 1996). Therefore although there is strong evidence

that fruit and vegetables have a protective effect, the responsible factor has not been clearly identified.

Cooked meat contains a number of mutagens and some products of protein metabolism in the gut have been implicated in colorectal carcinogenesis (Cummings and Bingham, 1987), but epidemiological support for involvement of meat intake in colorectal neoplasia is inconsistent (Bingham, 1990). Total calorific intake shows a positive association with colorectal cancer risk (Potter and McMichael, 1986). A reason for the inconsistency between case-control studies described above may be inadequate statistical adjustment for total calorific intake, to determine the specific effect of individual nutrients (Willett, 1989). Other dietary considerations include alcohol consumption, particularly beer which has been linked with rectal cancer (McMichael *et al.*, 1979). High doses of calcium have been shown to significantly reduce epithelial cell proliferation in the colorectum (Wargovich *et al.*, 1992). The mechanism is not clearly known but it has been hypothesized that free fatty acids and bile acids are converted into insoluble calcium compounds, thus mitigating their toxic effects (Newmark *et al.*, 1984).

Decreased colorectal cancer risk is associated with the use of nonsteroidal antiinflammatory drugs (NSAIDS) such as aspirin (Thun *et al.*, 1991) and sulindac, which has been found to cause polyp regression in patients with familial adenomatous polyposis (Gonzaga *et al.*, 1985). The antineoplastic effect of NSAIDS is not fully understood, however they are known to inhibit the synthesis of prostaglandins which are thought to be involved in tumour promotion (Verma *et al.*, 1980), and more recently have been demonstrated to block the cell cycle in the G_0-G_1 phase and induce apoptosis in colorectal cancer cells (Shiff *et al.*, 1995; Elder *et al.*, 1996).

The risk of colorectal cancer, particularly that of the colon, is greater in women than in men at younger ages. This risk is reversed postmenopausally, suggesting a hormonal effect (McMichael and Potter, 1983). In addition women have been found to have a slower bowel transit time and reduced stoolbulk compared with men which could contribute to their increased risk (McMichael and Potter, 1983). Finally, a higher risk of colorectal cancer is associated with a previous diagnosis of bowel cancer (Wynder and Shigematsu, 1967), and with other diseases of the bowel including ulcerative colitis (Devroed and Taylor, 1976) and Chrohn's disease (Kuster *et al.*, 1989).

1.2.6. Diagnosis and Treatment

The main symptoms of colorectal cancer are alterations in the frequency of bowel movements and stool consistency, blood and mucous in the stool and diarrhoea. Symptoms generally appear later with right-sided colon tumours as they not as exposed to damage by the stool (Hermanek *et al.*, 1983). The main treatment for colorectal cancer is surgery. Adjuvant chemotherapy is sometimes used especially to treat lymph node metastases but has not resulted in a significant improvement in survival rates (Hermanek *et al.*, 1983).

Almost half of all patients present at late stage of disease, prognosis is relatively poor with roughly one third of patients surviving to five years after diagnosis (Cancer Research Campaign Factsheet 18, 1993). Screening techniques for the detection of tumours at early stages are being assessed. In the United States it is currently recommended that asymptomatic individuals over the age of 50 at average risk, should be screened with an annual faecal occult blood test and sigmoidoscopy, preferably flexible, every 5 years (Lieberman and Sleisenger, 1996).

1.2.7. Heredity and colorectal cancer

There are a number of inherited conditions that are characterized by intestinal polyposis and a predisposition to develop colorectal cancer (Bussey, 1975). These diseases are uncommon and represent only a small portion of the total colorectal cancer burden. The two best defined colorectal cancer predisposition syndromes are Familial Adenomatous Polyposis (FAP) and Hereditary Nonpolyposis Colorectal Cancer (HNPCC).

FAP, also known as Familial Polyposis Coli (FPC) and Adenomatous Polyposis Coli (APC), is an autosomal dominantly inherited condition that affects nearly 0.01% of the British, American, and Japanese populations (Utsunomiya, 1990). This disease is characterized by the development of hundreds to thousands of adenomatous polyps throughout the colorectum, usually during adolescence or early adulthood. The high number of adenomas presents a high risk of colorectal cancer as some will inevitably progress towards malignancy (Bussey, 1975; Muto *et al.*, 1975). In addition patients may develop extracolonic lesions including osteomas, epidermoid cysts, desmoid tumours, upper gastrointestinal neoplasia, and congenital hypertrophy of the retinal pigment epithelium (CHRPE) (Bussey, 1975; Jagelman *et al.*, 1988; Chapman *et al.*, 1989).

HNPCC, like FAP, is inherited in an autosomal dominant fashion. Its prevalence is uncertain but it may account for up to 5% of all colorectal cancers (Marra and Boland, 1995). HNPCC has also been subdivided into Lynch I and Lynch II syndromes (Lynch *et al*, 1993). Lynch syndrome I is characterized by early age of onset of colorectal cancer (about 40-45 years), a tendency towards a proximal colon location, and an excess of synchronous and metachronous colorectal cancers. Lynch II syndrome (also known as the Cancer Family Syndrome) has all the above features and also shows extracolonic malignancies, primarily of the endometrium. Other organs that are affected mainly include the stomach, small intestine, ovary, pancreas, biliary tract, renal pelvis and ureter (Lynch *et al*, 1993). Another variant of the HNPCC syndrome is thought to be Muir-Torre syndrome, characterized by the occurrence of multiple skin tumours (Lynch *et al.*, 1985a).

Cancers in HNPCC develop from adenomas but these do not occur as profusely as in FAP hence the term 'nonpolyposis' is used. The adenomas are suggested to progress towards malignancy more rapidly than sporadic adenomas (Jass *et al.*, 1994), but patients with HNPCC cancers seem to have an improved survival compared to patients with sporadic colorectal cancers (Albano *et al.*, 1982). A lack of distinctive phenotypic stigmata means it can be difficult to diagnose HNPCC. The following minimum criteria, known as the 'Amsterdam criteria', have been proposed to assist with the diagnosis:

1) histologically verified colorectal cancer in three or more relatives, one of whom is a first degree relative of the other two.
2) at least two generations should be affected with colorectal cancer.
3) one or more of the colorectal cancer cases should be diagnosed before fifty years of age (Vasen *et al.*, 1991).

There have been many criticisms of the Amsterdam criteria, the main ones being exclusion of endometrial and other extracolonic cancers from the diagnosis, and small family size or poor documentation may mean that some HNPCC families are not recognised. Familial aggregation as a result of familial lifestyle factors for example, may lead to false-positive diagnoses (Lynch *et al.*, 1993; Marra and Boland, 1995).

It has been suggested that heritable factors are involved in the development of the majority of colorectal tumours too (ie. those not associated with FAP and HNPCC), and that there is a strong interaction of genetic with environmental factors (Cannon-Albright *et al.*, 1988; Willett, 1989). A history of colorectal cancer in a first degree relative increases the risk of this neoplasm two- to threefold (Macklin, 1960;

Boutron *et al.*, 1995). Analysis of 34 kindreds, selected because they included either familial clusters of cases of colorectal cancer or a single person with an adenomatous polyp, provided strong evidence for a dominantly inherited susceptibility to common colorectal polyps and cancers, with an estimated gene frequency of nineteen percent (Burt *et al.*, 1985; Cannon-Albright *et al.*, 1988).

1.3. Genetic studies in cancer

As early as 1914, Boveri proposed that cancer resulted as a direct consequence of chromosome abnormalities (cited in Evans, 1993). There is now conclusive evidence that cancer is indeed a genetic disease. The majority of cancers are caused by somatic mutation unlike most other human genetic disorders which are due to germline defects.

The belief that cancer has a genetic basis has stemmed from diverse observations. It is recognised that some individuals have an inherited predisposition to cancer. An autosomal dominant mode of inheritance of cancer predisposition is seen in some families, for example those with the conditions retinoblastoma and familial adenomatous polyposis (Knudson, 1993). Recessively inherited disorders that are characterized by the inability to process damaged DNA, such as xeroderma pigmentosum and ataxia telangiectasia, are associated with a higher cancer incidence (Hanawalt and Sarasin, 1986). Furthermore it has been observed that tumour cells of most cancers have chromosomal defects many of which are consistent (Yunis, 1983). As discussed in previous sections on gastric and colorectal cancer, environmental factors are implicated as a major cause of cancer. Additional evidence for a genetic role in neoplasia comes from the demonstration that most carcinogens are mutagenic (Ames, 1979).

The incidence of most cancers increases with age (10^3-10^7 times) (Vogelstein and Kinzler, 1993). This dramatic change of frequency is thought to have a genetic basis as it is believed that ageing provides the time needed for the accumulation of several separate mutations necessary for the development of malignancy (Miller, 1980; Vogelstein and Kinzler, 1993). Theories that carcinogenesis is a multistage process involving the occurrence of at least two and possibly as many as seven independent mutational events have been proposed by several authors (Nordling, 1953; Armitage and Doll, 1954; Ashley, 1969; Knudson, 1971). The number of mutations may vary in different tumour types (Ashley, 1969). The long latent period observed between initial exposure to a carcinogen and appearance of most tumours

may be explained by the multistage hypothesis as accordingly additional somatic mutations to that induced by the carcinogen are required for a cancer to form.

In hypotheses of multistage carcinogenesis it is suggested that succesive mutations occur in the same cell (eg. Nordling, 1953; Knudson, 1971). This is in accord with the theory that most tumours arise from the clonal progeny of a single cell of origin (Nowell, 1976). It is thought that this single cell contains genetic damage that all its progeny share. This genetic damage conferred a growth advantage on the cell by disrupting the control of cell proliferation. One of its progeny may acquire another genetic alteration that gives it an additional growth advantage allowing it to outgrow its sister cells. One of this cell's progeny may acquire a mutation that gives it a further growth advantage so that its progeny become the predominant cell population, thus a tumour progresses by a process of clonal expansion driven by mutation. A sufficient number of mutations may accumulate so that a tumour becomes malignant and can thus invade locally and metastasize to other organs.

Progress has now been made in identifying the genes in which these mutations occur. Furthermore it has been demonstrated that multiple genetic changes are indeed required for tumour formation. Two distinct classes of genes that are thought to regulate cell proliferation, growth-promoting oncogenes and growth-restraining tumour suppressor genes, will be reviewed in the following sections.

1.3.1. Oncogenes

Proto-oncogenes were originally discovered through the study of retroviruses. The genome of the Rous sarcoma virus contains the v-*src* gene which is responsible for the tumour inducing ability of the virus. This gene was shown to be actually derived from the genome of the chicken, the host of the virus (Stehelin *et al.*, 1976). This work thus demonstrated that a normal cellular gene, termed a proto-oncogene, is capable of causing neoplastic growth when activated, in this case by a retrovirus. Activated forms of proto-oncogenes are known as oncogenes. Studies of other retroviruses have identified a number of oncogenes and their cellular homologues in vertebrate genomes (Land *et al.*, 1983a).

Proto-oncogenes have been discovered through other lines of work too, including gene transfer studies in which transforming genes have been identified from

tumour cell DNA used to transfect cells in culture, and the analysis of genes that lie at breakpoints of chromosomal translocations and genes in amplified domains of DNA (Bishop, 1987). Chromosomal translocation and gene amplification result in the activation of proto-oncogenes, as do point mutations and small deletions and insertions (Solomon *et al.*, 1991).

Mutations of proto-oncogenes thus result in a gain of function and have a dominant effect as activated proto-oncogenes transform cells despite expression of the normal alleles. However it is thought that activation of a single proto-oncogene is insufficient for tumourigenesis. Land *et al.*, (1983b) demonstrated that *ras* and *myc* oncogenes when transfected together could fully transform primary rodent cells but could not do so individually. Such studies have led to the concept that oncogenes co-operate in producing a tumourigenic phenotype (Hunter, 1991).

Although a large number of proto-oncogenes have now been identified it has become apparent that each encodes a protein that is involved in signal transduction, the transmission of growth-regulatory messages from outside the cell to inside the nucleus (Hunter, 1991). Proto-oncogene products have been localized to the cytoplasmic membrane, cytoplasm, and nucleus and include growth factors, protein kinases, GTP-binding proteins, and transcription factors (Hunter, 1991).

Somatic alterations of proto-oncogenes have been reported in a wide variety of human cancers and with some consistency. For example, activation of the c-*myc* proto-oncogene by translocation is common to Burkitt's lymphoma (Dalla-Favera *et al.*, 1983) and point mutation of the K-*ras* gene is frequently observed in colorectal cancers (Bos *et al.*, 1987). Some consider it unlikely that dominant mutations in proto-oncogenes predispose to neoplasia because perhaps this would be lethal to foetal development (Bishop, 1991). However germline mutations in the *RET* proto-oncogene have now been identified in individuals with the dominantly inherited cancer predisposition syndrome, multiple endocrine neoplasia type 2 (Mulligan *et al.*, 1993; Donis-Keller *et al.*, 1993).

1.3.2. Tumour suppressor genes

In contrast to dominant oncogenes, tumour suppressor genes are recessive at the cellular level and it is their inactivation rather than activation that leads to tumour development.

1.3.2 (a) Cell hybrid studies

Early evidence for tumour suppressor genes came from somatic cell hybrid experiments. Fusion of tumourigenic cells with non-tumourigenic cells generated hybrid cells that no longer produced tumours when injected into animals. However a reversion to malignancy was sometimes observed which was determined to be due to a loss of chromosomes from the tumourigenic hybrid cell that were derived from the normal parent cells (Harris *et al.*, 1969; Kaebling and Klinger, 1986). Such studies indicated that chromosomes from normal cells contained genetic information that could suppress tumourigenicity, and that these were missing from the tumour cell genome. Thus a recessive genetic mechanism in tumourigenesis was implied.

Further support was provided when hybrids generated from the fusion of tumourigenic cell lines of differing cellular origin were also found to be non-tumourigenic indicating genetic complementation of loci controlling the suppression of neoplasia in the parent cell lines (Weissman and Stanbridge, 1983). These experiments suggested that a number of recessive tumour suppressor genes existed. Using the microcell transfer technique, the introduction of a single normal chromosome into a cell line of a particular tumour type resulted in suppresion of tumourigenicity only after transfer of a specific chromosome (Weissman *et al.*, 1987; Trent *et al.*, 1990) demonstrating that different tumours are acted on by different suppressor genes.

1.3.2 (b) Knudson's hypothesis and retinoblastoma

The existence of recessive tumour suppressor genes was further deduced from the study of hereditary cancer. In an epidemiological investigation of the childhood tumour, retinoblastoma, Knudson observed that the dominantly inherited form of this neoplasm was characterized by a high proportion of bilateral tumours while in the non-hereditary form tumours occurred in only one eye and had a later age of onset (Knudson, 1971). Knudson explained these differences by hypothesizing that two successive mutations were required for tumour formation: in the inherited form one mutation is inherited via the germline and the second mutation occurs as a somatic event in a retinal cell, whereas in the non-inherited form both mutations occur somatically in a single retinal cell, at the same genetic loci as in the inherited form. The fact that both mutations had to be acquired in non-hereditary tumours and the improbability of these mutational events occurring in more than one retinal cell, accounted for the late onset and invariability of unilateral tumours in sporadic retinoblastoma patients (Knudson, 1971).

It was later shown that the two mutational events involved inactivation of both copies of the same gene. Karyotypic investigations revealed a deletion of chromosomal region 13q14 in the germline of some patients with hereditary retinoblastoma (Lele *et al.*, 1963; Yunis and Ramsay, 1978) and in the tumours of sporadic retinoblastoma patients (Balaban *et al.*, 1982), suggesting a locus for the retinoblastoma gene. Cavenee *et al.* (1983) then pioneered the use of polymorphic DNA markers to look for allelic losses in tumours. For patients who were constitutionally heterozygous at a polymorphic locus, loss of one of the two corresponding alleles could be detected in matching tumour DNA. Consistent with a recessive mutational mechanism it was found that the majority of sporadic retinoblastoma tumours had lost heterozygosity for markers on one chromosome 13. This allele loss could occur by a number of mechanisms such as whole chromosome loss, mitotic recombination, deletion, or gene conversion (Cavenee *et al.*, 1983).

Most importantly, analysis of hereditary retinoblastoma patients demonstrated that it was the normal allele on the chromosome 13 inherited from the unaffected parent that was lost in the tumour (Cavenee *et al.*, 1985). From these studies it was concluded that the net result of loss of heterozygosity was to uncover the initial mutation (in the germline in hereditary cases), thus tumour formation was associated with complete dysfunction of the retinoblastoma gene. This was indeed shown to be the case once the retinoblastoma gene (*RB1*) was cloned using positional cloning techniques, when homozygous deletion of the *RB1* locus including internal deletion was detected in some retinoblastomas (Friend *et al.*, 1986; Lee *et al.*, 1987; Fung *et al.*, 1987).

The use of more sensitive mutation detection techniques identified germline and somatic mutations (eg. point mutations and small deletions or insertions) of the *RB1* gene in hereditary and nonhereditary retinoblastoma patients respectively. In some tumours, the other *RB1* allele was either lost or carried a second mutation (Dunn *et al.*, 1988; Yandell *et al.*, 1989; Dunn *et al.*, 1989). Thus the retinoblastoma gene was the first tumour suppressor gene to be cloned. Furthermore, when a cloned *RB1* gene was introduced into tumour cell lines that were mutant for *RB1* using a retroviral vector, these cells were no longer tumourigenic in mice (Huang *et al.*, 1988), corroborating the tumour suppressor status of the *RB1* gene.

1.3.2 (c) Loss of heterozygosity

Cavenee *et al.* (1983) recognized that allele loss could be a mechanism that is involved in the development of tumours other than retinoblastomas. Loss of heterozygosity (LOH) at a variety of chromosomal sites has been described in many different types of tumour (Lasko *et al.*, 1991; Rodriguez *et al.*, 1994). The consistent loss of heterozygosity in a particular chromosomal region is now taken as evidence of the existence of a nearby tumour suppressor gene. Indeed such observations have led to the isolation of several putative tumour suppressor genes, for example *WT1* at 11p13 and *APC* at 5q21 (reviewed by Levine, 1993). LOH at the same chromosomal site has been described in different tumour types (eg. 17p) (Rodriguez *et al.*, 1994), suggesting the involvement of the same locus in different tumours. Also many tumours exhibit LOH in more than one chromosomal region.

Traditionally LOH analysis has been performed using DNA probes for the detection of restriction fragment length polymorphisms (RFLPs) on Southern blots of restriction enzyme digested DNA (Lasko *et al.*, 1991). However this method is often limited by the low informativity and availability of RFLP markers and the requirement for relatively large amounts of DNA. PCR typing of polymorphic microsatellite repeat sequences (also known as short tandem repeats, STRs), especially those consisting of dinucleotide repeat units (Weber and May, 1989), is now widely used for the detection of LOH in tumours (eg. Gruis *et al.*, 1993; Osbourn and Leech, 1994) and has almost superseded Southern analysis. The dinucleotide repeat elements of the form $(dC\text{-}dA)_n\cdot(dG\text{-}dT)_n$ are one of the most abundant classes of repetitive DNA sequences in humans. There are 50,000 - 100,000 $(CA)_n$ repeats scattered throughout the genome, many exhibiting length polymorphisms (Weber and May, 1989). In general alleles at these sites are stably inherited from one generation to another and this class of DNA polymorphism is now frequently used in linkage analysis (Weissenbach *et al.*, 1992). The advantages of microsatellite PCR for LOH analysis are that large numbers of microsatellite markers are available that are highly informative and evenly distributed over the genome and very small amounts of DNA can be used.

Since the retinoblastoma gene was cloned, a number of tumour suppressor genes that are involved in dominantly inherited and sporadic cancers have been identified. The tumour suppressor gene that has been found to be the most commonly mutated in human cancer is *p53*.

1.3.2 (d) *p53*

The product of the *p53* gene was originally identified as a 53 kDa protein which formed an oligomeric complex with the large T-antigen of Simian virus 40 (SV40) (Lane and Crawford, 1979). The *p53* gene has been localized to the short arm of chromosome 17 at position 17p13 and it encodes a 393 amino acid nuclear phosphoprotein (Harlow *et al.*, 1985; McBride *et al.*, 1986; Miller *et al.*, 1986). A number of findings led to the proposal that *p53* was a dominant oncogene, for example its overexpression resulted in the immortalization of primary rodent cells (Jenkins *et al.*, 1984) and *p53* was demonstrated to co-operate with an activated *ras* gene in the transformation of rat embryo fibroblasts (Parada *et al.*, 1984).

However it was later found that wild-type *p53* was incapable of transforming cells. In the earlier studies transforming activity was exhibited by *p53* clones that were actually mutated forms of the gene (Finlay *et al.*, 1988; Eliyahu *et al.*, 1988). Moreover it was shown that wild-type p53 could suppress transformation mediated by oncogene co-operation between *myc* or adenovirus *E1A* and ras (Finlay *et al.*,1989; Eliyahu *et al.*, 1989). Further studies supporting a tumour suppressor gene role for *p53* included the demonstration that in colorectal carcinomas with allelic deletions of chromosome 17p, the remaining *p53* allele was mutated, a hallmark of tumour suppressor genes (Baker *et al.*, 1989). Transfection of a single wild-type *p53* gene into human tumour cell lines resulted in suppression of growth (Baker *et al.*, 1990b; Chen *et al.*, 1990). Furthermore, mice homozygous for *p53* null alleles, although developmentally normal, rapidly developed malignant tumours (Donehower *et al.*, 1992).

Somatic mutations of the *p53* are the most common genetic alterations observed in human cancer: more than 50% of human neoplasms, of diverse cell and tissue type, have been found to harbour mutations of this gene. Some cancers have a high percentage of *p53* mutations (eg. small cell lung cancers) while others rarely accumulate such alterations (eg. thyroid and nasopharyngeal carcinomas) (reviewed by Chang *et al.*, 1993). The *p53* gene has also been implicated in hereditary cancer. Germline transmission of *p53* mutations have been described in families affected with the Li-Fraumeni syndrome (Malkin *et al.*, 1990). This syndrome is characterized by early onset of a variety of cancers, notably soft tissue sarcomas, breast cancers, brain tumours, osteosarcomas, leukaemias, and adrenocortical carcinomas (Li *et al.*, 1988). Germline mutations of the *p53* gene have been detected in other non-Li-Fraumeni cancer families too (Toguchida *et al.*, 1992; Jolly *et al.*, 1994). In most tumours, both *p53* alleles are inactivated, one through point mutation and the other through a

deletion. Most mutations of the *p53* gene are missense mutations, giving rise to an altered protein. Chain-terminating mutations are also observed but more rarely (Hollstein *et al.*, 1991). The majority (>90%) of base substitutions that have been reported in *p53* are clustered between exons 5 and 8, where most of the evolutionarily conserved amino acids are concentrated (Hollstein *et al.*, 1991).

Functionally, the p53 protein has been divided into three domains. At the amino-terminus of the protein is an acidic transcription activation domain (Fields and Jang, 1990, Raycroft *et al.*, 1990). p53 can bind to DNA in a sequence-specific manner, a function that is mediated by the central domain of the protein (Pavletich *et al.*, 1993; Bargonetti *et al.*, 1993). Typically, p53 binding sites consist of four copies of the consensus pentamer sequence 5' - PuPuPuC(A/T) - 3' (El-Deiry *et al.*, 1992). It is thought that the p53 protein binds to such DNA sites as a tetramer (Pavletich *et al.*, 1993; Cho *et al.*, 1994). It has been shown that wild-type p53 can activate transcription of genes adjacent to its binding site (Kern *et al.*, 1992; Unger *et al.*, 1992). The majority of point mutations of *p53* occur in the conserved central domain (Hollstein *et al.*, 1991). The evidence from biochemical studies indicates that most of these mutations result in loss of the ability of p53 to bind to specific DNA sequences and activate the expression of adjacent genes (Unger *et al.*, 1992; Kern *et al.*, 1992; Cho *et al.*, 1994).

p53 thus acts as a transcription factor. A number of genes have now been identified that contain p53 binding sites and are transcriptionally activated by p53. These genes are likely to play a role in mediating the p53-dependent functions in a cell. The genes include *p21* (also known as *WAF1* or *CIP1*), *GADD45, bax, IGF-BP3*, which are all discussed later and *cyclin G* and *MDM2*. Cyclin G is a novel cyclin of unknown function (Okomoto and Beach, 1994). It has been demonstrated that the MDM2 protein binds to the transactivation domain of p53 to inhibit its transcriptional activity (Wu *et al.*, 1993; Kussie *et al.*, 1996). Thus MDM2 and p53 form a negative feedback loop that regulates both the activity of the p53 protein and expression of the MDM2 gene.

p53 forms a tetramer via an oligomerization domain at the carboxy-terminus (Pavletich *et al.*, 1993). This domain appears to stabilize the sequence-specific binding activity of p53 at its central domain (Shaulian *et al.*, 1993). Several other biochemical activities have been ascribed to the carboxy-terminus of p53, for example it helps to catalyze the reassociation of single-stranded DNA or RNA to double strands (Bakalkin *et al.*, 1994), binds to internal deletion loops in DNA resulting from DNA deletion/insertion mismatches (Lee *et al.*, 1995), and

demonstrates a DNA helicase activity (Brain and Jenkins, 1994). Thus p53 appears to play a role in DNA repair.

The major proposed function for the p53 protein is in mediating a response to DNA damage. In normal cells the p53 protein is not expressed at detectable levels. However it is stabilized and accumulates in cells undergoing DNA damage, mediated by ultraviolet or gamma irradiation or genotoxic drugs for example (Kastan *et al.*, 1991), and in cells responding to certain stresses, for example a lack of oxygen (hypoxia) or viral or cellular oncogene activation (Hermeking and Eick, 1994; Graeber *et al.*, 1996). Activation of p53 in this way leads to either arrest of the cell cycle at the G1 stage of the cell cycle (Kastan *et al.*, 1991; Lin *et al.*, 1992) or the induction of apoptosis (programmed cell death) (Yonish-Rouch *et al.*, 1991; Lowe *et al.*, 1993). It is not clear how a cell decides whether to undergo cell cycle arrest or apoptosis in response to p53 activation. It is thought that the choice depends on the cell type, cell environment, and other genetic alterations sustained by the cell (Bates and Vousden, 1996). Arrest at the G1 phase of the cell cycle would allow damaged DNA to be repaired before it is replicated, thereby avoiding the propagation of genetic lesions to progeny cells. If the damage is too extensive that DNA repair is not feasible, p53 may trigger apoptosis. It is thought this is the reason why so many cancerous cells select against wild-type p53 function (Levine, 1997). Fibroblast cells with no functional *p53* gene, which is often the situation in tumour cells, are genetically unstable, as seen by the increase in the frequency of gene amplification (Livingstone *et al.*, 1992).

It is now widely accepted that the main role of p53 is to preserve genomic integrity thus it has been dubbed the 'guardian of the genome' (Lane, 1992). At least one of the genes that are transcriptionally activated by p53, *p21* (also known as *WAF1* or *CIP1*), is thought to be a critical mediator of the p53-dependent G1 arrest. *p21* encodes a cyclin-dependent kinase inhibitor (Harper *et al.*, 1993; El-Deiry *et al.*, 1993; Xiong *et al.*, 1993). Cyclin-dependent kinases (CDKs) are a group of related enzymes that associate with regulatory subunits called cyclins to alter the activity of key proteins controlling entry into different stages of the cell cycle. p21 binds to a number of cyclin and CDK complexes including cyclin D1-CDK4 which is involved in a pathway that is central to the regulation of the G1 to S phase transition. Cyclin D1-CDK4 phosphorylates the retinoblastoma tumour suppressor gene product (Rb) which results in the release of E2F transcription factor complexes from an association with Rb. These transcription factor complexes regulate a number of genes required to initiate or propagate the S phase of the cell cycle (reviewed in Levine, 1997). Waldman *et al.* (1995) irradiated human colon tumour cells with a wild-type *p53* gene

and either two wild-type *p21* alleles or with deletions of both *p21* alleles, under conditions shown previously to induce a p53-dependent growth arrest and induction of p21. The $p21^{+/+}$ cells underwent a G1 arrest whereas the $p21^{-/-}$ cells did not. Deng *et al.* (1995) demonstrated that mouse embryonic fibroblasts derived from mice lacking p21 (null phenotype) were partially deficient in their ability to arrest cells in G1 in response to DNA damage. These studies showed that activation of p21 by p53 plays a major role in mediating the G1 cell cycle arrest following DNA damage.

GADD45 is another p53-responsive gene (Kastan *et al.*, 1992). It has been shown that overexpression of its gene product also results in cell cycle arrest at G1 (Smith *et al.*, 1994). Furhermore, it was shown that Gadd45 binds to PCNA (proliferating cell nuclear antigen), a subunit of DNA polymerase, to stimulate excision repair of damaged DNA (Smith *et al.*, 1994). There is evidence for a G2/M phase checkpoint for p53 too. Cross *et al.* (1995) reported that when mitotic spindle inhibitors are added to cells with wild-type *p53* the cells arrested in G2. However cells that lacked *p53* reinitiated DNA synthesis, resulting in an increase in ploidy.

The decision for a cell to undergo apoptosis in response to DNA damage or stressful situations could be influenced by two p53-regulated genes, *bax* and *IGF-BP3* (Miyashita *et al.*, 1994; Buckbinder *et al.*, 1995). The Bax protein promotes apoptosis in cultured cells. Growth of brain tumours in $TgT121$ transgenic mice is suppressed by the induction of p53-dependent apoptosis. Yin *et al.* (1997) showed that the expression of Bax was induced in these apoptotic cells and that this induction was p53-dependent. Moreover, in $TgT121bax^{-/-}$ mice, tumour growth was accelerated and apoptosis was reduced by 50% compared with $TgT121bax^{+/+}$ mice (Yin *et al.*, 1997). These results indicated that Bax was required for half of p53-mediated apoptosis. The residual p53-dependent apoptosis not requiring Bax could result from regulation of other genes by p53. The product of the p53-responsive gene *IGF-BP3* (insulin-like growth factor-binding protein-3) blocks the IGF mitotic signalling pathway by binding to IGF and preventing interaction with its receptor. It is thought that blocking of IGF activity could enhance apoptosis or lower the mitogenic response of cells (Buckbinder *et al.*, 1995). There is evidence that p53 can mediate apoptosis by a mechanism independent of its sequence-specific transcriptional activity. A p53 protein that is mutated in the transactivation domain and is unable to activate transcription still induces apoptosis in human tumour cells (Haupt *et al.*, 1995).

1.3.2 (e) Other tumour suppressor genes

Table 1.1 describes some cloned putative tumour suppressor genes and proposed function of the gene products. The genes involved in colorectal cancer, *APC* (adenomatous polyposis coli), *MCC* (mutated in colorectal cancer), and *DCC* (deleted in colorectal cancer) will be discussed more fully later. Heterozygous germline mutations in the majority of the tumour suppressor genes have been implicated in a dominantly inherited cancer syndrome as indicated in Table 1.1, except the *DCC*, *MCC*, *p16*, and *DPC-4* (deleted in pancreatic carcinoma-4) genes, for which only somatic alterations have been identified so far.

In most cases, the same gene that is germinally mutated in the hereditary form of a cancer is found to be somatically altered in the corresponding non-hereditary form, in accordance with Knudson's hypothesis (Knudson, 1971; 1985). The exceptions are *BRCA1* and *BRCA2* in which somatic mutations have very rarely been observed in sporadic breast or ovarian cancers (Stratton, 1996). The site of further putative tumour suppressor genes associated with a number of malignancies have been identified (eg. at 3p21 in small-cell lung carcinoma) but the genes have yet to be cloned.

1.3.3. Mutation detection

The detection of single base alterations in genomic DNA represents a critical step in the genetic analysis of human diseases, by identifying disease-causing mutations and polymorphic changes. A number of techniques have been developed to scan genes for unknown subtle mutations, the most commonly used include single-strand conformation polymorphism analysis, heteroduplex analysis, protein truncation test, denaturing gradient gel electrophoresis, Ribonuclease A protection analysis and chemical mismatch cleavage, and will be discussed below.

PCR (polymerase chain reaction) amplification of the sample DNA is carried out prior to analysis by one of these detection methods. The PCR is an *in vitro* technique that can isolate and amplify a specific segment of DNA by as much as a million-fold, in a short space of time (Saiki *et al.*, 1985; 1988). Two oligonucleotide primers are synthesized,typically 20-25 nucleotides in length, that flank the DNA segment to be amplified, each being complementary to opposite strands of DNA. Each cycle of the PCR involves three succesive steps: heat denaturation of DNA, annealing of primers to their complementary sequences, and extension of the annealed primers with DNA polymerase. The DNA template is heated to separate it

Table 1.1. Putative tumour suppressor genes.

Gene	Chromosomal location	Hereditary cancer syndrome	Proposed function	Reference
RB1	13q14	Retinoblastoma	Regulates transcription factors	Friend *et al.* (1986)
p53	17p13	Li-Fraumeni syndrome	Transcription factor	Malkin *et al.* (1990)
WT1	11p13	Wilms' tumour	Transcription factor	Call *et al.* (1990); Gessler *et al.* (1990)
NF1	17q11	Neurofibromatosis type 1	GTPase activating protein	Cawthon *et al.* (1990); Wallace *et al.* (1990)
NF2	22q12	Neurofibromatosis type 2	Cytoskeleton-plasma membrane link	Trofatter *et al.* (1993); Roulcau *et al.* (1993)
VH1	3p25	von Hippel-Lindau disease	Inhibition of transcription elongation	Latif *et al.* (1993)
APC	5q21-q22	Familial adenomatous polyposis	β-catenin binding	Groden *et al.* (1991); Nishisho *et al.* (1991)
MCC	5q21-q22	-	Regulating cell cycle progression	Kinzler *et al.* (1991a)
DCC	18q21	-	Netrin receptor	Fearon *et al.* (1990)
DPC4	18q21	-	Effector of TGF-β signalling	Hahn *et al.* (1996)
TSC1	9q34	Tuberous sclerosis	Not known	van Slegtenhorst *et al.* (1997)
TSC2	16p13	Tuberous sclerosis	GTPase activating protein	The European chromosome 16 Tuberous Sclerosis consortium (1993)
p16	9p21	Familial melanoma	Cyclin dependent kinase inhibitor	Hussussian *et al.* (1994); Kamb *et al.* (1994)
p15	9p21	-	Cyclin dependent kinase inhibitor	Hannon and Beach (1994); Jen *et al.* (1994a)
BRCA1	17q21	Familial breast cancer	Contains putative granin motif	Miki *et al.* (1994)
BRCA2	13q12-q13	Familial breast cancer	Contains putative granin motif	Wooster *et al.* (1995)
patched	9q22	Basal cell nevus syndrome	Transcription repressor	Hahn *et al.* (1996)

into single strands. The temperature is then lowered to an optimum to allow the primers to anneal, and then raised (typically to 72°C) for polymerase extension. The primers are oriented 5' to 3' so that DNA synthesis by the polymerase proceeds across the region between the primers, effectively doubling the amount of that DNA segment. This cycle of denaturation, annealing, and elongation is repeated 20-35 times, resulting in exponential accumulation of the target DNA sequence.

Although the aforementioned techniques can identify genetic variants within PCR products, these have to be sequenced in order to precisely define the nature of the change. Direct sequencing of PCR products is based on the Sanger dideoxy chain termination method (Sanger *et al.*, 1977). Direct sequencing itself has been used to scan genes for unknown mutations but this is laborious, time-consuming and expensive. Automation of direct sequencing involving the use of fluorescently labelled primers or dideoxy terminators is however making this method more efficient.

1.3.3 (a) Single-strand conformation polymorphism analysis

Single-strand conformation polymorphism (SSCP) analysis was first reported by Orita *et al.* (1989a and b). This technique is based on the principle that under non-denaturing conditions a single-stranded DNA molecule folds into a unique three-dimensional conformation that is stabilized by intramolecular interactions. Consequently the conformation, and therefore the mobility, is dependent on the primary sequence (Orita *et al.*, 1989 a and b). PCR products are heat denatured with formamide to attain single strands of DNA, and then electrophoresed through a non-denaturing polyacrylamide gel. The two complementary strands will each form a conformer of differing mobility, although under certain conditions a single strand may adopt more than one stable conformation. Even a single base substitution in a PCR fragment can induce a conformational change that is detectable as altered mobility of mutant strands in the polyacrylamide gel (Orita *et al.*, 1989 a and b). Some studies have used radiolabelled primers or nucleotides to generate a radioactive PCR product, followed by autoradiography to visualize SSCP bands (Orita *et al.*, 1989b; Sheffield *et al.*, 1993). Alternatively non-isotopic detection by silver staining (Ainsworth *et al.*, 1991) or ethidium bromide staining (Hongyo *et al.*, 1993) may be used.

SSCP is widely used as it is easy to set up and requires relatively less labour and expense than other methods. The reported sensitivity of this technique varies between authors but it is generally indicated that 70-95% of single base substitutions

are detectable in PCR products of 100-400 bp with an optimal size of approximately 150 bp (Orita *et al.*, 1990; Hayashi and Yandell, 1993; Sheffield *et al.*, 1993). A number of parameters can be varied to improve efficiency including electrophoresis temperature, buffer concentration, and gel content (Glavac and Dean, 1993). Modifications of the technique such as RNA-SSCP (Sarkar *et al.*, 1992a) and dideoxyfingerprinting (Sarkar *et al.*, 1992b) have been proposed to improve the detection level but these are more complex methods.

Double stranded DNA molecules are not usually completely eliminated during SSCP analysis, and so the formation of heteroduplex molecules, between mutant and wild-type fragments (see below), have been observed on gels used for SSCP screening where they migrate faster than single-stranded DNA (Ravnik-Glavac *et al.*, 1994). Their detection has increased the sensitivity of the SSCP technique particularly for detecting small insertions or deletions, which create stable heteroduplexes.

1.3.3 (b) Heteroduplex analysis

The direct detection of heteroduplex molecules alone on acrylamide or other vinyl polymer gels has been used to identify mutations in many genes (Glavac and Dean, 1995). Heteroduplexes can be formed during the late cycles of PCR if the target DNA contains two different alleles, or by mixing PCR products of mutant and wild-type DNAs. Heteroduplex molecules with just a single base substitution have been shown to migrate slower than their corresponding homoduplexes (White *et al.*, 1992). This phenomenom is thought to be due to a more 'open' double-stranded configuration surrounding the mismatched bases. Although other methods appear to be more sensitive than heteroduplex analysis (Glavac and Dean, 1995) this technique is attractive because of its simplicity and it can complement SSCP as discussed above.

1.3.3 (c) Protein truncation test

The protein truncation test or PTT (also known as in vitro synthesized-protein assay, IVSP) allows the detection of mutations that specifically result in premature termination of translation. These include nonsense mutations, splice-site alterations, and frame-shift causing deletions and insertions. Such a technique is useful for screening disease genes where translation-terminating mutations are frequent, for example in the *APC* tumour suppressor gene, or the dystrophin gene in Duchenne Muscular Dystrophy patients (Nagase and Nakamura, 1993; Roberts *et al.*, 1994).

PTT is based on isolation of RNA which is amplified by RT-PCR (reverse-transcription-polymerase chain reaction). The upstream PCR primer is modified to contain a T7-promoter sequence for the initiation of transcription and a eukaryotic translation initiation sequence. This modification allows *in vitro* coupled transcription and translation of the PCR product (Roest *et al.*, 1993). The resultant translation products are analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and a truncating mutation can be identified as a shortened polypeptide compared to the wild-type polypeptide. The size of the truncated polypeptide localizes the site of the mutation in the PCR fragment fairly precisely therefore only a small part of the fragment needs to be sequenced (Roest *et al.*, 1993). Incorporation of ^{3}H-labelled leucine or ^{35}S-labelled methionine has been used to detect the translation products after electrophoretic separation (Roest *et al.*, 1993; Powell *et al.*, 1993). More recently a non-radioactive method involving biotin labelling of proteins followed by chemiluminescent detection has been described (Becker *et al.*, 1996).

Large stretches of coding sequence can be analysed, fragments of up to 2.4 kb have been used as templates for the PTT (Powell *et al.*, 1993; Roest *et al.*, 1993). However missense mutations cannot be detected by this method. RNA needs to be isolated in order to generate cDNA templates for the transcription-translation reaction for genes with multiple, small exons. However for screening large exons in genes by PTT, for example those in the *APC* and *BRCA1* tumour suppressor genes, DNA template for the transcription-translation reaction can be generated by amplification of genomic DNA (Powell *et al.*, 1993; van der Luijt *et al.*, 1994; Plummer *et al.*, 1995).

1.3.3 (d) Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is based on the electrophoretic mobility of a double-stranded DNA fragment through linearly increasing concentrations of a denaturant (urea and/or formamide or temperature) (Fischer and Lerman, 1983; Myers *et al.*, 1985a; Wartell *et al.*, 1990). As the DNA molecule migrates through the gradient gel it remains double stranded until it reaches a position where the concentration of the denaturant is equivalent to the melting temperature (T_m) of its lowest melting domain, causing the strands to dissociate. This partial 'melting' of the double-stranded DNA leads to an abrupt decrease in its electrophoretic mobility. The T_m of a melting domain is dependent on its nucleotide sequence thus DNA fragments differing by only a single base substitution in their lowest melting domain will have a differing T_m and consequently their mobility will

be retarded at different positions in the gel, allowing their separation (Myers *et al.*, 1988).

A base change in the highest temperature melting domain of a DNA fragment cannot be resolved because complete strand dissociation causes loss of sequence-dependent mobility. The introduction of a GC rich sequence (termed a GC-clamp) into the DNA fragment, as a tail to one of the PCR primers, can serve as a high T_m domain for the DGGE analysis, thus preventing complete denaturation of the DNA fragment (Sheffield *et al.*, 1989). The sensitivity of DGGE is also improved if heteroduplexes are generated between wild-type and mutant DNA fragments as the presence of a mismatch makes them easier to melt allowing separation from homoduplexes and aiding visual detection of mutants (Sheffield *et al.*, 1989). In addition computer programs that predict melting behaviour of a target DNA fragment can optimise the analysis (Lerman and Siverstein, 1987). GC-clamped DGGE has a high sensitivity of detection (about 99% in a 500 bp fragment) (Fodde and Losekoot, 1994). A modification of the technique, constant denaturing gel electrophoresis, CDGE, has been described (Borresen *et al.*, 1991).

1.3.3 (e) Ribonuclease A protection analysis

In Ribonuclease A (RNase A) protection analysis (also known as Ribonuclease A cleavage analysis), the target DNA fragment is hybridised to a single-stranded radioactive RNA probe, corresponding to the normal genomic sequence. The RNA:DNA hybrid is treated with RNase A which can cleave at a single base pair mismatch within the hybrid, generated if the DNA sample contains a base change. The products are analysed by denaturing gel electrophoresis followed by autoradiography. If the test DNA is complementary to the RNA strand, a single band is observed, however if there is a base change in the test DNA and the mismatch in the RNA:DNA hybrid is efficiently cleaved, two bands are observed (Myers *et al.*, 1985b; 1988). The size of DNA fragment that can be analysed is 100-1000 bp. RNase protection analysis can detect about 35% of all possible mismatches within a fragment but if two separate cleavage analyses are performed, one with a sense probe, the other with the antisense probe, 60-70% of all base changes in the fragment can be detected (Myers *et al.*, 1988).

1.3.3 (f) Chemical mismatch cleavage

In chemical mismatch cleavage (CMC) a heteroduplex is generated between a radiolabelled wild-type DNA strand and a test DNA strand. Any mismatched base in

this double-stranded molecule is then chemically modified by treatment with either hydroxylamine (modifies mismatched cytosine) or osmium tetroxide (modifies mismatched thymine) or both. Treament with piperdine then cleaves at the site of modification. As with RNase cleavage analysis, the cleavage products are detected by autoradiography after denaturing gel electrophoresis (Cotton *et al.*, 1988). When both DNA fragments in the heteroduplex are labelled and modification is performed using both hydroxylamine and osmium tetroxide, CMC can identify 100% mismatches (Forrest *et al.*, 1991) thus it is highly sensitive. The precise localization and nature of the sequence alteration is also indicated by the size of the cleavage band and the cleaving reagent. DNA fragments up to 1200 bp in length have been successfully analysed (Grompe *et al.*, 1989).

Genetic alterations in colorectal and gastric neoplasia will be reviewed in the following sections. As some genes relevant to gastric cancer were first identified through the study of colorectal cancer, the latter malignancy will be discussed first.

1.4. Genetic studies in colorectal cancer

The genetics of colorectal cancer is better characterized than that of most other neoplasms. The availability of tumours from both hereditary and sporadic colorectal cancer patients, of various stages of development from very small adenomas to large metastatic carcinomas, has provided a unique opportunity to study the genetic alterations involved in the development and progression of a common human malignancy.

1.4.1. Genetic alterations in oncogenes

One of the most significant somatic alterations in colorectal tumours is thought to be mutation of *ras* genes. The H-, K-, and N-*ras* genes encode closely related 21 kDa membrane bound proteins (p21) with intrinsic GTPase activity (Marshall, 1988). They are most commonly activated to transforming genes by point mutations at codons 12, 13, and 61. Mutations of *ras* genes have been found in approximately 50% of colorectal carcinomas, most of them were detected at codon 12 of K-*ras* (Bos *et al.*, 1987; Forrester *et al.*, 1987; Vogelstein *et al.*, 1988). Vogelstein *et al.* (1988) found *ras* mutations to be similarly prevalent in adenomas greater than 1cm, but present in less than 10% of smaller adenomas, indicating that *ras* gene mutation is a relatively early but not initiating event in colorectal tumourigenesis. In a genetic model of colorectal tumourigenesis (Fearon and Vogelstein, 1990) it was hypothesized that K-*ras* gene mutation would occur in one cell of a preexisting small

adenoma and through clonal expansion produce a larger and more dysplastic tumour, which would progress to carcinoma as a result of other genetic alterations.

More recently a high frequency of K-*ras* codon 12 mutations has been reported in aberrant crypt foci (ACF) of the colorectum (Pretlow *et al.*, 1993; Smith *et al.*, 1994), a lesion which is thought to precede the development of an adenoma and is therefore the earliest identifiable precursor of a colorectal cancer (Pretlow *et al.*, 1991). But Jen *et al.* (1994b) found that K-*ras* mutations were present not in dysplastic ACF but in hyperplastic ACF, which are thought to have little or no malignant potential to progress to a clinically important tumour (Shpitz *et al.*, 1996), and concluded that such mutations are not initiators of colorectal neoplasia.

Other oncogenes that are proposed to be involved in colorectal tumourigenesis include c-*myc*: overexpression of c-*myc* mRNA has been observed in colonic polyps and carcinomas (Stewart *et al.*, 1986; Smith *et al.*, 1993). Activation of protooncogenes by amplification has been found to be rare in colorectal tumours, the few reported cases include c-*myb* and c-*erb*B-2 gene amplification (Alitalo *et al.*, 1984; Gutman *et al.*, 1989).

1.4.2. Allele loss studies

In order to determine the extent and variation of allelic loss in colorectal cancer, Vogelstein *et al.* (1989) studied polymorphic DNA markers from every non-acrocentric autosomal arm in 56 paired colorectal carcinoma and normal mucosa specimens. Loss of heterozygosity (LOH) was found to be a common event, being detected with each marker tested. However of most significance was the finding that alleles from chromosomal arms 17p and 18q were lost in more than 75% of tumours (Vogelstein *et al.*, 1989). Other studies also reported a high frequency of LOH on chromosome 17p and 18q in colorectal carcinomas from both sporadic colorectal cancer patients and patients with the dominantly inherited colorectal cancer predisposition syndrome, familial adenomatous polyposis (FAP) (Law *et al.*, 1988; Vogelstein *et al.*, 1988; Sasaki *et al.*, 1989; Fey *et al.*, 1989). In addition to studying colorectal carcinomas, Vogelstein *et al.* (1988) examined colorectal adenomas representing different stages of neoplastic development, from sporadic and FAP patients. Three classes of adenomas were described with size, grade of dysplasia, and villous component generally increasing from Class I to Class III. Class I adenomas were generally small tubular adenomas with low grade dysplasia, Class II adenomas consisted of lesions without associated foci of carcinoma, and Class III adenomas were more advanced adenomas that had given rise to areas of invasive

adenocarcinoma. On chromosome 18q, allele loss was detected frequently in carcinomas and in Class III adenomas but infrequently in the earlier stage Class I and II adenomas. On chromosome 17p, LOH was observed frequently in carcinomas but infrequently in all 3 classes of adenomas (Vogelstein *et al.*, 1988).

Allelic loss on chromosome 5q also appeared to be significant in FAP and sporadic colorectal cancers. The first clue as to the importance of this chromosome came from cytogenetic analysis of an individual with both FAP and mental retardation which revealed a constitutional deletion in the long arm of chromosome 5 (del (5) (q13q15) or (q15q22) (Herrera *et al.*, 1986). Following this observation linkage analyses in FAP families demonstrated tight linkage of the disease to polymorphic DNA markers on chromosome 5q21 (Bodmer *et al.*, 1987; Leppert *et a*l., 1987). Thus a chromosomal location for the gene responsible for FAP was suggested. Loss of heterozygosity studies were carried out using polymorphic markers from this region in FAP and sporadic colorectal tumours. In accordance with the 'recessive' hypothesis for tumour suppressor genes, in individuals with a germline mutation in the FAP gene there would be a requirement for inactivation of the remaining wild-type allele (for example by loss or intragenic mutation) at the predisposition locus for tumour formation, while in sporadic colorectal cancer cases both mutational events would occur somatically (Knudson 1971; 1985). Allelic loss of chromosome 5q was reported in 20-50% of colorectal carcinomas from patients with sporadic colorectal cancer and FAP (Solomon *et al.*, 1987; Vogelstein *et al.*, 1988; Sasaki *et al.*, 1989; Vogelstein *et al.*, 1989; Okomoto *et al.*, 1990). Vogelstein *et al.* (1988) also detected LOH in about 30% of sporadic colorectal adenomas, even Class II adenomas less than 1cm in size. However in adenomas from patients with FAP, allelic losses of chromosome 5q were rarely observed (Solomon *et al.*, 1987; Vogelstein *et al.*, 1988; Rees *et al.*, 1989). This finding contrasted with the pattern of allelic loss seen in other inherited tumour predisposition syndromes such as retinoblastoma (Cavenee *et al.*, 1983; 1985).

Allelic losses on many other chromosomes have been observed in colorectal tumours. A high rate of loss on chromosome 1p, particularly in the region 1p36, has been reported in sporadic colorectal carcinomas (42-84%) (Leister *et al.*, 1990; Praml *et al.*, 1995a) and sporadic colorectal adenomas (20%) (Lothe *et al.*, 1995) leading to the suggestion that inactivation of a tumour suppressor gene in this chromosomal region is an early event in the adenoma-carcinoma sequence (Bardi *et al.*, 1993; Lothe *et al.*, 1995). LOH on chromosome 8p has been detected in approximately one-half of colorectal carcinomas (Emi *et al.*, 1992; Fujiwara *et al.*, 1993; Gustafson *et al.*, 1996). Furthermore there is evidence for the existence of two tumour

suppressor genes on chromosome 8p for colorectal carcinoma (Fujiwara *et al.*, 1993). The association of chromosome 8p LOH with advanced stage colorectal carcinomas (Fujiwara *et al.*, 1993) and the low rate of 8p LOH in colorectal adenomas (Cunningham *et al.*, 1994; Gustafson *et al.*, 1996) suggested that inactivation of the putative chromosome 8p tumour suppressor genes is important in the later stages of colorectal tumourigenesis. Other chromosomes showing a high frequency of allele loss in colorectal cancer include chromosome 14 (30-53% colorectal carinomas) (Sasaki *et al.*, 1989; Young *et al.*, 1993a) and chromosome 22 (19-40% colorectal carcinomas) (Vogelstein *et al.*, 1989; Sasaki *et al.*, 1989; Yana *et al.*, 1995). LOH on these two chromosomes was associated with advanced Dukes' stages (Dukes' C1 and C2) (Young *et al.*, 1993; Yana *et al.*, 1995).

In their genetic model of colorectal tumourigenesis, Fearon and Vogelstein (1990) proposed that loss or inactivation of the familial adenomatous polyposis gene on chromosome 5q may be the initiating event, leading to the formation of precancerous adenomatous polyps. Allelic deletions on chromosomes 18q and 17p were proposed to respectively occur at later stages of tumourigenesis, loss of 17p being associated with the progression from advanced adenoma to carcinoma. Loss of tumour suppressor genes on other chromosomes also occurs and the accumulated loss correlates with the ability of the carcinomas to metastasize and cause death (Fearon and Vogelstein, 1990). Genes on chromosomes 5, 17, and 18 involved in colorectal cancer development have been identified and will be described in the following sections.

1.4.3. Genetic alterations in tumour suppressor genes

1.4.3 (a) Adenomatous Polyposis Coli (*APC*) gene

The gene on chromosome 5q21-22 responsible for FAP, named *APC*, was isolated by positional cloning methods by two groups in 1991 (Joslyn *et al.*, 1991; Kinzler *et al.*, 1991b). Co-segregation of inactivating mutations of the *APC* gene in affected kindreds was demonstrated (Groden *et al.*, 1991; Nishisho *et al.*, 1991). Groden *et al.* analysed the majority of the coding region of *APC* using single-strand conformation polymorphism (SSCP) analysis and detected variant conformers in 4 of 61 unrelated FAP patients that were not observed in DNA samples from unaffected controls. Sequencing analysis revealed two of the variants to be the result of point mutations which created stop codons (nonsense mutations), while the two other variants were characterized as small deletions that altered the reading frame and created new stop codons immediately downstream of the mutations. Thus all 4

mutations were predicted to result in truncation of the *APC* gene product. Furthermore transmission of a frameshift mutation detected in one of the FAP patients to two of his children was demonstrated.

Nishisho *et al.* (1991) similarly detected mainly inactivating mutations when 3 exons of the *APC* gene were analysed in 103 FAP kindreds using an RNase A protection assay. Five variants were observed all of which were characterized as being due to single base substitutions on sequencing. Four of these mutations resulted in the creation of stop codons and one resulted in a missense mutation. Moreover, in one kindred the stop codon precisely co-segregated with disease phenotype in 21 members including 12 patients with FAP. Thus these studies showed that the *APC* gene caused FAP. In addition to identifying germline *APC* mutations, Nishisho *et al.* (1991) detected somatic mutations in sporadic colorectal carcinomas. Examining the same region of the *APC* gene as in the FAP patients, mutations were observed in 3 of 158 colorectal tumours. One tumour contained a 5 bp insertion that resulted in a stop codon due to a frameshift, another tumour contained a point mutation that created a stop codon, while the third tumour harboured a point mutation that altered a splice donor site. Thus all the mutations were predicted to have profound effects on the *APC* gene product.

The *APC* gene is approximately 8,500 bp long, consisting of 15 exons. Exons 1 to 14 are small, ranging in size from between 79 and 379 bp. The most 3' exon of *APC*, exon 15, is very large being 6.5 kb in length (Groden *et al.*, 1991). The gene was found to be expressed in a variety of tissue types including normal colon mucosa, brain, lung, aorta, spleen, heart, kidney, liver, stomach, placenta, peripheral white blood cells, and skin (Groden *et al.*, 1991; Kinzler *et al.*, 1991b). It was predicted from the cDNA sequence of *APC* that the gene codes for a 2843 amino acid peptide with a mass of approximately 311 kDa. Database searches with the *APC* gene product for amino acid similarities with known proteins gave little indication as to its function. In the amino-terminal domain of the APC protein local sequence similarities to intermediate filament proteins such as myosins and keratins and also to the Drosophila armadillo protein (human plakoglobin) were identified. The similarities with the former type of protein were largely due to a series of heptad repeats. These regions can form uninterrupted α helices and are thought to be able to facilitate protein-protein interactions by allowing hydrophobic interaction between α helices on two polypeptides, so the two helices associate through the formation of a coiled coil (Cohen and Parry, 1986). Thus APC could potentially form homo- or heterodimers. Another putative coiled coil region was identified in the central portion of APC (Groden *et al.*, 1991; Kinzler *et al.*, 1991b). In addition to heptad repeats,

multiple potential serine phosphorylation motifs, glycosylation motifs, and myristoylation sites were noted. No hydrophobic regions indicating potential signal peptides or transmembrane domains were found in the APC protein and its overall hydrophilic nature suggested a cytoplasmic localization (Kinzler *et al.*, 1991b; Groden *et al.*, 1991).

The *APC* gene was analysed for mutations by Groden *et al.* (1991) and Nishisho *et al.* (1991) using PCR followed by SSCP and RNase protection analysis respectively. Exons 1 - 14 were of suitable size to be amplified individually by PCR. As exon 15 was so large, representing 77% of the coding region (codons 653-2843), it was divided into 17 to 23 overlapping segments which were of suitable length for PCR amplification and for analysis with the mutation detection techniques used (Groden *et al.*, 1991; Nishisho *et al.*, 1991; Miyoshi *et al.*, 1992a). Following the studies on the identification of the *APC* gene, there were a number of reports describing the detection of germline and somatic mutations using a variety of mutation detection techniques including denaturing gradient gel electrophoresis (DGGE) (Fodde *et al.*, 1992; Wallis *et al.*, 1992), direct DNA sequencing (Powell *et al.*, 1992), as well as SSCP (Cottrell *et al.*, 1992; Groden *et al.*, 1993) and RNase protection analysis (Miyoshi *et al.*, 1992a and b; Nagase *et al.*, 1992a).

In the largest study, one group screened the entire coding region of the *APC* gene in 150 unrelated FAP patients and detected germline alterations in 97 (65%) individuals (Miyoshi *et al.*, 1992a; Nagase *et al.*, 1992a). It was found that the great majority of mutations (over 90%) were predicted to lead to truncation of the *APC* gene product due to the creation of new stop codons as a result of either a point mutation or a frameshift caused by small deletions or insertions. Another interesting finding was that 97% of the germline mutations were located within the 5' half of the coding region of the gene. In addition, greater than 70% of the mutations were clustered in the 5' half of exon 15, between codons 713 and 1597 (Miyoshi *et al.*, 1992a; Nagase *et al.*, 1992a). The above findings were supported by another group who also examined the entire *APC* coding region in 60 unrelated FAP patients by SSCP analysis and observed that 100% (16 of 16) germline mutations were truncating mutations, and 69% of these were located in the 5' half of exon 15 (Groden *et al.*, 1991; 1993). Two 5 base pair deletions, at codons 1061 and 1309 respectively, were observed in more than one patient. Results from these and other studies showed that these two mutations accounted for more than a quarter of the total germline mutations detected in FAP patients from varying ethnic backgrounds, indicating that these sites represented mutational hotspots (Miyoshi *et al.*, 1992a; Nagase *et al.*, 1992a; Cottrell *et al.*, 1992; Wallis *et al.*, 1992; Varesco *et al.*, 1993). Mutations in the 3' half of the

APC gene have been described in FAP patients although they appear to be relatively rare (Miyoshi *et al.*, 1992a; Scott *et al.*, 1995; van der Luijt *et al.*, 1996).

The high proportion of translation-terminating mutations observed in the *APC* gene led to the use of the protein truncation test (also known as in vitro synthesized-protein assay) in screening FAP patients, a technique that specifically detects truncating mutations (Powell *et al.*, 1993; van der Luijt *et al.*, 1994). The other methods used to screen the *APC* gene for mutations such as SSCP, DGGE, and RNase protection analysis typically involved analysis of PCR fragments of < 400 bp in length making mutation detection a labour-intensive process (Groden *et al.*, 1993; Miyoshi *et al.*, 1992a; Fodde *et al.*, 1992). Larger stretches of coding region could be examined with the PTT. For example Powell *et al.* (1993) analysed the first 14 exons of *APC* in a single coupled transcription-translation reaction, having generated DNA template for the reaction by amplifying these exons together by RT-PCR. Exon 15 could be amplified directly from genomic DNA in 4 overlapping segments which were each then subjected to the in vitro synthesized-protein assay. Truncating mutations were detected in 51 of 62 (82%) FAP patients. van der Luijt *et al.* (1994) described protein truncation test (PTT) analysis of a 2 Kb fragment of exon 15 of *APC*, representing 24% of the coding region, generated by genomic DNA amplification. The part of the gene analysed included a mutation dense region where somatic mutations in particular are found to cluster (Miyoshi *et al.*, 1992b).

As well as screening FAP patients for germline *APC* mutations, in additional studies colorectal tumours were investigated for somatic mutations (Miyoshi *et al.*, 1992b; Powell *et al.*, 1992). In both reports the entire coding region of the *APC* gene was analysed. Miyoshi *et al.* examined 16 adenomas and 47 carcinomas developed in 48 sporadic colorectal cancer patients and 5 FAP patients, and identified somatic *APC* mutations in 37 (59%) tumours using RNase protection analysis. Powell *et al.* observed a similar frequency of somatic mutation using DNA sequencing, detecting alterations in 15 of 25 (60%) sporadic colorectal carcinomas and 10 of 16 (63%) sporadic colorectal adenomas. In common with germline mutations observed in FAP patients, the vast majority (>95%) of somatic mutations reported in both of these studies were predicted to cause truncation of the *APC* gene product.

All the somatic mutations were located in the first half of the coding region, again similar to germline mutations. Miyoshi *et al.* (1992b) reported that not only more than three-quarters of mutations occur in exon 15 of *APC* but 65% of total somatic mutations were clustered in a small part of this exon, spanning codons 1286 to 1513, which was thus designated the mutation cluster region (MCR). This area of

the gene represented less than 10% of the coding region of *APC*. Powell *et al.* (1992) observed that approximately 50% of somatic mutations occurred in a 722 bp region that encompassed codons 1281 to 1554, and thus corresponded to the mutation cluster region described by Miyoshi *et al.*

Two separate genetic alterations at the *APC* locus, either two intragenic mutations or an intragenic mutation and allelic loss, were observed in 32 of 63 (51%) colorectal tumours inclusive of 9 adenomas by Miyoshi *et al.* (1992b). Among the 32 tumours it could be determined in 20 tumours that the *APC* gene on both alleles was inactivated. Powell *et al.* (1992) also detected two alterations at the *APC* locus in 14 of 41 (34%) sporadic colorectal tumours inclusive of 5 adenomas. In 8 tumours it could be demonstrated that both copies of the *APC* gene were inactivated. Finally both Miyoshi *et al.* and Powell *et al.* observed that somatic *APC* mutations occurred at a similar frequency in colorectal adenomas as in colorectal carcinomas and were even detected in sporadic adenomas as small as 0.5cm in diameter. These results therefore supported the hypothesis that *APC* mutation is an early event in the development of colorectal tumours (Fearon and Vogelstein, 1990). In conclusion, these studies strongly suggested that mutations of the *APC* gene play a major role in the development of most sporadic colorectal tumours.

1.4.3 (b) Mutated in colorectal cancer (*MCC*) gene

The *MCC* gene on chromosome 5q21-22 was isolated prior to *APC* during attempts to identify the gene responsible for FAP (Kinzler *et al.*, 1991a). *MCC* was regarded as a candidate gene however no mutations of *MCC* were detected in the germline of FAP patients in this study (Kinzler *et al.*, 1991a) nor in a subsequent report from this group (Nishisho *et al.*, 1991). Later when the *APC* gene was cloned it was found to lie close to *MCC* at chromosome 5q21-22, the 3' ends of the two genes being separated by approximately 150 kb (Kinzler *et al.*, 1991b). Somatic mutations of the *MCC* gene were however detected in sporadic colorectal carcinomas. On screening the entire coding region of *MCC* in 90 sporadic colorectal cancers by RNase protection analysis, somatic point mutations were observed in 6 tumours (Kinzler *et al.*, 1991b; Nishisho *et al.*, 1991). Two of the point mutations were alterations at splice sites while 4 were missense mutations resulting in amino acid substitutions (Kinzler *et al.*, 1991b; Nishisho *et al.*, 1991).

Curtis *et al.* (1994) however failed to detect mutations in *MCC* on screening the entire coding region of the gene in 21 sporadic colorectal carcinomas that had exhibited LOH (loss of heterozygosity) at *MCC* intragenic polymorphic loci. Thus

MCC did not appear to function as a conventional tumour suppressor gene with mutation in the retained allele (Knudson, 1971). Curtis *et al.* analysed the sporadic colorectal carcinomas for LOH at intragenic polymorphic loci in the *APC* gene as well as in the *MCC* gene and at polymorphic loci flanking the two genes. In informative tumours exhibiting allele loss, loss of *MCC* independent of *APC* (and vice versa) was not observed. It was suggested that perhaps the mechanism which resulted in *APC* loss coincidentally deleted *MCC* (Curtis *et al.*, 1994). The role of the *MCC* gene in colorectal tumourigenesis remains unclear.

Analysis of the *MCC* protein may suggest a potential tumour suppressor role. Database searches with the predicted *MCC* gene product revealed the protein shares a short region (19 amino acid) of similarity with the G protein-coupled m3 muscarinic acetylcholine receptor (Kinzler *et al.*, 1991a). This finding was interesting as G proteins had previously been linked to neoplasia, for example the *ras* proto-oncogenes are members of the G protein family (Marshall, 1988) and the *NF1* tumour suppressor gene product has been shown to activate the guanine triphosphatase activity of the ras protein (Shen *et al.*, 1996). The MCC protein like the APC protein was found to contain regions that have a high probability of forming coiled-coil structures (Bourne, 1991; Kinzler *et al.*, 1991b). Such structures often mediate homo- and hetero-oligomerization (Cohen and Parry, 1986) and so it was suggested that MCC and APC may interact with each other (Nishisho *et al.*, 1991; Groden *et al.*, 1991). However subsequent studies have failed to provide evidence in support of this hypothesis (Su *et al.*, 1993; Matsumine *et al.*, 1996a). More recently it was shown that overexpression of the MCC protein blocked cell cycle progresssion from G1 to S phase in mouse cells whereas a mutant MCC protein did not exhibit this activity (Matsumine *et al.*, 1996a). It was proposed that MCC may have a role in the negative regulation of cell cycle progression as has been suggested for the products of the *p53* and retinoblastoma tumour suppressor genes (Lin *et al.*, 1992; Hollingsworth *et al.*, 1993).

1.4.3 (c) *p53* gene

As previously described allelic deletion of chromosome 17p was found to be a frequent event in colorectal carcinoma (Vogelstein *et al.*, 1988; 1989). The common region of deletion was localized and found to encompass the *p53* gene (Baker *et al.*, 1989). On examination of the *p53* coding region in two colorectal tumours with allelic deletions of chromosome 17p, the remaining *p53* allele was found to be mutated in both tumours (Baker *et al.*, 1989). In subsequent studies it was demonstrated that the majority of colorectal tumours with allelic deletions of

chromosome 17p had mutations in the remaining *p53* allele, while tumours that retained both copies of chromosome 17p infrequently harboured *p53* mutations (Nigro *et al.*, 1989; Baker *et al.*, 1990a; Yamaguchi *et al.*, 1997). This was consistent with Knudson's two-hit hypothesis as both copies of the *p53* gene were inactivated in tumourigenesis (Knudson, 1971; 1985).

Baker *et al.* (1990a) found that *p53* gene mutations rarely occurred in adenomas, consistent with earlier studies demonstrating that allelic deletions of chromosome 17p were rarely seen before the carcinoma stage (Vogelstein *et al.*, 1988). Thus alteration of the *p53* gene appeared to be a late step in the progression of colorectal cancer. Baker *et al.* (1990a) suggested that at some point in colorectal tumourigenesis, probably after adenoma development, expression of wild-type *p53* may become rate-limiting for cell growth perhaps because of other genetic alterations that have accumulated and selection for *p53* point mutation followed by allelic deletion would then occur. Support for this hypothesis came in an *in vitro* transfection study in which wild-type *p53* gene suppressed growth of colorectal carcinoma but not adenoma cells (Baker *et al.*, 1990b).

1.4.3 (d) Deleted in colon carcinoma (*DCC*) gene

The high frequency of allelic deletion on chromosome 18q observed in colorectal carcinomas (Vogelstein *et al.*, 1988; 1989) led to the search for a tumour suppressor gene on this chromosome. The common region of loss was localized and a candidate tumour suppressor gene from this region (18q21.2), termed *DCC*, was isolated (Fearon *et al.*, 1990). Somatic mutations of the *DCC* gene detectable by Southern blotting were observed in 12 of 94 (13%) colorectal carcinomas (Fearon *et al.*, 1990). A homozygous deletion at the 5' end of the gene was observed in one tumour, an intronic point mutation was observed in another, while insertions ranging from 120 to 300 bp in a microsatellite sequence located downstream of one of the *DCC* exons were detected in ten further tumours. The effects of these alterations on *DCC* expression were not known. Cho *et al.* (1994) further characterized the genomic structure of the *DCC* gene. *DCC* was found to be a very large gene, spanning approximately 1.4 Mb, and included at least 29 exons. Due to the large size and complexity of *DCC* (29 exons with alternative splicing), extensive mutational analysis of the gene has not been carried out. Cho *et al.* (1994) however analysed several *DCC* exons and flanking intronic sequences in colorectal carcinomas, and identified a somatic point mutation in an intron in one tumour and a somatic missense mutation in exon 28 in another tumour. Again the functional significance of these alterations was not known.

The *DCC* gene was found to be expressed in most normal adult tissues at low levels, with highest expression being observed in the brain and central nervous system (Fearon *et al.*, 1990; Reale *et al.*, 1994). Using a sensitive RT-PCR assay for the detection of *DCC* transcripts, the level of *DCC* expression seen in normal colonic mucosa was found to be significantly reduced or absent in the majority of colorectal cancer cell lines (Fearon *et al.*, 1990). Further studies also indicated that DCC mRNA expression was reduced during colorectal cancer progression and that the absence of DCC expression was associated with metastasis of these tumours (Itoh *et al.*, 1993; Iino *et al.*, 1994). In a later study it was suggested that expression of the DCC protein was a prognostic marker in patients with TNM stage II or stage III colorectal cancer (Shibata *et al.*, 1996). In patients with colorectal cancers of stage II (Dukes' stage B) and with an absence of DCC expression the clinical outcome was similar to that in patients with stage III disease. In contrast, patients with DCC-positive stage II tumours had a significantly longer overall survival (Shibata *et al.*, 1996). The mechanism of loss or reduced expression of *DCC* remains to be resolved. Loss of heterozygosity and loss of expression of *DCC* have been observed in many other tumour types too, including prostate carcinoma (Gao *et al.*, 1993), oesophageal carcinoma (Huang *et al.*, 1992), pancreatic carcinoma (Hohne *et al.*, 1992), breast carcinoma (Thompson *et al.*, 1993), and brain tumours (Ekstrand *et al.*, 1995).

Further support for a tumour suppressor function for *DCC* was suggested in a study in which it was found that transfection of full length but not truncated *DCC* cDNA constructs into transformed human kearatinocytes lacking *DCC* expression, suppressed tumourigenicity of these cells in nude mice (Klingelhultz *et al.*, 1995). The *DCC* gene was found to encode a transmembrane protein that belongs to a class of cell adhesion molecules that are members of the immunoglobulin (Ig) superfamily characterized by the presence of four Ig domains and six fibronectin type III repeats in their extracellular domains (Fearon *et al.*, 1990; Reale *et al.*, 1994). Fearon *et al.* (1990) suggested a role for *DCC* in colorectal cancer progression involving alterations of normal cell-cell interactions controlling growth. In later studies it was demonstrated that proteins identified in *Caenorhabditis elegans* and *Drosophila melanogaster* which are thought to be components of netrin receptors were homologues of the vertebrate DCC protein (Chan *et al.*, 1996; Kolodziej *et al.*, 1996). Netrins are proteins that guide axons to the correct position during the development of the nervous system (Kolodziej *et al.*, 1996). In a further study in rats it was demonstrated that the DCC protein itself possesses netrin-1-binding activity and is probably a component of a mammalian netrin receptor involved in the guidance of developing axons (Keino-Masu *et al.*, 1996).

In transgenic mice studies, mice with a single functional copy of the *DCC* gene ($Dcc^{+/-}$ mice) surprisingly did not show an increased tumour predisposition compared to wild-type $Dcc^{+/+}$ mice, only 1 of 200 $Dcc^{+/-}$ mice developed gastrointestinal tumours (Fazeli *et al.*, 1997). However homozygote knockout mice ($Dcc^{-/-}$ mice) embryos had defects in axonal projections and in brain development similar to those seen in netrin-1-deficient mice (Serafini *et al.*, 1996), thus the results supported the hypothesis that DCC is a component of a netrin receptor (Keino-Masu *et al.*, 1996). This study thus failed to support a tumour suppressor function for *DCC* but indicated a role in the development of the nervous system. It has been suggested that the above reports raise doubts about whether it is inactivation of the *DCC* gene on chromosome 18q21 in colorectal tumour cells that leads to tumour progression or another gene that lies close to it. Other genes on chromosome 18q may be responsible for the high frequency of alleleic loss observed on this chromosome in colorectal carcinomas (Fazeli *et al.*, 1997; Roush, 1997).

At least two other candidate tumour suppressor genes on chromosome 18q21 have been identified, *DPC4* and *JV18-1* (Hahn *et al.*, 1996; Thiagalingam *et al.*, 1996; Riggins *et al.*, 1996). By examining a panel of highly polymorphic microsatellite repeat markers from chromosome 18 in colorectal tumour and normal tissue pairs, Thiagalingam *et al.* (1996) detected allelic deletions in the tumours and identified a minimally lost region (MLR) on chromosome 18q21.1-2, spanning 16 Mb. Over two-thirds of colorectal cancers exhibited allele loss in this region. The MLR was found to contain the *DCC*, *DPC4*, and *JV18-1* genes (Thiagalingam *et al.*, 1996; Riggins *et al.*, 1996). *DPC4* was identified through studies of pancreatic carcinomas which exhibit a high frequency of allelic loss on chromosome 18q (Hahn *et al.*, 1995; 1996). *DPC4* was homozygously deleted in 30% of pancreatic carcinomas and inactivated by intragenic mutation in another 20% of the tumours (Hahn *et al.*, 1996). *DPC4* and *JV18-1* are human homologues of the *Drosophila* gene *Mad*, implicated in a Transforming Growth Factor-β (TGF-β) signalling pathway (Hahn *et al.*, 1996; Riggins *et al.*, 1996). TGF-β inhibits the growth of many epithelial cell types including colon epithelial cells (Polyak, 1996).

Eighteen colorectal carcinomas that each contained an allelic loss of the MLR on chromosome 18q21 were investigated for mutations in the remaining copies of the *DPC4* and *JV18-1* genes in studies by Thiagalingam *et al.* (1996) and Riggins *et al.* (1996). Somatic mutations of *DPC4* were identified in 5 tumours and of *JV18-1* in 2 tumours. The authors concluded that although these genes may be important in a subset of colorectal cancers, they were not the target of allelic loss in the majority of colorectal cancers, other gene(s) must be targets too (Thiagalingam *et al.*, 1996;

Riggins *et al.*, 1996). Thiagalingam *et al.* also evaluated the *DCC* gene in the eighteen colorectal carcinomas. Low or aberrant expression of *DCC* was detected in all 18 tumours, retaining *DCC* as a viable candidate for the target of inactivation during colorectal tumourigenesis, but it was suggested that this may reflect the expected expression level in the stem cells or be a consequence of events affecting a linked gene (Thiagalingam *et al.*, 1996).

1.4.4. The genetic basis of Hereditary Nonpolyposis Colorectal Cancer

As described in section 1.2.7. there is a second comparatively common colorectal cancer predisposition syndrome in addition to FAP that is also inherited as an autosomal dominant, Hereditary Nonpolyposis Colorectal Cancer (HNPCC). The genetic basis of this disease eluded investigators for many years. In 1985, gene linkage studies using blood group markers had suggested linkage of the Kidd blood group locus on chromosome 18 to HNPCC (Lynch *et al.*, 1985b). However no subsequent studies reported linkage between chromosome 18 markers and HNPCC and the *DCC* tumour suppressor gene on chromosome 18q was specifically excluded as the locus for susceptibility to HNPCC (Peltomaki *et al.*, 1991). Other tumour suppressor genes that were known to be of importance in sporadic colorectal cancer were also considered as candidates for the HNPCC gene but no linkage was found between HNPCC and the *APC / MCC* region on chromosome 5 (Peltomaki *et al.*, 1992) nor the *p53* gene locus on chromosome 17 (Lynch *et al.*, 1993).

Peltomaki *et al.* (1993) then reported their study of two large HNPCC kindreds. They searched the genome for genetic linkage using highly informative microsatellite markers (Weber and May, 1989; Weissenbach *et al.*, 1992). A total of 345 microsatellite markers were analysed before close linkage of the disease to markers on chromosome 2p15-16 was demonstrated. This group then studied 14 additional smaller HNPCC kindreds (Aaltonen *et al.*, 1993), performing linkage analyses with the closely linked $(CA)_n$ repeat marker from chromosome 2, *D2S123* (Peltomaki *et al.*, 1993). Linkage was excluded in 3 families but was possible in half the families. The findings indicated that HNPCC was either genetically heterogenous or that some sporadic cases of colorectal cancer may have confounded the linkage analysis (Aaltonen *et al.*, 1993). However these studies proved the existence of a genetically determined predisposition to HNPCC.

Aaltonen *et al.* further investigated the molecular pathogenesis of HNPCC. Putative tumour suppressor genes responsible for cancer predisposition are characteristically associated with a high frequency of allele loss in the region of the

gene in tumours (Knudson, 1993). Fourteen tumours derived from HNPCC patients and 46 sporadic colorectal tumours were analysed for loss of heterozygosity (LOH) at the *D2S123* microsatellite repeat locus. However this locus was found to be deleted in only 1 of 46 sporadic tumours (Aaltonen *et al.*, 1993). This indicated that the HNPCC gene on chromosome 2 did not act as a classic tumour suppressor gene. However, a surprising observation during analysis of the *D2S123* marker was that the majority of HNPCC tumours contained changes consisting of shifts in the electrophoretic mobility of the $(CA)_n$ repeat fragments. Some of the shifts resulted in alleles larger than those present in the normal tissue while other shifts resulted in smaller alleles. Five more $(CA)_n$ markers as well as a $(CAG)_n$ trinucleotide repeat were analysed. Extra allele bands in tumour DNA compared with corresponding normal DNA were observed with each marker. Eleven of the 14 HNPCC tumours (79%) demonstrated alterations in at least 2 of the markers and as the shifts suggested that replication errors (RER) had occurred in the repeat sequences during tumour development these tumours were classified as RER+. The RER+ tumours included 9 colorectal carcinomas, 1 colorectal adenoma, and 1 ovarian carcinoma. No mobility shifts were observed in the other 3 tumours which were classified as RER- (Aaltonen *et al.*, 1993).

The 46 sporadic colorectal carcinoma and corresponding normal tissues were also evaluated with the seven markers. Electrophoretic shifts in at least two markers were also observed in the sporadic tumours but at a much lower incidence than in the HNPCC tumours, 6 of 46 (13%) sporadic colorectal carcinomas being found to be RER+ (Aaltonen *et al.*, 1993). Two additional studies reporting such instability at simple repeat sequences in sporadic colorectal carcinomas were published at the same time as this study (Thibodeau *et al.*, 1993; Ionov *et al.*, 1993). Thibodeau *et al.* (1993) studied four $(CA)_n$ repeat markers in 90 colorectal tumour and normal pairs and identified electrophoretic shifts resulting in larger or smaller alleles in 25 (28%) tumours. Fifteen tumours exhibited alterations at multiple loci while ten tumours exhibited alterations at 1 locus only. Ionov *et al.* (1993) used a genomic fingerprinting approach, the arbitrarily primed polymerase chain reaction (AP-PCR), in which a large number of anonymous DNA fragments are amplified, to detect genetic alterations in tumours. Twelve percent of colorectal carcinomas exhibited somatic deletions in poly (dA.dT) sequences and other simple repeats. Ionov *et al.* (1993) and Aaltonen *et al.* (1993) suggested that extrapolation of their results would predict that the cancer cell genome in tumours showing the alterations at repeat sequences would contain thousands of such changes and thus represented widespread genetic instability.

Instability at repeat sequences in sporadic colorectal carcinomas was found to be correlated with location of sporadic tumours in the right side of the colon (proximal colon), increased patient survival, and diploidy, biologic features often shown by HNPCC tumours (Aaltonen *et al.*, 1993; Thibodeau *et al.*, 1993; Ionov *et al.*, 1993). These findings suggested that related mechanisms may be responsible for the instability in hereditary and sporadic tumours. Thibodeau *et al.* (1993) observed an inverse correlation between the colorectal tumours with electrophoretic shifts at multiple repeat loci and allele loss on chromosomes 5q, 17p, and 18q, while Ionov *et al.* (1993) noted an inverse correlation between the tumours showing such changes and *p53* and K-*ras* gene mutations. However colorectal tumours from HNPCC patients, which show a high incidence of shifts at microsatellite repeats, exhibited similar frequencies of *APC*, *p53*, and K-*ras* mutation to those observed in sporadic colorectal carcinomas in previous studies (Aaltonen *et al.*, 1993).

The observation of alterations at repeat sequences in colorectal tumours described above is referred to by various authors as replication error (RER) (Aaltonen *et al.*, 1993), microsatellite instability (MI or MIN) (Thibodeau *et al.*, 1993), or ubiquitous somatic mutation (USM) (Ionov *et al.*, 1993). In this thesis such changes will be referred to as replication error (RER). RER has now been observed in most extracolonic tumours from HNPCC patients (Aaltonen *et al.*, 1994; Risinger *et al.*, 1993). Moreover RER has been reported in a variety of sporadic tumours including gastric cancers (Mironov *et al.*, 1994), lung cancers (Merlo *et al.*, 1994), breast cancers (Yee *et al.*, 1994), and ovarian cancers (King *et al.*, 1995). The high incidence of RER observed in tumours from HNPCC patients led Aaltonen *et al.* (1993) to propose that the tendency to form such alterations can be inherited and may be associated with a defect of a gene responsible for HNPCC on chromosome 2 (Peltomaki *et al.*, 1993). It was suggested that this gene may code for a replication factor.

However workers on mismatch repair systems in bacteria and yeast recognised that the replication error observed in the tumours was similar to that observed in *Escherichia coli* harbouring mutations in DNA mismatch repair genes (Levinson and Gutman, 1987; Strand *et al.*, 1993). An elevated rate of contraction and expansion of dinucleotide repeat sequences was observed in yeast with mismatch repair gene mutations (Strand *et al.*, 1993). The authors suggested that the instability of these sequences was due to slippage of DNA polymerase during replication and the resulting errors not being corrected due to a defective mismatch repair system. The tandem nature of elemental units within microsatellite repeat sequences is thought to make them particularly prone to strand slippage during replication and hence prone to

insertion and deletion mutagenesis (Kunkel, 1993). Strand *et al.* (1993) further proposed that the putative HNPCC gene on chromosome 2 may be a human homologue of a bacterial and yeast mismatch repair gene. In fact this was found to be the case when cloning of the HNPCC gene on chromosome 2p16 was reported by two groups in 1993 (Fishel *et al.*, 1993; Leach *et al.*, 1993). Fishel *et al.* used a degenerate PCR approach targeted at the most highly conserved region of the bacterial mutS mismatch repair protein to clone the human homologue *hMSH2*, while Leach *et al.* used positonal cloning techniques and identified the same gene. Leach et al. (1993) detected germline mutations of hMSH2 in 3 large HNPCC kindreds: two of the mutations were truncating mutations while one mutation was a missense mutation located in a highly conserved region of the gene. Fishel *et al.* (1993) detected the same intronic T to C transition mutation in sporadic colorectal tumours and in affected individuals of two small HNPCC kindreds.

The bacterial mismatch repair (MMR) system repairs single base mismatches and small insertion and deletions in DNA. The system requires 10 components including 3 mismatch repair proteins mutS, mutL, and mutH (Fishel and Kolodner, 1995). In eukaryotes the system appears to be more complex, 6 *mutS* gene homologues and 4 *mutL* homologues having been described in yeast (Fishel and Kolodner, 1995). In humans, the mapping of a second locus predisposing to HNPCC at chromosome 3p21-23 (Lindblom *et al.*, 1993) led to the hypothesis that another homologue of a bacterial MMR gene resided at this locus. In March 1994 the same two groups that had identified the *hMSH2* gene reported the cloning of a human homologue of the bacterial *mutL* MMR gene, located at 3p21-23, and detected germline mutations of this gene, named *hMLH1*, in several HNPCC families (Bronner *et al.*, 1994; Papadopoulos *et al.*, 1994). Since the *hMSH2* and *hMLH1* genes were cloned other human homologues of bacterial MMR genes have been identified, including two further *mutL* homologues, *hPMS1* and *hPMS2* (post-meiotic segregation 1 and 2), located on chromosomes 2q31-33 and 7p22 respectively (Papadopoulos *et al.*, 1994; Nicolaides *et al.*, 1994), and two *mutS* homologues, *hMSH6* (also called *GTBP*) (Palombo *et al.*, 1995; Drummond *et al.*, 1995) and *hMSH3* (also called *DUG*) (Fujii and Shimada, 1989) which are located on chromosomes 2p16 and 5q respectively.

Mutation analyses of the MMR genes in HNPCC families indicate that the *hMLH1* and *hMSH2* genes account for the great majority (>90%) of germline mutations in these kindreds (literature reviewed by Papadopoulos and Lindblom, 1997 and Peltomaki *et al.*, 1997). The *hPMS2*, *hPMS1*, and *hMSH6* genes have been found to be mutated in only a few families (Nicolaides *et al.*, 1994; Hamilton *et al.*,

1995; Akiyama *et al.*, 1997a; Miyaki *et al.*, 1997). No germline mutatons of the *hMSH3* gene have yet been described although somatic mutations of this gene have been detected in HNPCC and sporadic tumours (Malkhosyan *et al.*, 1996; Akiyama *et al.*, 1997b). Somatic mutations of other MMR genes have been detected in sporadic RER+ tumours (Papadopoulos and Lindblom, 1997), the studies will be described in the Discussion chapter of this thesis (Chapter 4).

Parsons *et al.* (1993) demonstrated that the mutation rate of $(CA)_n$ repeats was 100-fold greater in RER+ colorectal tumour cells than in RER- cells. Biochemical assays demonstrated that the hypermutability of RER+ tumour cells was asssociated with a defect in strand-specific mismatch repair (Parsons *et al.*, 1993; Umar *et al.*, 1994). Transfer of a single human chromosome 3 harbouring a normal copy of *hMLH1* into a colon tumour cell line with homozygous *hMLH1* mutation completely restored mismatch repair activity and reversed the RER at microsatellite repeats (Koi *et al.*, 1994). This study supported the hypothesis that mutations in both alleles of MMR genes is necessary for manifestation of defective mismatch repair and RER (Leach *et al.*, 1993). It has been found that individuals with a germline mutation in a MMR gene generally exhibit somatic mutation or loss of the residual wild-type allele in their tumours (Leach *et al.*, 1993; Nicolaides *et al.*, 1994; Hemminki *et al.*, 1994; Miyaki *et al.*, 1997). Thus MMR genes appear to demonstrate two-hit inactivation like tumour suppressor genes. However in one report MMR deficiency and RER was detected in phenotypically normal cells from 3 patients (from 2 families) with truncating mutations in *hMLH1* and *hPMS2* (Parsons *et al.*, 1995a). Transgenic mice that are homozygous for mutation in either the *PMS2* or *MLH1* genes demonstrate RER in normal tissues (Baker *et al.*, 1995; Edelmann *et al.*, 1996). Parsons *et al.* (1995) hypothesized that some MMR gene mutations may be dominant negative mutations, whereby the mutant gene product inhibits the function of the normal gene product by interacting with it.

Figure 1.4 illustrates a proposed pathway of mismatch repair in human cells based on evidence from biochemical experiments. Single base mismatches or insertion/deletion loop-type mismatches are recognised by mutS homologues. It has been demonstrated that the hMSH2 protein functionally interacts with hMSH6 (GTBP) and with hMSH3 to form complexes that bind to mismatches in DNA (Drummond *et al.*, 1995; Palombo *et al.*, 1995; Acharya *et al.*, 1996). The evidence suggests that repair of single base substitution mispairs requires the hMSH2-hMSH6 complex but not the hMSH2-hMSH3 complex, that repair of single base insertion/deletion mismatches can utilize both the hMSH2-hMSH6 or hMSH2-hMSH3 complexes, and that repair of larger insertion/deletion mismatches utilizes the

Figure 1.4.

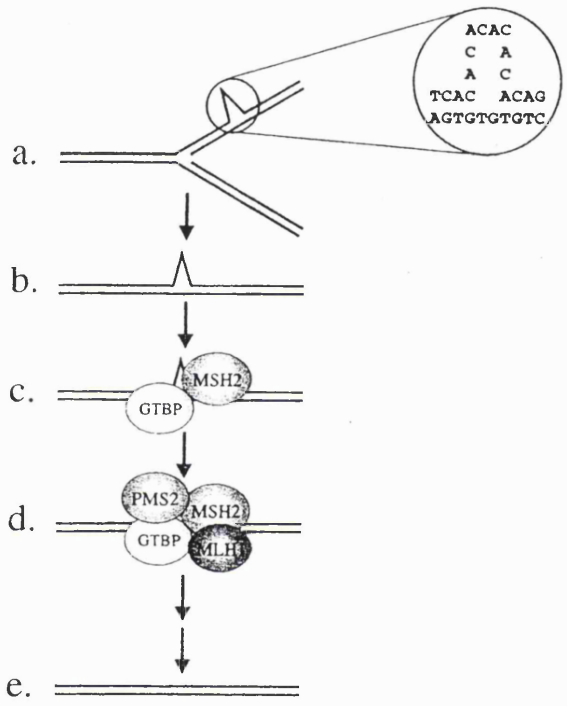


Figure 1.4. Mismatch repair pathway in human cells (taken from Kinzler and Vogelstein, 1996). (a and b) During DNA replication single base mismatches may result from misincorporation by polymerases (not shown) and larger insertion/deletion mismatches may result from strand slippage as shown in (a). (c) A heterodimer of hMSH2 and GTBP (hMSH6) recognizes and binds to the mismatch. Another mutS homologue, hMSH3, may substitute for GTBP in certain cases (see text). (d and e) MutL homologues are then recruited (d) to the complex and (e) the mismatch is repaired by a process that in bacteria involves an exonuclease, helicase, DNA polymerase III, single-stranded binding protein, and DNA ligase.

hMSH2-hMSH3 complex more frequently than the hMSH2-hMSH6 complex (Acharya *et al.*, 1996; Drummond *et al.*, 1995; Palombo *et al.*, 1995). Thus hMSH3 and hMSH6 appear to have partially redundant functions. The results were consistent with the observation of a lack of dinucleotide repeat instability and reduced mononucleotide repeat instability in *hMSH6*-deficient human tumour cell lines compared to cell lines defective in other MMR genes (Papadopoulos *et al.*, 1995). Furthermore, an increase in the rate of base substitutions in the *hprt* (hypoxanthine phosphoribosyl transferase) gene is seen in some MMR deficient colorectal cancer cell lines including one line with homozygous mutation of *hMSH6* (Bhattacharyya *et al.*, 1995). It has been suggested that hMSH2 is central to all mismatch recognition thereby providing an explanation for the high prevalance of *hMSH2* gene mutations in HNPCC patients compared with *hMSH6* (Acharya *et al.*, 1996).

It has been shown that hMLH1 forms a heterodimer with hPMS2 (Li and Modrich, 1995). This heterodimer did not exhibit mismatch-specific binding capability but was essential for repair proficiency. Based on studies in bacteria and yeast it is thought that mutL homologues may couple the binding of DNA mismatches by mutS homologues to the other excision/repair components of the MMR system (Li and Modrich, 1995; Kinzler and Vogelstein, 1997). Finally there is evidence that the MMR gene products perform functions other than the repair of errors made during replication. A role for the MMR system has been suggested in transcription-coupled excision repair (Mellon *et al.*, 1996), in mediating the G2 cell cycle checkpoint (Hawn *et al.*, 1995), and in meiosis (Baker *et al.*, 1995; Edelmann *et al.*, 1996).

1.5. Genetic studies in gastric cancer

In contrast to colorectal tumourigenesis no clear picture has emerged to indicate which are the significant genetic alterations in gastric tumourigenesis. Studies describing alterations of oncogenes, allele loss, mutations in tumour suppressor genes, and replication error (RER) in gastric carcinomas will be summarized in the following sections.

1.5.1. Genetic alterations in oncogenes

Gastric cancer involves alterations in multiple oncogenes at varying frequencies. The c-*erb*B-2 proto-oncogene (also known as *HER-2* or *neu*) encodes a transmembrane tyrosine kinase receptor (Hunter, 1991). Amplification of c-*erb*B-2 and overexpression of its gene product has been reported in 10-26% of gastric

carcinomas (Yonemura *et al.*, 1991; Lemoine *et al.*, 1991; Uchino *et al.*, 1993a; Mizutani *et al.*, 1993; Ohguri *et al.*, 1993). Most studies have observed that amplification or overexpression of c-*erb*B-2 is largely confined to the intestinal histological type (Lemoine *et al.*, 1991; Uchino *et al.*, 1993a; Mizutani *et al.*, 1993). In addition c-*erb*B-2 is thought to play a role at an advanced stage of gastric tumourigenesis as overexpression of its gene product has been associated with lymph node and liver metastasis (Yonemura *et al.*, 1991; Ohguri *et al.*, 1993; Mizutani *et al.*, 1993). Furthermore some studies have found that patients with c-*erb*B-2 protein-positive gastric tumours have poorer survival rates than those with c-*erb*B-2 protein-negative gastric tumours (Yonemura *et al.*, 1991; Uchino *et al.*, 1993a; Mizutani *et al.*, 1993) leading to the suggestion that c-*erb*B-2 protein expression could be a useful independent prognostic indicator in gastric cancer (Yonemura *et al.*, 1991; Uchino *et al.*, 1993a). However some authors report no association between c-*erb*B-2 protein expression and prognosis (Tateishi *et al.*, 1992; Ohguri *et al.*, 1993). The c-*erb*B-2 gene product is highly homologous to the product of the c-*erb* proto-oncogene product, the epidermal growth factor (EGF) receptor. Overexpression of EGF receptor has been detected in 18-35% gastric carcinomas (Yasui *et al.*, 1988; Lemoine *et al.*, 1991). The expression of this receptor and its ligand, EGF, in the same tumour correlated with disease progression and a poor prognosis (Yasui *et al.*, 1988).

The *TPR-MET* oncogenic rearrangement is thought to be involved in the early stages of gastric carcinogenesis (Soman *et al.*, 1991). This rearrangement involves fusion of the translocated promoter region (*TPR*) locus on chromosome 1 to the 5' region of the c-*met* proto-oncogene on chromosome 7, thus resulting in activation of the proto-oncogene, and was first described in a human osteosarcoma cell line transformed by N-methyl-N'-nitrosoguanidine, a strong gastric carcinogen in experimental animals (Mirvish, 1983). Soman *et al.* (1991) found that expression of *TPR-MET* RNA was found not only in intestinal type gastric carcinomas but in all of the precursor stages, including the earliest identifiable stage, superficial gastritis. In contrast amplification and overexpression of the c-*met* proto-oncogene, which was found to be highly frequent in gastric carcinomas, was associated with advanced tumour stage (Kuniyasu *et al.*, 1992; 1993). A more recent study implied a tumour suppressor role for the *TPR* gene or a gene close to it on chromosome 1q25. Of 7 gastric carcinoma cases informative for a *TPR* probe, loss of heterozygosity was observed in 3 cases while a further 3 cases showed homozygous deletion for the *TPR* probe (Cunningham *et al.*, 1997).

The *myc* proto-oncogenes encode a p62 nuclear protein that may have a role in transcription, proliferation, and cell cycle control (Hunter, 1991). As with the

TPR-MET rearrangement, increased expression of the c-*myc* proto-oncogene product has been detected in patients with the intestinal type gastric cancer-associated pre-malignant lesions, atrophic gastritis, intestinal metaplasia, and dysplasia (Macdonald *et al.*, 1987; Ciclitira *et al.*, 1987). Moreover Macdonald *et al.* (1987) observed overexpression of the c-myc protein in 42% intestinal type gastric cancers compared with 23% diffuse type cancers.

In contrast to colorectal cancer, most studies have reported that the incidence of mutations at codons 12, 13, or 61 of the H-*ras,* K-*ras*, and N-*ras* genes is low in gastric carcinomas (0-12%) (Nanus *et al.*, 1990; Miki *et al.*, 1991; Kihana *et al.*, 1991; Lee *et al.*, 1995). Miki *et al.* (1991) and Kihana *et al.* (1991) found K-*ras* gene mutations to be more frequent in intestinal type gastric carcinomas than in the diffuse type, however Lee *et al.* (1995) found no difference between the two histological types. *Ras* gene mutations have also been described in the precursor lesions intestinal metaplasia and gastric adenoma (Kihana *et al.*, 1991; Soman *et al.*, 1991). Amplification of the K-*sam* gene on the other hand was detected in 10 of 48 (21%) undifferentiated (diffuse) gastric carcinomas compared with none of 35 well differentiated (intestinal) gastric carcinomas (Nakatini *et al.*, 1990). K-*sam* is homologous to genes encoding receptors of the fibroblast growth factor receptor family.

1.5.2. Allele loss studies

Allele loss on chromosomes 5q, 17p, and 18q has been observed in gastric carcinomas. These chromosomes harbour the *APC* and *MCC*, *p53*, and *DCC* genes respectively. Alterations of these genes and the LOH studies are described in sections 1.5.3a-c. LOH studies have identified a number of other chromosomal regions that demonstrate allele loss in gastric tumours. Frequent LOH has been reported on 1p, 1q, 3p, 6q, 7q, 11p, 11q, 12q, and 13q.

LOH on chromosome 1p has been described in 30-50% informative gastric carcinomas in some studies (Sano *et al.*, 1991, Ezaki *et al.*, 1996; Gleeson *et al.*, 1997). Ezaki *et al.* (1996) identified a 13cM commonly deleted region between two microsatellite repeat loci which mapped to 1p35 and 1p34.3 respectively. The commonly deleted region overlapped with the commonly deleted regions on chromosome 1p reported in various other tumour types, for example colorectal cancer (Leister *et al.*, 1990), neuroblastoma (Fong *et al.*, 1989), and phaeochromocytoma (Moley *et al.*, 1992), suggesting that the short arm of chromosome 1 may harbour tumour suppressor gene(s) associated with more than one type of cancer. On the long

arm of chromosome 1, some studies report LOH frequencies of between 30% and 50% (Fey *et al.*, 1989; Tamura *et al.*, 1996a; Gleeson *et al.*, 1997). Sano *et al.* (1991) observed LOH on chromosome 1q in 67% well differentiated gastric carcinomas but not in poorly differentiated carcinomas. Chromosome 1q harbours the *TPR* (translocated promoter region) gene which has been implicated in gastric tumourigenesis as described in section 1.5.1.

Chromosome 3p harbours a number of genes implicated in tumourigenesis. The *VH1* (von Hippel-Lindau disease 1) tumour suppressor gene and the *hMLH1* mismatch repair gene are locted on this chromosome arm (Latif *et al.*, 1993; Bronner *et al.*, 1994). The region 3p21.3-p21.2 is believed to harbour another tumour suppressor gene that is implicated in small cell lung cancer (Daly *et al.*, 1993). In gastric cancer, allele loss on chromosome 3p was detected in 25%-36% tumours in the studies of Schneider *et al.* (1995) and Tamura *et al.* (1996a) and in 60% of tumours in the study of Gleeson *et al.* (1997).

In a cytogenetic study of 10 gastric carcinomas, Seruca *et al.* (1993) noted that deletion of the region 6q21-22 to qter was the most consistent structural cytogenetic abnormality in these tumours. To determine the deleted region more precisely, in a later study these authors carried out LOH analysis using 9 polymorphic markers on this chromosome arm (Queimado *et al.*, 1995). An overall frequency of LOH at 6q of 39% was observed. LOH was found in all histological types of gastric carcinoma. A deletion map was constructed and two regions of frequent allele loss were identified, one between 6q16.3-q21 and 6q22.3-q23.1 and the other distal to 6q23-q24. This study was carried out in gastric carcinomas from Portuguese patients. In two other studies on gastric carcinomas from Western patients, LOH of chromosome 6q was detected in 29-53% tumours (Schneider *et al.*, 1995; Gleeson *et al.*, 1997). Interestingly in some studies of Japanese gastric carcinomas, LOH on chromosome 6q has not been detected in informative tumours (Motomura *et al.*, 1988; Sano *et al.*, 1991; Tamura *et al.*, 1996a), which suggests that some genetic as well as epidemiological differences may exist between Eastern and Western gastric cancers.

Allele loss on chromosome 7q has been observed in both Western and Eastern gastric carcinomas (30-45%) (Sano *et al.*, 1991; Kuniyasu *et al.*, 1994; Tamura *et al.*, 1996a; Gleeson *et al.*, 1997; Nishizuka *et al.*, 1997). Sano *et al.* (1991) and Kuniyasu *et al.* (1994) reported that 7q LOH was frequent in advanced gastric carcinomas but not in early tumours suggesting an association with tumour progression. Nishizuka *et al.* (1997) determined the smallest common deleted region to be at 7q31.1. On chromosome 11p, Ranzani *et al.* (1993) detected frequent LOH at chromosome 11p in

gastric carcinomas (37% tumours). Subsequent deletion mapping analysis using microsatellite repeat markers revealed a minimum region of deletion at 11p15..5 and also identified a second region of deletion at 11q22-23 (Baffa *et al.*, 1996). Some additional studies also report frequent allele loss at 11p in gastric tumours (Tamura *et al.*, 1996a; Gleeson *et al.*, 1997) although others have detected few or no 11p deletions (Sano *et al.*, 1991; Schneider *et al.*, 1995). On chromosome 11q most studies report the occurence of LOH in about 30% gastric carcinomas (Schneider *et al.*, 1995; Tamura *et al.*, 1996a; Baffa *et al.*, 1996). The commonly deleted regions identified on chromosomes 6q, 7q, 11p, and 11q in gastric carcinomas correspond to regions showing frequent allele loss in other tumour types, suggesting the existence of tumour suppressor genes in these regions that may be involved in the pathogenesis of a wide range of human malignancies.

Several groups have observed a high rate of LOH on chromosome 12q (30-58%) (Fey *et al.*, 1989; Sano *et al.*, 1991; Schneider *et al.*, 1995; Gleeson *et al.*, 1997; Schmutte et al., 1997) suggesting evidence for a tumour suppressor gene involved in gastric tumourigenesis on this chromosome arm. On chromosome 13q, some studies report frequent LOH in gastric tumours, ranging from 30% to 53% (Motomura *et al.*, 1988; Uchino *et al.*, 1992; Schneider *et al.*, 1995; Gleeson *et al.*, 1997), while others report a lower rate (7-19%) (Ranzani *et al.*, 1993; Tamura *et al.*, 1996a). The *RB1* (retinoblastoma 1) tumour suppressor gene is located on chromosome 13q. In their analysis of the *RB1* gene and protein in gastric carcinomas, Constancia *et al.* (1994) however did not detect gross alterations of *RB1*, such as homozygous deletions or rearrangements, using Southern blot analysis nor did they detect any abnormal patterns of pRB using Western blot analysis.

1.5.3. Genetic alterations in tumour suppressor genes

1.5.3 (a) *APC* and *MCC*

The *APC* (Adenomatous Polyposis Coli) and *MCC* (Mutated in Colorectal Cancer) genes lie in close proximity to one another at chromosome 5q21-22. In the studies reporting the investigation of mutations of the *APC* gene in gastric tumours, only the 5' half of exon 15 has been analysed. This part of the gene includes the region where somatic mutations in colorectal carcinomas have been found to cluster (Miyoshi *et al.*, 1992b). The studies of *APC* gene mutations in gastric carcinomas report conflicting results. Nakatsuru *et al.* (1992) detected *APC* mutations in 12 of 57 (21%) gastric cancers. The mutations were detected in well differentiated gastric carcinomas and signet-ring cell carcinomas but not in poorly differentiated

carcinomas. In contrast Ogasawara *et al.* (1994) did not detect *APC* gene mutations in 24 gastric carcinomas, nor did Powell *et al.* (1996) in 30 gastric carcinomas. In a later study the former group examined 72 gastric carcinomas for mutations of the *APC* gene (Maesawa *et al.*, 1995). A mutation was identified in just one tumour (1.4%), a signet-ring cell carcinoma.

Mutations of *APC* have also been investigated in precancerous lesions of the stomach. It is thought that gastric adenoma arising from intestinal metaplasia is a prcursor lesion of some differentiated (intestinal) type gastric carcinomas. Mutations of the *APC* gene have been identified in 20-40% of gastric adenomas (Nakatsuru *et al.*, 1993; Tamura *et al.*, 1994; Nishimura *et al.*, 1995). Nishimura *et al.* (1995) also examined 16 intestinal metaplasia mucosae and detected a mutation of *APC* in one case (6%).

Loss of markers on chromosome 5q has been reported in 10-58% of gastric carcinomas in various studies (Fey *et al.*, 1989; Sano *et al.*, 1991; Schneider *et al.*, 1995; Tamura *et al.*, 1996; Gleeson *et al.*, 1997). Some investigators have analysed gastric carcinomas for LOH at polymorphic loci within the *APC* and *MCC* genes (Tamura *et al.*, 1993; McKie *et al.*, 1993; Rhyu *et al.*, 1994a). McKie *et al.* (1993) and Rhyu *et al.* (1994a) detected LOH at both *APC* and *MCC* in about 30% tumours. Tamura *et al.* (1993) carried out flow cytometric cell sorting analysis to enrich for aneuploid gastric tumour cells. LOH at *APC* was observed in 86% (12/14) informative cases and at *MCC* in 100% (7/7) informative cases. Sano *et al.* (1991) identified 5q LOH in well differentiated gastric carcinomas but not in poorly differentiated gastric carcinomas, whereas in other studies no difference between histological types has been reported (Tamura *et al.*, 1993; Rhyu *et al.*, 1994a; Gleeson *et al.*, 1997).

1.5.3 (b) *DCC*

The reported frequency of allele loss on chromosome 18q in gastric cancer varies considerably between authors (22%-68%) (Uchino *et al.*, 1992; Ranzani *et al.*, 1993; Schneider *et al.*, 1995; Gleeson *et al.*, 1997). In the study of Uchino *et al.* (1992) the putative common region showing LOH on chromosome 18q included the locus of the *DCC* (Deleted in Colon Carcinoma) gene. Using PCR-based techniques, LOH has been investigated at polymorphic loci within the *DCC* gene in gastric carcinomas. In these studies the reported incidence of LOH varies from 27% to 58% (Maesawa *et al.*, 1995; Cho *et al.*, 1996; Wu *et al.*, 1997a).

Published studies report conflicting results regarding an association of 18q LOH with histological type. A similar frequency of LOH has been observed in both intestinal and diffuse type gastric carcinomas in some studies (Maesawa *et al.*, 1995; Cho *et al.*, 1996; Gleeson *et al.*, 1997). However Wu *et al.* (1997a) reported that *DCC* LOH was significantly more frequent in advanced stage intestinal type gastric carcinoma (32.1%) than in advanced diffuse type carcinoma (4%). Although Uchino *et al.* (1992) did not examine any cases of diffuse gastric carcinoma, a high rate of LOH was observed in intestinal type gastric carcinomas (61%).

Maesawa *et al.* (1995) also investigated the incidence of *DCC* LOH in gastric adenomas. *DCC* LOH was observed infrequently in gastric adenomas (14%) compared to gastric carcinomas (58%) (Maesawa *et al.*, 1995). Wu *et al.* (1997a) failed to observe DCC LOH in both diffuse and intestinal type early gastric cancers. Similarly Ranzani *et al.* (1993) reported that loss on chromosome 18q was more frequent in advanced than in earlier-staged gastric carcinomas. Thus 18q LOH may be more important in the late stages of gastric tumourigenesis.

1.5.3 (c) *p53*

Allelic loss of chromosome 17p loci has been reported to be frequent in gastric carcinomas (45% to 78%) (Sano *et al.*, 1991; Rhyu *et al.*, 1994a; Schneider *et al.*, 1995; Tamura *et al.*, 1996a; Gleeson *et al.*, 1997). Most published studies investigating mutations of p53 in gastric cancer have examined exons 5-8 of the gene, which correspond to the highly conserved domains of the protein. Reported frequencies of *p53* gene mutation in gastric carcinomas vary from 33% to 65% (Tamura *et al.*, 1991; Kim *et al.*, 1991; Seruca *et al.*, 1992; Imazeki *et al.*, 1992; Matozaki *et al.*, 1992a; Renault *et al.*, 1993; Uchino *et al.*, 1993b; Schneider *et al.*, 1994; Poremba *et al.*, 1995; Hongyo *et al.*, 1995). The majority of mutations detected in these studies were missense mutations. Mutations of the *p53* gene have been observed in more than 50% of human malignancies. In other tumour types too, the majority of *p53* mutations are missense mutations (Hollstein *et al.*, 1991). However the nature of *p53* point mutations varies among different neoplasms and appears to be related to organ site and specific aetiological agents. These findings will be discussed in Chapter 4.

Most reports describing mutations of the *p53* gene in gastric cancer have not compared their incidence in the different histological types although Tamura *et al.* (1991) and Imazeki *et al.* (1992) found no correlation between *p53* mutation and histological type. Uchino *et al.* (1993b) however reported that *p53* mutations were

associated with the differentiated (intestinal) type. Mutations of *p53* appear to be an early event in gastric tumourigenesis. Shiao *et al.* (1994) detected *p53* mutations in 37.5% of intestinal metaplasia cases and in 58.3% gastric dysplasia cases, while Tohdo *et al.* (1993) found that 30% of gastric adenomas harboured mutations of *p53*.

Alterations of *p53* in tumours have been investigated using immunohistochemistry (IHC) to detect p53 protein. Because of its short half-life, wild-type p53 protein does not normally accumulate in amounts so that it can be detected by IHC. However missense mutations induce conformational changes that stabilize the p53 protein, increasing its half-life. Therefore the detection of p53 expression by IHC is used as an indicator of *p53* mutation. However a positive IHC reaction may not always reflect accumulation of mutant protein. The wild-type protein can be stabilized by association with viral oncoproteins or cellular proteins, for example MDM2, or in response to DNA damage. In gastric cancer, overexpression of p53 has been observed in 40-50% tumours (Martin *et al.*, 1992; Joypaul *et al.*, 1994; Gabbert *et al.*, 1995; Muller and Borchard, 1996; Starzynska *et al.*, 1996). Gabbert et al. (1995) reported that p53 expression was significantly less frequent in the diffuse type than in the intestinal type of gastric carcinoma but most other studies report no difference between the two histological types with regard to p53 expression (Martin *et al.*, 1992; Joypaul *et al.*, 1994; Muller and Borchard, 1996; Starzynska *et al.*, 1996). Some but not all studies report that patients with p53-positive tumours have a reduced survival time compared to patients with p53-negative tumours leading to the suggestion that detection of p53 expression could be a predictor of outcome in gastric cancer patients (Martin *et al.*, 1992; Joypaul *et al.*, 1994; Starzynska *et al.*, 1996).

Further evidence of a role for p53 in gastric tumourigenesis was provided by a study in which it was found that transfection of wild-type but not mutant *p53* cDNA into gastric cancer cell lines suppressed growth of these cells (Matozaki *et al.*, 1992b).

1.5.3 (d) E-cadherin

Cadherins are cell surface molecules that are responsible for calcium-dependent cell-cell adhesion. E-cadherin is the member of the cadherin family that is predominantly expressed in epithelial cells and plays an important role in the maintenance of epithelial cell polarity and the organization of epithelial tissues (Takeichi, 1991). The E-cadherin (also known as uvomorulin) gene has been mapped to chromosome 16q22 (Natt *et al.*, 1989). The E-cadherin gene is regarded as an

invasion or metastasis suppressor subtype of tumour suppressor gene for several reasons. Firstly, E-cadherin has been shown to suppress the invasiveness of epithelial tumour cells *in vitro* (Behrens *et al.*, 1989). Secondly, reduced or absent expression of E-cadherin has been observed in many tumour types including carcinoma of the bladder (Bringuier *et al.*, 1993), oesophagus (Bongiorno *et al.*, 1995), colorectum (Dorudi *et al.*, 1993), and breast (Oka *et al.*, 1993). Most investigations have shown that decreased E-cadherin expression is associated with a decreased state of differentiation and the presence of metastasis. Thirdly, somatic mutation of the E-cadherin gene concomitant with allelic loss has been reported in endometrial and ovarian carcinomas (Risinger *et al.*, 1994).

Reduced or absent expression of the E-cadherin protein has been described in gastric cancer too. The percentage of tumours with reduced or absent E-cadherin expression varies from 32% in the study of Shino *et al.* (1995) to about 70% in the studies of Yonemura *et al.* (1995) and Gabbert *et al.* (1996) and 92% in the study of Mayer *et al.* (1993). In all these studies decreased E-cadherin expression was correlated with tumour dedifferentiation. Mayer *et al.* (1993) and Gabbert *et al.* (1996) reported that E-cadherin expression was significantly more often reduced in diffuse type carcinomas than in intestinal type carcinomas. Reduced expression of E-cadherin has been associated with a poor prognosis in gastric cancer patients (Mayer *et al.*, 1993; Shino *et al.*, 1995; Yonemura *et al.*, 1995; Gabbert *et al.*, 1996), an association that has been reported in other tumour types too (Bringuier *et al.*, 1993; Dorudi *et al.*, 1995).

Mutations of the E-cadherin gene have also been investigated in gastric carcinomas. In one study, E-cadherin gene mutations that were predicted to alter the protein structure were found in 50% of diffuse type gastric carcinomas and in 14% of mixed type gastric carcinomas (Becker *et al.*, 1994). In addition, LOH with retention of the mutated E-cadherin allele was detected in one diffuse type tumour. In contrast silent point mutations were detected in two (10%) intestinal type gastric carcinomas (Becker *et al.*, 1994). In a second report, E-cadherin gene mutations that resulted in mRNA splicing defects were detected in two poorly differentiated gastric carcinoma cell lines (Oda *et al.*, 1994). Furthermore loss of the wild-type allele was observed in both cell lines.

The above studies suggest that alterations of E-cadherin may play an important role in the development of the diffuse type of gastric carcinoma in particular.

1.5.4. Replication error

As described in section 1.4.4, replication error (RER) at microsatellite repeat sequences was first described in tumours from HNPCC (hereditary nonpolyposis colorectal cancer) patients and in tumours from a subset of sporadic colorectal cancer patients, and is manifest as electrophoretic shifts in the repeat fragments in tumour compared with matching normal DNA. In HNPCC patients at least, this genetic instability is thought to reflect a defect in the DNA mismatch repair system. Gastric cancer is regarded as the second most common extracolonic malignancy occurring in the HNPCC syndrome. RER at short repeat sequences has been observed in gastric tumours from HNPCC patients (Aaltonen *et al.*, 1994). RER has been reported in a variety of sporadic tumour types too, including gastric carcinomas. The frequency of RER in gastric cancer varies between authors, but most report that approximately 30% of gastric carcinomas exhibit RER, at one or more microsatellite repeat locus (Han *et al.*, 1993; Chong *et al.*, 1994; Rhyu *et al.*, 1994b; Seruca *et al.*, 1995; Lin *et al.*, 1995; Dos Santos *et al.*, 1996; Renault *et al.*, 1996; Wu *et al.*, 1997b; Ottini *et al.*, 1997). However some authors report a lower incidence of RER in gastric cancer (14%-18% tumours) (Mironov *et al.*, 1994; Strickler *et al.*, 1994; Nakashima *et al.*, 1995; Tamura *et al.*, 1995; Buonsanti *et al.*, 1997). In a further study RER was not detected in gastric carcinomas from British patients younger than 40 years (Hayden *et al.*, 1997).

With regard to the different histological types of gastric carcinoma, in an early Japanese study RER was found to be significantly more prevalent in poorly differentiated (diffuse) type gastric carcinomas than in differentiated (intestinal) type tumours (Han *et al.*, 1993). However some studies have reported that the presence of RER is associated with the intestinal type (Dos Santos *et al.*, 1996; Ottini *et al.*, 1997; Buonsanti *et al.*, 1997) while others find no difference between the intestinal and diffuse types (Rhyu *et al.*, 1994b; Lin *et al.*, 1995; Nakashima *et al.*, 1995; Renault *et al.*, 1996; Wu *et al.*, 1997b). Several groups have reported that RER is more prevalent in distally located gastric tumours, particularly in those in the antrum, than in carcinomas in the gastric cardia (Strickler *et al.*, 1994; Rhyu *et al.*, 1994b; Seruca *et al.*, 1995; Dos Santos *et al.*, 1996; Gleeson *et al.*, 1996; Ottini *et al.*, 1997). Interestingly intestinal type gastric carcinomas predominantly occur in the distal stomach (Lehtola, 1978).

There is much debate in the literature as to when RER occurs in gastric carcinogenesis. It is thought by some that RER plays a role in tumour progression.

RER has been detected in early gastric cancer, and some workers have compared its frequency in early and advanced stage carcinomas. These studies showed that the frequency of RER increased from early (12-21%) to advanced (20-41%) stage of disease (Chong *et al.*, 1994; Lin *et al.*, 1995; Wu *et al.*, 1997b; Buonsanti *et al.*, 1997). RER has been detected in premalignant lesions too, for example in metaplastic and dysplastic lesions adjacent to carcinomas (Rhyu *et al.*, 1994b; Buonsanti *et al.*, 1997; Ottini *et al.*, 1997). Tamura *et al.* (1995) found that the incidence of RER was low in gastric adenomas as it was detected in just 1 of 13 (8%) adenomas, at one microsatellite repeat locus only. In contrast, Semba *et al.* (1996) observed RER in 42% gastric adenomas, 33% intestinal metaplasia, and 33% gastric carcinomas, and concluded that genetic instability is an early event in multistep gastric carcinogenesis.

Patients with RER+ colorectal carcinomas have been reported to have better survival rates than patients with RER- colorectal carcinomas (Aaltonen *et al.*, 1993; Thibodeau *et al.*, 1993; Ionov *et al.*, 1993). Seruca *et al.* (1995) and Dos Santos *et al.* (1996) reported that patients with RER+ gastric carcinomas also had better survival rates than patients with RER- gastric carcinomas, but Ottini *et al.* (1997) reported that survival analyses showed no difference between RER+ and RER- gastric carcinomas. However lymph node metastasis has been found to be significantly less prevalent in RER+ gastric carcinomas than in RER- gastric carcinomas in other studies (Nakashima *et al.*, 1995; Renault *et al.*, 1996) which suggests that RER+ gastric carcinomas may behave in a less malignant manner and consequently have a better prognosis. Finally, investigation of an association of the RER+ phenotype and family history of gastric cancer has yielded contradictory results: in two studies no correlation between RER and family history was found (Chong *et al.*, 1994; Strickler *et al.*, 1994) while two further studies reported that the presence of RER was associated with a gastric cancer-positive family history (Keller *et al.*, 1995; Ottini *et al.*, 1997).

1.6. Aims of this study

There were two main aims of this study. The first was to determine the significance of alterations of the *APC*, *MCC*, *DCC*, *p53*, and *hMSH2* genes and of alterations at microsatellite repeat loci (replication error and loss of heterozygosity) in gastric tumourigenesis. The second was to determine the importance of alterations of the *APC* and *hMSH2* genes and of alterations at microsatellite repeat loci in colorectal

tumourigenesis. Twenty-six gastric carcinoma and corresponding normal tissues were investigated for genetic alterations as follows:

(1) Mutations of the *APC* gene were investigated using three different methods. Firstly, 43% of the coding region of *APC* was screened for mutations using a single-strand conformation polymorphism (SSCP) and heteroduplex assay. The region of the gene analysed was inclusive of the 5' half of exon 15 (codons 653-1700). This part of *APC* was chosen for analysis as it included the region where somatic mutations have been found to cluster in colorectal tumours in other studies (Miyoshi *et al.*, 1992b; Nagase and Nakamura, 1993). This region is known as the mutation cluster region or MCR and spans codons 1286 to 1513. Exons 6, 8, 11, and 14 were also analysed by SSCP and heteroduplex analysis in the present study. Secondly, a segment of exon 15 of the *APC* gene, inclusive of codons 1028-1700, was screened for mutations using the protein truncation test (PTT). The region analysed encompassed the mutation cluster region. Thirdly, certain relatively common mutations in exons 6, 8, and 14 of the *APC* gene result in either the generation or loss of recognition sites of specific restriction enzymes (Ando *et al.*, 1993). These mutations were investigated in the gastric carcinoma cases by restriction enzyme digest analysis of PCR products. Additionally, loss of heterozygosity (LOH) was investigated at intragenic polymorphic loci of *APC*.

(2) LOH involving the *MCC* gene was investigated at intragenic polymorphic loci.

(3) LOH at the *DCC* gene locus was investigated at intragenic polymorphic loci.

(4) The entire coding region of the *p53* gene was screened for mutations by SSCP and heteroduplex analysis. In addition, LOH was investigated at intragenic polymorphic loci of *p53*.

(5) Twelve microsatellite repeat loci were analysed for replicaton error (RER) and LOH.

(6) Exons 12, 13, and 14 of the *hMSH2* gene were analysed for mutations by SSCP and heteroduplex analysis.

The gastric carcinomas analysed in the present study included those of the intestinal type and those of the diffuse type. The frequency of genetic alterations in each histological type were compared. Furthermore, some of the genetic alterations that were investigated in gastric carcinoma cases were viewed in comparison with

colorectal carcinoma cases. Forty-three colorectal carcinoma and corresponding normal tissues were analysed for the following genetic alterations:

(1) Mutations in exons 6, 8, 11, 14, and the 5' half of exon 15 of the *APC* gene, using SSCP and heteroduplex analysis.

(2) LOH at polymorphic loci within the *APC* gene.

(3) RER and LOH at twelve microsatellite repeat loci.

(4) Mutations in exons 12, 13, and 14 of the *hMSH2* gene, using SSCP and heteroduplex analysis.

Chapter 2

Materials and Methods

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals

General laboratory chemicals and chemicals used for silver staining of polyacrylamide gels were obtained from British Drug House (BDH), Poole, Dorset. Diethyl pyrocarbonate (DEPC) and NNN'N' tetramethylethylenediamine (TEMED) were also purchased from BDH.

Standard agarose (Type I), ethidium bromide, and β-mercaptoethanol were obtained from Sigma Chemical Company, Poole, Dorset. NuSieve GTG low melting temperature agarose was purchased from FMC BioProducts, Rockland, USA. A 6% acrylamide solution (19:1 acrylamide:bisacrylamide) was supplied by Severn Biotech Limited, Kidderminster, Worcs. A 30% acrylamide solution (37.5:1 acrylamide: bisacrylamide) as well as ammonium persulphate were supplied by Biorad Laboratories Limited, Herts.

The radiochemical [α-^{35}S] dATP (1415Ci/mmol) was obtained from NEN/Dupont, Stevenage, Herts., while ^{35}S-methionine (1000Ci/mmol) was obtained from Amersham International plc, Amersham, Bucks.

2.1.2. Enzymes

Restriction endonucleases (supplied with the appropriate reaction buffer) were purchased from Gibco BRL, Paisley, Scotland. Proteinase K was obtained from Boehringer Mannheim GmbH and was made to a final concentration of 25mg/ml with sterile deionised distilled water and stored at -20°C. *Taq* DNA polymerase was supplied by HT Biotechnology Limited, Cambridge, at a concentration of 5 units/µl. Exonuclease I and Shrimp Alkaline Phosphatase (available as Reagent Pack for use with Sequenase PCR Product Sequencing Kit) were obtained from Amersham International plc.

2.1.3. DNA and protein molecular weight markers

The 100bp and 1kb ladders were purchased from Gibco BRL. The GeneScan™ 500-TAMRA size standard (range 35-500 bp) was supplied by Perkin-

Elmer Corporation, California, USA. The Rainbow™ coloured protein molecular weight marker (molecular weight range: 14300-220000) was supplied by Amersham International plc.

2.1.4. Tissue and DNA samples

Twenty-six gastric carcinoma specimens were obtained from seven hospitals in London: Homerton hospital, Whittington Hospital, The London Clinic, Newham General Hospital, The Royal London Hospital, Oldchurch Hospital, and the Royal Free Hospital. Normal stomach mucosa specimens were also obtained from each patient. The tissue samples were either flash frozen in liquid nitrogen immediately after surgery or transported back to the laboratory in phosphate buffered saline (appendix 1) and flash frozen there. The tissues were then stored at -70°C until use.

Table 2.1 gives the clinicopathological and family history data of the 26 gastric carcinoma cases. Twenty-two of the cases were U.K. residents. Three cases (GACA16, GACA18, and GACA20) were from the Middle East, and one case (GACA5) was from Brazil. Sections were removed from each tumour specimen from which DNA was to be extracted for histological analysis which was kindly performed by Mr. Ian Talbot of St. Mark's Hospital, London. Cryostat sections were made and stained with haematoxylin and eosin. Tumour samples used in this study contained at least 50% neoplastic cells. The tumours were classified according to Lauren (1965): 17 were of the intestinal histological type and 9 were of the diffuse type. Information regarding the tumour site in the stomach was not obtained for seven of the carcinomas. Information about family history of cancer was obtained by written request of the surgeons. In 3 cases this information was not obtained hence NK (Not Known) is written in table 2.1. In one case (GACA22) information on family history was obtained from the patient's family doctor.

Forty-three colorectal carcinoma and corresponding normal colon mucosa specimens were provided by St. Mark's Hospital, London. Tissue samples were flash frozen in liquid nitrogen shortly after surgery, and stored at -70°C until use. Clinicopathological and family history data of the colorectal carcinoma cases is shown in table 2.2. Information on age, sex, tumour site, and Dukes' stage was obtained from the Pathology Department at St. Mark's Hospital. Information on family history of cancer was obtained by looking at patient hospital records. If the records were not available or if no mention was made in the records about whether there was a family history or not, NK (Not Known) is written in table 2.2.

Table 2.1. Clinicopathological data and family history from twenty-six gastric carcinoma cases.

Case	Age	Sex	Tumour site	Histological type	Family history
GACA1	72	F	Cardia	Diffuse	Sister-breast cancer
GACA2	54	M	Antrum	Diffuse	None
GACA3	42	M	NK	Intestinal	NK
GACA4	79	F	NK	Intestinal	None
GACA5	67	M	Body	Diffuse	None
GACA6	35	F	Cardia	Diffuse	None
GACA7	65	M	NK	Diffuse	NK
GACA8	72	M	NK	Intestinal	None
GACA9	83	F	Antrum	Diffuse	None
GACA10	60	M	Cardia	Intestinal	None
GACA11	74	F	Cardia	Intestinal	None
GACA12	65	M	NK	Intestinal	None
GACA13	47	M	Cardia	Intestinal	None
GACA14	77	M	Cardia	Intestinal	NK
GACA15	71	F	Cardia	Intestinal	None
GACA16	75	M	Body	Intestinal	None
GACA17	67	F	NK	Intestinal	Sister-breast cancer
GACA18	70	F	Body	Diffuse	None
GACA19	49	M	Fundus	Diffuse	Father-gastric cancer
GACA20	55	M	Antrum	Intestinal	None
GACA21	85	F	Cardia	Intestinal	Brother-cancer
GACA22	84	F	Pylorus	Intestinal	None
GACA23	60	M	Cardia	Intestinal	Father-cancer; brother-CRC
GACA24	66	M	Body	Diffuse	None
GACA25	81	F	Cardia	Intestinal	None
GACA26	61	M	NK	Intestinal	None

NK, not known; CRC, colorectal cancer.

Table 2.2. Clinicopathological data and family history from forty-three colorectal carcinoma cases.

Case	Age	Sex	Tumour site	Dukes' stage	Family history
COCA1	48	M	Sigmoid colon	C2	NK
COCA2	72	M	Recto-sigmoid	C1	None
COCA3	63	M	Rectum	B	None
COCA4	85	F	Splenic flexure	B	Sister-cancer
COCA5	71	M	Asc. colon	C2	Father-CRC
COCA6	53	M	Rectum	B	Mother-CRC
COCA7	73	F	Rectum	B	None
COCA8	61	F	Sigmoid colon	C2	None
COCA9	74	F	Recto-sigmoid	B	HNPCC-type
COCA10	48	M	Sigmoid colon	B	None
COCA11	72	M	Sigmoid colon	C1	None
COCA12	72	M	Caecum	C2	None
COCA13	66	F	Des. colon	B	NK
COCA14	56	M	Rectum	A	NK
COCA15	80	M	Rectum	C1	NK
COCA16	70	M	Sigmoid colon	B	Both parents-cancer
COCA17	76	M	Rectum	C1	None
COCA18	60	M	Caecum	C2	NK
COCA19	83	F	Rectum	C1	NK
COCA20	43	F	Sigmoid colon	B	Grandfather-CRC
COCA21	65	M	Trans colon	B	None
COCA22	65	M	Rectum	B	None
COCA23	75	M	Rectum	B	None
COCA24	53	F	Splenic flexure	B	NK
COCA25	83	M	Rectum	B	NK
COCA26	49	M	Sigmoid colon	B	HNPCC-type
COCA27	49	M	Rectum	B	NK
COCA28	60	M	Rectum	C1	None
COCA29	66	M	Rectum	C1	None
COCA30	58	F	Ileocaecal valve	C1	NK
COCA31	42	F	Rectum	B	None
COCA32	78	M	Rectum	A	NK
COCA33	67	F	Rectum	A	None
COCA34	56	M	Caecum	C1	None
COCA35	60	M	Rectum	B	NK
COCA36	48	M	Caecum	C2	None
COCA37	74	F	Hepatic flexure	B	None
COCA38	70	M	Rectum	B	None
COCA39	71	F	Sigmoid colon	C2	NK
COCA40	52	M	Rectum	C2	NK
COCA41	43	M	Rectum	C1	None
COCA42	69	M	Rectum	B	Brother-CRC
COCA43	67	M	Rectum	C1	None

Asc. colon, Ascending colon; Des. colon, Descending colon; Trans. colon, Transverse colon; NK, Not known; CRC, Colorectal cancer.

DNA samples from fifty unrelated individuals, who were spouses of FAP (familial adenomatous polyposis) patients who were being screened in our laboratory for germline *APC* gene mutations, were available for further characterization of a sequence variation in the *hMSH2* gene.

2.1.5. Buffers and solutions

Solutions and buffers were prepared using distilled deionised water. Sterilisation was by autoclaving at 15lbs psi 121°C for 30 minutes. The components of standard buffers and solutions are given in appendix 1.

2.2. Methods

2.2.1. Extraction of genomic DNA

DNA was extracted from frozen carcinoma and normal mucosa tissue samples by either a standard phenol-choroform method (Maniatis *et al.*, 1982) or by using the Nucleon™ II DNA extraction kit (Scotlab, Strathclyde, Scotland).

2.2.1 (a) Standard phenol-chloroform extraction method

Frozen tissue was removed from -70°C storage, placed in a petri dish, and chopped finely with a scalpel. The chopped tissue was transferred to a centrifuge tube containing 10ml of STE (appendix 1), 80µl 25mg/ml proteinase K, and 500µl 10% SDS. This mixture was incubated at 37°C for at least 16 hours. When digestion was complete, 10ml of phenol (appendix 2) was added to the mixture which was then shaken in a daisy wheel for 10 minutes, followed by centrifugation in a bench-top centrifuge at 800g for 10 minutes, to separate the organic and the aqueous phases. The aqueous (upper) phase was then removed and the phenol extraction was repeated once more, after which the aqueous phase was extracted once with 10ml chloroform (appendix 2). Two volumes of ice cold absolute ethanol was added to the final aqueous phase to precipitate the DNA. The DNA was hooked from the liquid with a plastic inoculating loop and washed in 70% ethanol. The DNA was then allowed to air dry, and finally dissolved in an appropriate volume of 1x TE buffer (appendix 1). The concentration of the DNA was determined spectrophotometrically. The absorbance of a dilution (1/100) of the DNA sample was measured at 260nm with a path length of 1cm. The concentration of the DNA sample was calculated using one A_{260} unit = 50µg/ml double-stranded DNA.

2.2.1 (b) DNA extraction kit method

Protocol b of the Nucleon™ II DNA extraction kit (Scotlab) for genomic DNA extraction from whole blood and cell cultures was adapted for the extraction of DNA from solid tissue as recommended by the manufacturers. This method is more rapid than the standard method described above and does not involve the use of phenol. Frozen tissue was chopped finely with a scalpel in a petri dish and then transferred to a 15ml screw-capped polypropylene centrifuge tube containing 6ml of Reagent B (appendix 2) and 1.5ml 5M sodium perchlorate. Both of these solutions were supplied by the manufacturer. Reagent B is a lysis buffer and sodium perchlorate denatures proteins. The mixture was shaken in a daisy wheel for 15 minutes, followed by incubation in a water bath at 65°C, for 30 minutes to 2 hours. The centrifuge tube was inverted occasionally during this incubation period. 5.5ml of chloroform that had been stored at -20°C was then added, the mixture was shaken in a daisy wheel for 10 minutes, followed by centrifugation at 800g for 1 minute. 800µl of Nucleon™ silica suspension was added to the mixture which was then centrifuged at 1400g for 3 minutes. The Nucleon™ silica binds proteins by the formation of an imide bond and forms a solid 'stratum' at the interface between the chloroform and aqueous upper phase. The solid Nucleon™ stratum traps both bound and unbound proteinaceous material in the lower organic phase. The aqueous phase was transferred into a fresh centrifuge tube and centrifuged at 1300g for 30 seconds to pellet any residual Nucleon silica. The DNA containing supernatant was decanted to a fresh tube and two volumes of ice cold absolute ethanol was added to precipitate the DNA. The DNA was hooked from the liquid with a plastic inoculating loop, washed in 70% ethanol, allowed to air dry, and then dissolved in an appropriate volume of 1x TE buffer. The concentration of the DNA sample was determined as described in section 2.2.1 (a).

2.2.2. Agarose gel electrophoresis

Polymerase chain reaction (PCR) products and restriction enzyme digestion products were analysed by agarose gel electrophoresis. A 2% (w/v) or 3% (w/v) agarose gel was prepared by mixing an appropriate amount of agarose (Sigma) with 1x TBE buffer (appendix 1), adding 1µg/ml ethidium bromide, and heating in a microwave oven at full power for 1 minute. The agarose solution was allowed to cool, poured into a mini-gel mould (UniScience), and a 16-well comb was inserted to create wells for sample loading. The gel was allowed to set at room temperature for 30 minutes after which the comb was removed. DNA samples were mixed with a one-tenth volume of agarose gel loading buffer (appendix 1) and loaded into wells

submerged under 1x TBE electrophoretic running buffer. Electrophoresis was carried out at 50V. The duration of electrophoresis depended on the size of the DNA fragments being analysed. Following electrophoresis the DNA was visualized under ultraviolet transillumination.

2.2.3. The polymerase chain reaction (PCR)

2.2.3 (a) Oligonucleotide primers

Oligonucleotide DNA primers were purchased from Oswel DNA service, University of Southampton, Southampton with the exception of the primers for the *p53* gene which were kindly donated by Dr. Margaret Knowles of the Marie Curie Institute, Surrey. A total of 44 oligonucleotide primer pairs were used in this study. For each pair, primer sequences, optimal annealing temperature used, and size of the polymerase chain reaction product generated are given in sections 1-6 of appendix 3.1. The melting temperature (T_m) of each primer was calculated by using the formula Tm = [69.3 + (0.41 x G+C%)] - 650 / L, where G+C% is the percentage of guanine and cytosine residues in the primer, and L = total number of bases in the primer. A range of temperatures were tested to determine the optimal annealing temperature for each primer pair, which was generally 2-5°C below the calculated T_m.

2.2.3 (b) Polymerase chain reaction

Polymerase chain reaction (PCR) mixtures were prepared on ice under sterile conditions in a laminar hood using sterile pipette tips and Gilson pipettors that were kept exclusively for use in setting up PCR, to minimise DNA contamination. Each reaction mixture consisted of 50pM of each oligonucleotide primer, 0.2mM each deoxynucleoside triphosphate (dNTP) (appendix 3.2), 1 unit of *Taq* DNA polymerase (Super *Taq*, from HT Biotechnology Limited), 1x Super *Taq* reaction buffer (appendix 3.3), 200-500ng genomic DNA, and sterile distilled deionised water to make up to a final volume of 25µl or 100µl. A negative control consisting of the mixture described above but with sterile distilled deionised water instead of DNA template was also prepared. Reaction mixtures in sterile 0.5ml centrifuge tubes were centrifuged briefly (3 seconds) in a microcentrifuge to mix, overlaid with 50µl of mineral oil to prevent evaporation, and placed in an Omnigene thermal cycler (Hybaid Limited, Teddington, Middlesex). The conditions used for amplification with the majority of primer sets were an initial denaturation step at 94°C for 4.5 minutes, followed by 35 cycles of:

94°C for 30 seconds (denaturation step)
appropriate annealing temperature for 45 seconds (primer annealing step)
72°C for 45 seconds (elongation step).

On completion of the final cycle a further elongation at 72°C for 10 minutes was carried out, to ensure complete extension of all amplified molecules. If different reaction conditions were used for a particular primer pair, these are described in the appropriate section of appendix 3.1. After completion of the PCR, the products were stored at 4°C.

2.2.3 (c) Agarose gel electrophoresis of PCR products

The PCR products and negative control were analysed by agarose gel electrophoresis to determine that the product of expected size had been generated and to determine whether or not the negative control was contaminated with amplified DNA. For 25µl PCR mixtures, a 4.5µl aliquot of PCR product or negative control was mixed with 0.5µl of agarose gel loading buffer (appendix 1). For 100µl PCR mixtures, a 9µl aliquot of PCR product or negative control was mixed with 1µl of agarose gel loading buffer. The products were analysed by electrophoresis in a 2% (w/v) agarose gel as described in section 2.2.2. The duration of electrophoresis depended on the size of the PCR products to be analysed but was generally 30-60 minutes. In order to estimate the size of the PCR products, a DNA molecular weight marker (100 bp or 1 kb ladder, diluted 1 in 10 with sterile distilled deionised water and with agarose gel loading buffer) was electrophoresed alongside the products.

2.2.4. Single-strand conformation polymorphism (SSCP) and heteroduplex analysis

Single-strand conformation polymorphism (SSCP) and heteroduplex analysis was carried out using the PhastSystem™ (Pharmacia, St. Albans, Herts.), an automated gel electrophoresis and staining system. Separation of DNA fragments is performed on commercially available ultra-thin precast polyacrylamide gels (PhastGel®) (Pharmacia). In the PhastSystem™ the running conditions of electrophoresis (temperature, voltage, and amperage) are accurately controlled by a microprocessor. The PhastSystem™ also has a development chamber in which the gels are stained.

An aliquot of 0.75µl of PCR product was diluted with an equal volume of sterile distilled deionised water and mixed with 1.5µl of 95% formamide. This

mixture was denatured in an Omnigene thermal cycler (Hybaid Limited) at 95°C for 5 minutes to attain single strands of DNA and then cooled on ice for 5 minutes. The denatured PCR products were electrophoresed through precast 20% non-denaturing polyacrylamide gels (PhastGel homogenous 20) (Pharmacia). The gels were run with PhastGel® Native Buffer Strips (Pharmacia) according to the manufacturer's instructions. The buffer strips transfer current and voltage to the gel. Denatured PCR products were loaded onto the PhastGel® using a 12-well sample applicator (Pharmacia) (each applicator can load up to 12 DNA samples). A well template was formed with Nescofilm™ (Bando Chemical Industry Limited) using a 12-well template mould (Pharmacia). 2µl of each denatured PCR product was pipetted into the wells and the sample applicator was lowered to the samples which were drawn up into the capillaries of the applicator. This was then placed in the applicator arm in the electrophoresis chamber of the PhastSystem™, the electrophoresis programme was started, and the samples were loaded automatically onto the PhastGel®. Prior to sample loading PhastGels were pre-run to generate a continuous buffer system, at 400V, 20mA, 2W, for 10Vh. Samples were applied at 25V, 5mA, 2W, for 2Vh. Samples were separated at 400V, 20mA, 2W, for 200-400Vh. Electrophoresis was carried out at 4, 10, and 15°C.

Following electrophoresis, the PhastGels were stained with silver in the development chamber of the PhastSystem™ according to the manufacturer's instructions. The staining was an automated procedure. The staining programme, with the solutions and conditions used, is described in appendix 4. In the PhastSystem™, two gels can be run simultaneously, and also stained at the same time in the development chamber.

2.2.5. DNA sequencing

DNA sequencing reactions were performed using either the Thermo Sequenase™ cycle sequencing kit (Amersham International plc) or the CircumVent™ thermal cycle dideoxy DNA sequencing kit (New England Biolabs). Both protocols were based on the chain termination method described by Sanger *et al.* (1977).

2.2.5 (a) Preparation of PCR generated DNA template for sequencing

Excess primers and nucleotides must be removed from PCR mixtures before performing sequencing reactions. Two different methods were used to prepare PCR products for sequencing. One of the methods involved enzymatic treatment of PCR products with Exonuclease I and Shrimp Alkaline Phosphatase (Amersham). The

Exonuclease I removes residual single-stranded primers and any extraneous single-stranded DNA produced by the PCR. The Shrimp Alkaline Phosphatase removes the remaining dNTPs from the PCR mixture which would interfere with the labelling step of the sequencing process. 10 units of Exonuclease I and 2 units of Shrimp Alkaline Phosphatase were added to 5µl of PCR product and mixed. The mixture was incubated at 37°C for 15 minutes in an Omnigene thermal cycler (Hybaid Limited). The mixture was then heated to 80°C for 15 minutes, to inactivate the two enzymes. This method of preparing DNA template was used when sequencing reactions were performed using the Thermo Sequenase™ cycle sequencing kit.

The other method involved purification of PCR products using the Wizard™ PCR Preps DNA purification system (Promega Corporation, Wisconsin, USA). PCR products were firstly separated in a 2% (w/v) agarose gel, prepared using a low melting temperature agarose (NuSieve GTG agarose, FMC BioProducts). The gel was prepared essentially as described for a standard agarose gel in section 2.2.2, except 1x TAE buffer (appendix 1) was used instead of 1x TBE buffer and a midi-gel mould (Bethesda Research Laboratories, Rockville, USA) was used. 1x TAE was also the electrophoretic running buffer. 100µl of PCR product was diluted with a one-tenth volume of agarose gel loading buffer and loaded onto the gel. Electrophoresis was carried out at 4°C and 125V for between 2 and 4 hours. The desired DNA band was then excised from the gel using a scalpel. The DNA-containing gel slice was transferred to a 1.5ml microcentrifuge tube and incubated at 70°C until the agarose had completely melted. 1ml of PCR Preps resin (Promega) was added and mixed. The DNA was purified from this mixture using a Wizard™ minicolumn (Promega) according to the manufacturer's instructions. DNA was finally eluted in 50µl sterile distilled deionised water, and stored at 4°C. This method of preparing DNA template for sequencing was used when sequencing reactions were performed using the CircumVent™ thermal cycle dideoxy DNA sequencing kit.

2.2.5 (b) DNA sequencing reactions performed using the Thermo Sequenase™ cycle sequencing kit

The 3-dNTP Internal label cycle sequencing protocol available with the kit was used. In the first step of this method (labelling step) the primer used in the sequencing reaction is labelled to high specific radioactivity. To achieve this, prior knowledge of downstream template sequence is required. The primer is extended using only 3 of the 4 deoxynucleoside triphosphates (dNTPs), including an α - labelled dNTP, in a thermal cycling reaction. The extension is terminated when the fourth (omitted) nucleotide is needed. For example, the sequence of the forward

primer used for amplifying exon 4 of the *p53* gene and the corresponding template sequence is as follows:

Primer 5' - ATCTACAGTCCCCCTTGCCG - 3'
Template GGTAGATGTCAGGGGGAACGGCAGGGTTCGT

Polymerization in the presence of dTTP, dCTP, and labelled dATP, but omitting dGTP from the reaction, will result in extension of the primer by 6 nucleotides:

**
Primer 5' - ATCTACAGTCCCCCTTGCCGTCCCAA - 3'
Template GGTAGATGTCAGGGGGAACGGCAGGGTTCGT

Repeated thermal cycling of this step will result in the production of a significant amount of labelled, extended primer whose length is dependent on the template sequence.

In the labelling step of the sequencing reaction, the following components were combined in a 0.5ml microcentrifuge tube on ice:

2µl treated PCR product
0.5pmol/µl primer
2µl reaction buffer
1µl dNTP cycle mix 1*
1µl dNTP cycle mix 2*
0.5µl [α-^{35}S] dATP
8 units of Thermo Sequenase™ DNA polymerase
distilled deionised water to a total volume of 17.5µl.

* either dCTP, dGTP, or dTTP cycle mix were used depending on the sequence.

The solution was mixed by gentle pipetting, and the mixture was overlaid with 15µl of mineral oil. The tube was placed in an Omnigene thermal cycler (Hybaid Limited) and the cycling programme was started. The cycling parameters used were 50 cycles of:

95°C for 15 seconds
annealing temperature of primer for 30 seconds.

On completion of the final cycle, the reaction mixture was transferred to a fresh microcentrifuge tube, taking care to transfer as little of the mineral oil overlay as possible, and the termination step of the sequencing reaction was prepared. Four microcentrifuge tubes were labelled G, A, T, and C. 4µl of ddGTP termination mix was dispensed into the tube labelled G. Similarly, 4µl of ddATP, ddTTP, and ddCTP termination mixes were pipetted into the tubes labelled A, T, and C respectively. 3.5µl of the labelling step reaction mixture was added to each of the termination

mixes, and the solutions were mixed by gentle pipetting. The mixtures were overlaid with 15µl mineral oil, placed in a thermal cycler and the termination step cycling programme was started. The cycling parameters used were 50 cycles of:
95°C for 30 seconds
extension temperature* for 90 seconds.
* The extension temperature was chosen to be low enough to allow annealing of the extended, labelled primer but not low enough for annealing of the unextended primer.
On completion of the final cycle, 4µl of formamide stop solution (appendix 1) was added to each termination reaction and mixed. The reaction products were then centrifuged briefly (5 seconds) in a microcentrifuge to separate the oil from the aqueous phase, and stored at -20°C for up to one week.

2.2.5 (c) DNA sequencing reactions performed using the CircumVent™ Thermal Dideoxy DNA sequencing kit

Four 0.5ml microcentrifuge tubes were labelled G, A, T, and C. 3µl of G deoxy/dideoxy sequencing mix was dispensed into the tube labelled G. Similarly 3µl of of the A, T, and C deoxy/dideoxy sequencing mixes were dispensed into the tubes labelled A, T, and C respectively. The following reaction components were mixed in another microcentrifuge tube on ice:

1µl purified PCR product
1.2pmol primer
1.5µl 10x CircumVent™ Sequencing buffer
1µl 3% Triton X-100
distilled deionised water to a total volume of 12µl.

To this mixture was added 2µl of [α-^{35}S] dATP and 2 units of Vent$_R$™ (exo-) DNA polymerase, and the solution was mixed by gentle pipetting. 3.2µl of this mixture was then added to each of the four deoxy/dideoxy sequencing mixes and mixed by gentle pipetting. Each reaction mixture was then overlaid with 15µl of mineral oil. The tubes were placed in an Omnigene thermal cycler and the cycling programme was started. The cycling parameters used were 20 cycles of:
95°C for 20 seconds
annealing temperature of primer for 20 seconds
72°C for 20 seconds.
The reactions were terminated by the addition of 4µl of formamide stop solution. The reaction products were centrifuged briefly (5 seconds) in a microcentrifuge to separate the oil from the aqueous phase and then stored at -20°C.

2.2.5 (d) Polyacrylamide gel electrophoresis

The products of DNA sequencing reactions were electrophoresed in 6% denaturing polyacrylamide gels. The sequencing gel mix was supplied by Severn Biotech Limited and consisted of 6% (w/v) acrylamide ratio 19:1 bisacrylamide, 7M urea, 1x TBE. The gels were prepared and run in a 21cm x 40cm sequencing gel apparatus (Sequi-Gen DNA Sequencing Cell) supplied by Biorad Laboratories Limited and were of 0.4mm thickness. To 40ml of the sequencing gel mix was added 60µl of NNN'N' tetramethylethylenediamine (TEMED) and 60µl of 25% (w/v) ammonium persulphate, which initiate and catalyse polymerisation. This solution was mixed and poured between the glass plates of the sequencing gel apparatus using a 50ml syringe. A flat edge was obtained at the top of the gel by inserting a 48-well sharks tooth comb upside down between the plates, and the gel was allowed to polymerize for one hour at room temperature. The comb was then removed, the sequencing apparatus was assembled, and the comb was inserted into the gel the right way up. The gel was prerun for between 30 and 60 minutes in 1x TBE running buffer (appendix 1) at 2000V, 45W, to allow the gel to reach a temperature of 50-55°C, before the samples were loaded. This is the temperature at which best resolution of sequence is achieved. The sequencing reaction products were denatured by heating to 80°C for 3 minutes in an Omnigene thermal cycler (Hybaid Limited) and 2µl was loaded onto the gel. The samples were electrophoresed for the desired length of time, determined by the relative positions of the bromophenol blue and xylene cyanol dyes.

2.2.5 (e) Drying of polyacrylamide gels and autoradiography

On completion of electrophoresis the sequencing apparatus was disassembled with the polyacrylamide gel remaining on the inner glass plate. The gel was transferred to 3mm filter paper (Whatman), covered with Saran Wrap (Dow Chemical Company), and dried in an oven at 80°C for 1 hour. The Saran Wrap was removed and autoradiography was performed by exposing the gel to Hyperfilm^{TM}-βmax (Amersham International plc) for 16-72 hours.

2.2.6. Protein truncation test (PTT)

2.2.6 (a) Generation of DNA template for the PTT

Twenty-six gastric carcinoma and corresponding normal DNAs were analysed for translation-terminating mutations in exon 15 of the *APC* gene by the protein truncation test (PTT). As this exon is very large (6.5 kb), template for the *in vitro*

transcription and translation reaction could be generated by PCR amplification of genomic DNA, thus RNA isolation and RT-PCR (reverse transcription-polymerase chain reaction) did not have to be performed. The region of *APC* exon 15 anlysed included codons 1028-1700. The primers used to amplify this region from genomic DNA were those described by van der Luijt *et al.* (1994) (appendix 3.1.1b). The sense (forward) primer was modified to contain a T7 bacteriophage promoter sequence for the initiation of transcription as well as a eukaryotic consensus sequence for the initiation of translation at the 5' end, in frame with APC unique sequence. The 2kb products generated as a result of PCR were used directly without purification as templates in coupled transcription-translation reactions.

2.2.6 (b) *In vitro* coupled transcription-translation reactions

The coupled transcription-translation reactions were carried out using the TNT™ T7 Coupled Reticulocyte Lysate Kit (Promega Corporation, Wisconsin, USA). RNA is very sensitive to nuclease degradation. To reduce the chance of introducing ribonuclease contamination, the reactions were set up using sterile pipette tips and microcentrifuge tubes that had been treated with the ribonuclease inhibitor diethyl pyrocarbonate (DEPC), thus inactivating any nucleases. The pipette tips and microcentrifuge tubes were soaked in a 0.1% (v/v) solution of DEPC, prepared using distilled deionised water and placed in a 37°C incubator for 12 hours. The DEPC solution was poured off, and the pipette tips and microcentrifuge tubes were autoclaved to remove the remaining DEPC. Reactions with a final volume of 12µl were set up. A premix of the following volumes of reaction components was first prepared on ice:

TNT rabbit reticulocyte lysate[a]	6.25µl
TNT reaction buffer[a]	0.5µl
TNT T7 RNA polymerase[a]	0.25µl
Amino acid mixture minus methionine[a], 1mM	0.25µl
^{35}S-methionine (1000Ci/mmol)	0.5µl
RNasin ribonuclease inhibitor (40U/µl)	0.25µl
Nuclease-free water[b]	2µl

[a] These reaction components were all supplied with the TNT™ T7 coupled reticulocyte lysate kit from Promega. The other components were obtained separately. The RNasin ribonuclease inhibitor was also purchased from Promega.

[b] The nuclease-free water was prepared by adding DEPC to distilled deionised water to a concentration of 0.1%, mixing thoroughly. The solution was left overnight at room temperature and then autoclaved to remove any trace DEPC.

The reaction mixture was pipetted up and down gently to mix, and 10µl was dispensed into microcentrifuge tubes containing 2µl of PCR product, and mixed. The reaction mixtures were incubated in a waterbath at 30°C for 90 minutes, and then stored at -20°C.

2.2.6 (c) SDS-polyacrylamide gel electrophoresis (SDS -PAGE)

The translation products were separated by electrophoresis on a sodium dodecyl sulphate (SDS) - polyacrylamide gel with a gradient of 10 to 20 percent. A 10% acrylamide gel mix and a 20% acrylamide gel mix were prepared as described in appendix 5. A stacking gel mix was also prepared (appendix 5) to pour on top of the gradient gel. The translation products are applied to the stacking gel and are concentrated during electrophoresis through the stacking zone so that when the separating zone is entered, the sample components are packed into a very narrow starting zone. Separation does not begin until the samples enter the gradient gel. The volumes of gel mixes given were sufficient for a 160mm x 195mm gel, of 1.5mm thickness. The 10% and 20% acrylamide gel mixes were prepared first. The two gel solutions were mixed using a Masterflex® Economy Drive pump so that a gradient gel would be produced. Gel mixture of progressively lower acrylamide concentration was pumped between two vertically assembled glass plates (Bethesda Research Laboratories), leaving sufficient space above the gel for the stacking gel to be added later. The gel mixture was overlaid with 3ml distilled deionised water. The gel was then allowed to polymerize for 30 minutes at room temperature. After this time the overlay was removed and the surface of the gradient gel was rinsed with water to remove any unpolymerized acrylamide. The stacking gel mix was then prepared and poured on top of the gradient gel. A 12 well comb was inserted and the gel was allowed to polymerize for 30 minutes. The comb was then removed. The gel was assembled in the SDS-PAGE apparatus (Vertical gel electrophoresis system, Bethesda Research Laboratories) and immersed in 1l of Tris-glycine electrophoretic running buffer (appendix 1).

5µl of each translation product was mixed with 20µl of SDS-polyacrylamide gel loading buffer (appendix 1) in a 0.5ml microcentrifuge tube. The mixtures were heated at 100°C for 2 minutes to denature the proteins, and 20µl was loaded into the wells of the stacking gel. A protein molecular weight marker (Rainbow™ coloured protein molecular weight marker-high molecular weight range, Amersham International plc) was electrophoresed alongside the translation products, so that the size of the products could be estimated. 7µl of the Rainbow™ marker was mixed

with 10µl of SDS-polyacrylamide gel loading buffer (appendix 1) and 3µl of distilled deionised water, and all 20µl was loaded onto the gel. Electrophoresis was then carried out at 200V, 100mA, until the bromophenol blue dye reached the bottom of the gel (approximately 3 hours).

2.2.6 (d) Fixing and drying of SDS-PAGE gels and autoradiography

On completion of gel electrophoresis, the stacking gel was removed and the gradient gel was fixed in 30:30:40 acetic acid:methanol:water for 30 minutes. The gel was then soaked in 5:10:85 glycerol:methanol:water for 12-16 hours. After this period the gel was sandwiched between Saran Wrap and Whatman 3mm paper and dried at 80°C for 2 hours in a Biorad gel dryer. The Saran Wrap was removed and autoradiography was performed by exposing the gel to Hyperfilm^{TM}-MP (Amersham International plc) at room temperature for 24-72 hours.

2.2.7. Restriction enzyme digestion of PCR products

PCR products were digested with the appropriate restriction enzyme either for the detection of specific mutations of the *APC* gene or for the detection of restriction fragment length polymorphisms (RFLPs) in loss of heterozygosity (LOH) analysis (sectons 2.2.8 and 2.2.9). Restriction enzyme digests were set up as follows in sterile 0.5ml microcentrifuge tubes:

10µl PCR product
4µl 10x restriction enzyme buffer
10 units restriction enzyme
Sterile distilled deionised water to 40µl.

The mixtures were briefly centrifuged (5 seconds) in a microcentrifuge to mix, followed by incubation in a water bath at 37°C overnight. The reactions were stopped by the addition of 4µl agarose gel loading buffer (appendix 1). A 15µl aliquot of each digestion product was analysed by electrophoresis in a 2% (w/v) or 3% (w/v) agarose gel, as described in section 2.2.2. A 100bp ladder (diluted 1 in 10 with sterile distilled deionised water and with agarose gel loading buffer) was electrophoresed alongside the digests, so that the size of the digestion products could be estimated.

2.2.8. Restriction enzyme digest analysis for the detection of mutations of the *APC* gene

Some relatively common point mutations in exons 6, 8, and 14 of the *APC* gene result in either the generation or loss of recognition sites of certain restriction enzymes (Ando *et al.*, 1993). Thus the mutation can be detected by the appearance of smaller fragment(s) or retention of an undigested fragment on an agarose gel after enzyme digestion of PCR products. The specific mutation detected together with the restriction enzyme used and size of fragments expected after digestion of PCR products are given in table 2.3. The mutations in exon 6, 8, and at codon 622 in exon 14 result in loss of recognition sites of the enzymes *Acc* I, *Taq* I, and *Msp* I respectively. Thus the PCR products are not cleaved into the smaller fragments following treatment with the restriction enzymes, if the mutation is present. The mutation at codon 625 in exon 14 creates a recognition site of *Mae* I, thus the PCR product is cleaved into smaller fragments following treatment with the restriction enzyme, if the mutation is present. The 26 gastric carcinoma and matching normal DNAs were investigated for these specific mutations. Exons 6, 8, and 14 of *APC* were amplified using primers described in appendix 3.1.1 (a). The digests were set up as described in section 2.2.7 and then analysed by electrophoresis in 2% (w/v) agarose gels.

Table 2.3. Detection of mutations in the *APC* gene by PCR and restriction enzyme digestion.

Exon	Codon	Mutation	Size of fragments derived from normal alleles	Size of fragments derived from mutant alleles	Enzyme
6	232	CGA→TGA	137 bp, 98 bp	235 bp	*Acc* I
8	302	CGA→TGA	134 bp, 81 bp	215 bp	*Taq* I
14	622	TAC→TAA	163 bp, 140 bp	303 bp	*Msp* I
14	625	CAG→TAG	266 bp, 37 bp	135 bp, 131 bp, 37 bp	*Mae* I

2.2.9. PCR-LOH analysis at polymorphic loci of the *APC*, *MCC*, *DCC*, and *p53* genes

Table 2.4 shows the polymorphic loci of the *APC*, *MCC*, *DCC*, and *p53* genes at which loss of heterozygosity (LOH) was investigated. Primers used for

amplification of the polymorphic sites by PCR are described in appendix 3.1. For LOH analysis at the RFLP sites, PCR products were digested with the appropriate restriction enzyme and electrophoresed on 3% (w/v) agarose gels as described in section 2.2.7. For analysis at the VNTR type polymorphic site in exon 10 of *MCC*, PCR products were electrophoresed directly in 3% (w/v) agarose gels as described in section 2.2.2. The polymorphisms in *APC* exon 15 regions I and J, *MCC* 3' UTR, *p53* intron 2, *p53* exon 4, and the *p53CA* dinucleotide repeat polymorphism were all detected by SSCP analysis in PhastGel homogenous 20 using the PhastSystem™, as described in section 2.2.4.

All the polymorphic sites shown in table 2.4 were analysed in the 26 gastric carcinoma and corresponding normal DNAs, except the *Msp* I RFLP site in exon 15 region N of *APC*. The 43 colorectal carcinoma and corresponding normal DNAs were only investigated for LOH at the 5 polymorphic loci of the *APC* gene. Only cases where analysis of normal DNA showed constitutional heterozygosity at a given locus were considered to be informative for LOH analysis. LOH was assessed visually and defined as a change in allele:allele ratio in tumour relative to corresponding normal DNA. A reduction in intensity of an allele as well as complete loss of an allele was seen, and scored as LOH.

Table 2.4. Polymorphic loci of the *APC*, *MCC*, *DCC*, and *p53* genes analysed for LOH

Polymorphic site[a]	Allele sizes
APC exon 11, *Rsa* I RFLP	A1=215 bp, A2=130/85 bp
APC exon 15I, codon 1493	A1/A2 = 507 bp
APC exon 15J, codon 1678	A1/A2 = 317 bp
APC exon 15N, *Msp* I RFLP	A1= 541 bp, A2=270 bp
APC 3' UTR, *Ssp* I RFLP	$A1^b$ = 270bp, A2=135 bp
MCC 3' UTR	A1/A2 = 210 bp
MCC exon 10, VNTR polymorphism	A1= 79 bp, A2=93 bp
DCC M2, *Msp* I RFLP	A1= 396 bp, A2=257/139 bp
DCC M3, *Msp* I RFLP	A1= 240 bp, A2=137/103 bp
p53 intron 2, position 11827	A1/A2 = 271 bp
p53 exon 4, codon 72	A1/A2 = 293 bp
p53CA	103 - 135 bp

[a] RFLP, restriction fragment length polymorphism; 3' UTR, 3' untranslated region; VNTR, variable number of tandem repeats.

[b] Digestion with *Ssp* I also gave a band of 580 bp which thus served as an inbuilt control for complete digestion.

2.2.10. Analysis of alterations at microsatellite repeat loci

Twelve microsatellite repeat loci were analysed for replication error (RER) and loss of heterozygosity (LOH) in paired tumour and normal DNA from 26 gastric carcinoma cases and 43 colorectal carcinoma cases. Details of the loci studied with primer sequences are given in appendix 3.1.6. All twelve repeat sequences are of the dinucleotide $(CA)_n$ type. The microsatellite repeat polymorphisms were analysed by PCR-SSCP analysis using the PhastSystem™, as described in section 2.2.4. The *D22S351* marker was also analysed under denaturing conditions by capillary electrophoresis of fluorescent PCR products using an automated laser DNA analyser, in the colorectal carcinoma and corresponding normal DNAs, as will be described in the next section. An alteration was regarded as RER when additional bands were observed in tumour DNA which were not seen in corresponding normal DNA, and as LOH when a band corresponding to one allele of the normal DNA was lost in the corresponding tumour DNA. Only cases where analysis of normal DNA showed constitutional heterozygosity at a given locus were considered to be informative for LOH analysis. If tumour DNA from a case showed RER at a given locus it was excluded from the LOH analysis at that locus and deemed uninformative.

2.2.11. Fluorescent PCR and capillary electrophoresis using an automated laser DNA analyser

The forward *D22S351* primer (appendix 3.1.6) was 5'-end-labelled with 6-carboxyfluoroscein (6-FAM) (blue). PCR was performed as described in section 2.2.3 (b). The conditions used for amplification were an initial denaturation step at 94°C for 4.5 minutes, followed by 21 cycles of:

94°C for 30 seconds (denaturation)
60°C for 45 seconds (primer annealing)
72°C for 45 seconds (elongation)

On completion of the final cycle a further elongation at 72°C for 10 minutes was carried out.

The fluorescent PCR products were separated under fully denaturing conditions by capillary electrophoresis in an automated laser DNA analyser (ABI Prism™ 310 Genetic Analyzer, Perkin-Elmer Corporation) exactly according to the manufacturer's instructions. 1µl of PCR product was mixed with 12µl of formamide

and 0.5µl of TAMRA-labelled size standard (GeneScan™ 500-TAMRA [red], Perkin-Elmer Corporation) in a 0.5ml sample tube. The mixture was denatured by heating at 95°C for 2 minutes in an Omnigene thermal cycler (Hybaid Limited). The denatured PCR product was then run through a capillary filled with GeneScan™ polymer solution (Perkin-Elmer Corporation) at 15kV, 60°C, for 24 minutes. The data collected by the ABI Prism™ 310 Genetic Analyzer were automatically analysed using GeneScan™ Analysis software (Version 2.0.2) (Perkin-Elmer Corporation), to size and quantify the DNA fragments.

2.2.12. Statistical analysis

Statistical analysis was performed using the Fisher's exact test (Fish6 Fisher's exact test program), Student's t test, or chi-squared test. A p value of <0.05 was considered to be statistically significant.

Chapter 3

Results

3. RESULTS

3.1. Genetic alterations in colorectal cancer

3.1.1. Mutational analysis of the *APC* gene

The *APC* (adenomatous polyposis coli) tumour suppressor gene has been localized to chromosome 5q21-22 (Joslyn *et al.*, 1991; Kinzler *et al.*, 1991b). Forty-three percent of the coding region of the *APC* gene was analysed for mutations using a single-strand conformation polymorphism (SSCP) and heteroduplex assay in DNA derived from 43 colorectal carcinoma and matching normal tissues. The region of *APC* analysed was inclusive of exons 6, 8, 11, 14, and the 5' half of exon 15 (codons 653-1700). The 5' half of exon 15 was amplified by PCR in 10 overlapping segments, termed 15A-J, using primers described by Groden *et al.* (1991) (see appendix 3.1.1a) and each segment was subjected to the SSCP and heteroduplex assay. SSCP analysis involves separation of the positive and negative strands of DNA by heat denaturation with formamide. The denatured PCR products are then separated in native polyacrylamide gels. Double-stranded DNA molecules are not usually completely eliminated thus heteroduplex DNA may be observed on the gels. The exact nature of any variants identified using SSCP and heteroduplex analysis was then determined by sequencing. Somatic mutations were detected in 16 of 43 (37.2%) colorectal carcinomas, the results are summarized in table 3.1. All mutations were confirmed as somatic changes by simultaneous analysis of DNA isolated from corresponding normal tissues. In addition a number of germline variants were observed, these are described in table 3.2.

Figure 3.1a illustrates SSCP analysis with intronic primers flanking exon 8 of *APC* for case COCA35. Unique single-stranded DNA conformers were detected in the carcinoma that were not present in corresponding normal DNA. Sequence analysis (figure 3.1b) revealed the electrophoretic variation to be the result of a single base substitution of g for a in the intronic sequence, at a position eight base pairs upstream of the start of exon 8 (taatttttag/GGT to tagtttttag/GGT, with upper and lower case letters indicating exonic and intronic sequence respectively). Sequencing of corresponding normal DNA confirmed this to be a somatic event. The change resulted in the creation of a cryptic 3' splice site sequence (ag) which could lead to a frameshift in the *APC* transcript. A second SSCP variation was detected in case COCA35 in amplicon 15G of *APC*, and was present in both tumour and normal DNA

Table 3.1. Somatic mutations of the *APC* gene detected in colorectal carcinomas.

Case	Exon	Codon	Nucleotide change	Nature of mutation
COCA35	8	279*	aatttttag/GGT → agtttttag/GGT	Splice acceptor
COCA19	8	302	CGA → TGA	Arg → Stop
COCA34	15B	772-773	CTTTTG → CTTG	2 bp deletion
COCA33	15B	789	CAG → TAG	Gln → Stop
COCA32	15B	793-794	CAAAG → CAG	2 bp deletion
COCA2	15G	1307-1311	AAAAGAAAAGAT → AAAAGAT	5 bp deletion
COCA40	15G	1307-1311	AAAAGAAAAGAT → AAAAGAT	5 bp deletion
COCA42	15G	1307-1311	AAAAGAAAAGAT → AAAAGAT	5 bp deletion
COCA7	15G	1322	GAA → TAA	Glu → Stop
COCA23	15G	1322	GAA → TAA	Glu → Stop
COCA26	15G	1331	AGA → TGA	Arg → Stop
COCA28	15G	1331	AGA → TGA	Arg → Stop
COCA19	15G	1357-1359	GGAGCGAAA → GGAGCGAGCGAAA	4 bp insertion
COCA38	15H	1395	AGT → ATATCGT	4 bp insertion
COCA32	15H	1395-1405	AGTTTTGAGAGTCGTTCGATTGCCAGCTCCGTTCA → AGTTCA	29 bp deletion
COCA9	15H	1405	GTT → ATT	Val → Ile
COCA33	15H	1429	CAA → TAA	Gln → Stop
COCA24	15H	1462-1465	AAAAGAGAGAGAGT → AAAAGAGAGAGT	2 bp deletion
COCA8	15I	1494-1495	GAAAGT → GAAGT	1 bp deletion

* To the nearest codon.

Table 3.2. Germline variants of the *APC* gene detected in colorectal carcinoma cases.

Exon	Codon	Nucleotide change	Coding change
15A	653	AGG/AGA	Arg/Arg
15G	1317	GAA/CAA	Glu/Gln
15I	1493	ACA/ACG	Thr/Thr
15J	1678	GGA/GGG	Gly/Gly

(figure 3.2). The variant was sequenced and found to be due to a G to C change at the first nucleotide of codon 1317, resulting in an amino acid change from glutamic acid (GAA) to glutamine (CAA). This alteration has previously been characterized as a rare polymorphism (Groden *et al.*, 1993).

Two independent somatic variants were detected in the carcinoma DNA from case COCA19, in PCR amplicons exon 8 and exon 15G, as shown in figure 3.3. Single-stranded DNA variants were detected in the exon 8 amplicon. This was not the case for the 15G amplicon in which double-stranded heteroduplex molecules migrating faster than single-stranded DNA were observed as two well separated bands in the tumour DNA. Sequence analysis of amplicon exon 8 demonstrated a nonsense mutation from CGA (arginine) to TGA (stop codon) at codon 302, which is predicted to result in truncation of the gene product (figure 3.4a). Sequencing of amplicon 15G revealed a 4 bp duplication in the tumour DNA of GAGC or AGCG or GCGA at codon 1357-1359, the nature of the sequence making it difficult to determine exactly which four bases had been duplicated (figure 3.4b). This change is also predicted to result in truncation of the APC product because of the creation of a new stop codon downstream at codon 1373, due to frameshift.

Figure 3.5a shows SSCP analysis of the exon 15A amplicon in carcinoma and normal DNA from case COCA33. The same variant single strand band pattern was detected in both DNA samples. Single-stranded DNA variants were also detected in amplicons 15B and 15H in case COCA33 but these were somatic alterations, being found in the carcinoma DNA but not in corresponding normal DNA (figures 3.5b and c). Sequencing of amplicon 15A in normal DNA from COCA33 revealed the SSCP variation to be the result of a single base substitution of adenine for guanine at the third nucleotide of codon 653, the first codon of exon 15 (figure 3.6a). This was found to be a silent change of AGG (Arg) to AGA (Arg). An identical sequence change was detected in the carcinoma DNA from this case. In contrast, sequence

Figure 3.1.

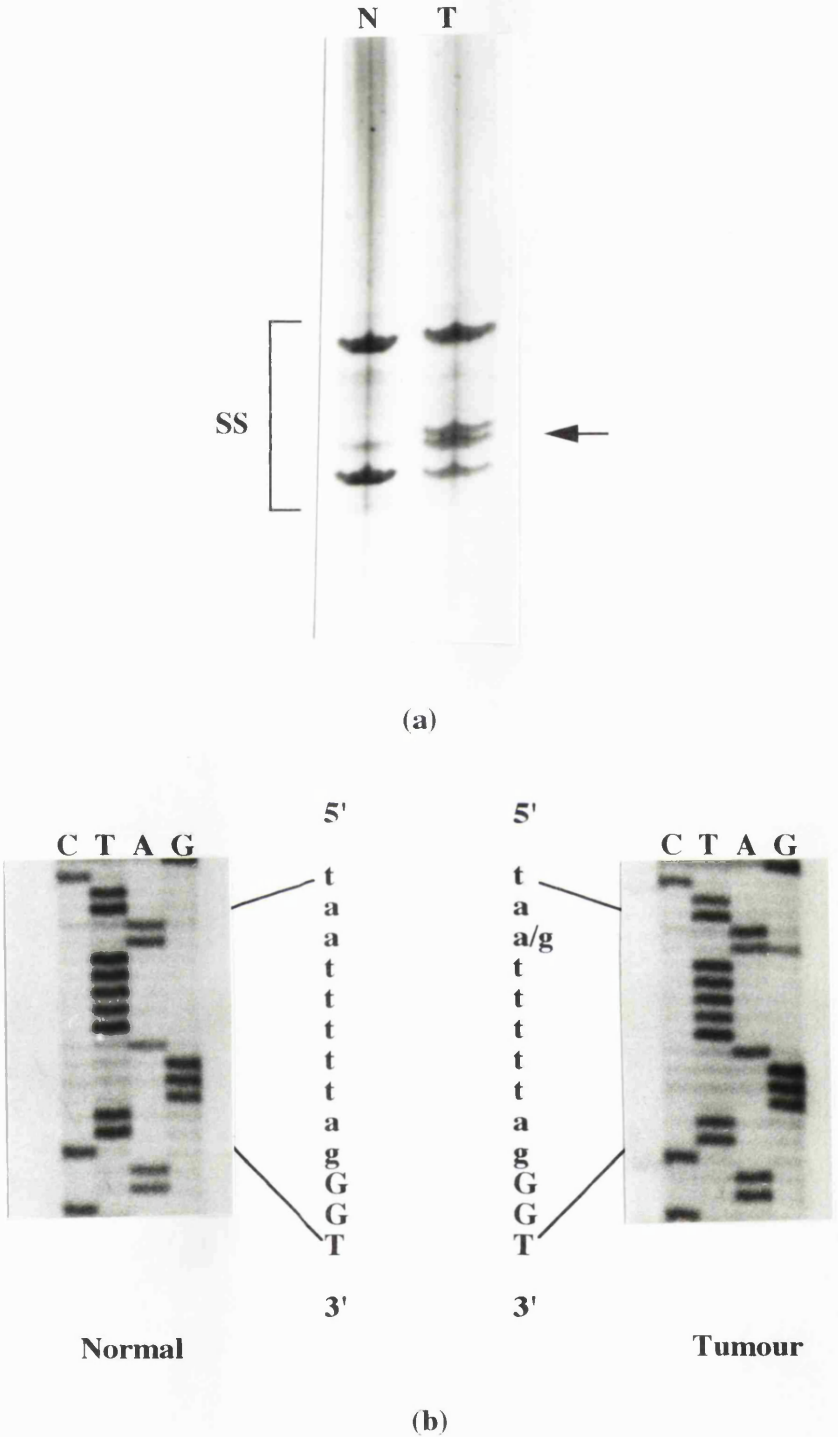
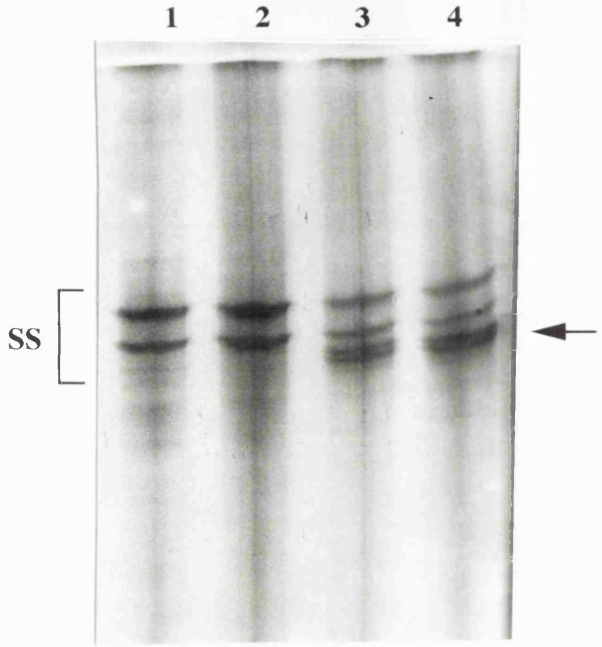
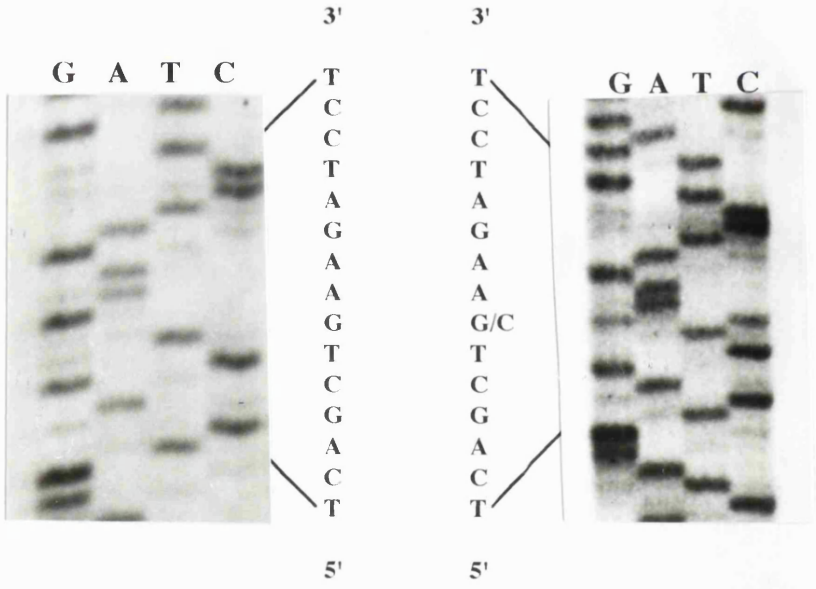


Figure 3.1. Detection of somatic mutation in exon 8 amplicon of *APC* in case COCA35. (**a**) SSCP analysis. N and T denote DNA samples from normal and corresponding tumour tissues respectively. In addition to the wild-type single strand (SS) band pattern, single strands of a differing mobility were observed in the tumour as indicated by the arrow. (**b**) Sequence analysis of exon 8 amplicon in tumour and normal tissue DNA from case COCA35. Lower and upper case letters represent intronic and exonic sequences respectively. An a to g transition was identified eight base pairs upstream of exon 8 at the splice acceptor site in the tumour DNA, the wild-type sequence is also seen. The sequence of the antisense strand is shown.

Figure 3.2.



(a)



Normal DNA from COCA34 **Normal DNA from COCA35**

(b)

Figure 3.2. Germline variant of the *APC* gene detected in the exon 15G amplicon in case COCA35. **(a)** SSCP analysis. Lanes 1 and 2 show SSCP of tumour and normal DNA respectively from case COCA34. Both samples demonstrated a normal single strand (SS) band pattern for amplicon 15G. Lanes 3 and 4 show SSCP of tumour and normal DNA respectively from case COCA35. Extra single-stranded DNA bands were detected on the gel in addition to the wild-type band pattern in both samples, as indicated by the arrow. **(b)** Sequence analysis of amplicon 15G (sense strand). Normal tissue DNA from case COCA34 contains only the wild-type *APC* sequence whereas normal DNA from case COCA35 contains a single base substitution (G to C) resulting in a coding change of Glu (GAA) to Gln (CAA) at codon 1317. An identical sequence change was identified in tumour tissue DNA from case COCA35 (not shown).

Figure 3.3.

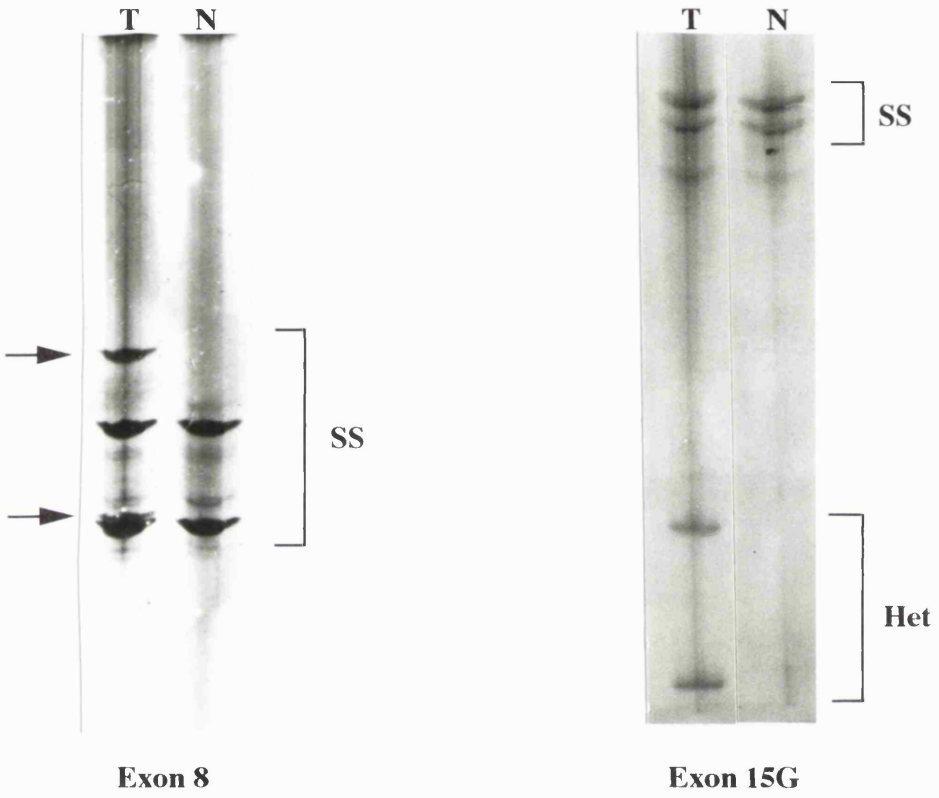
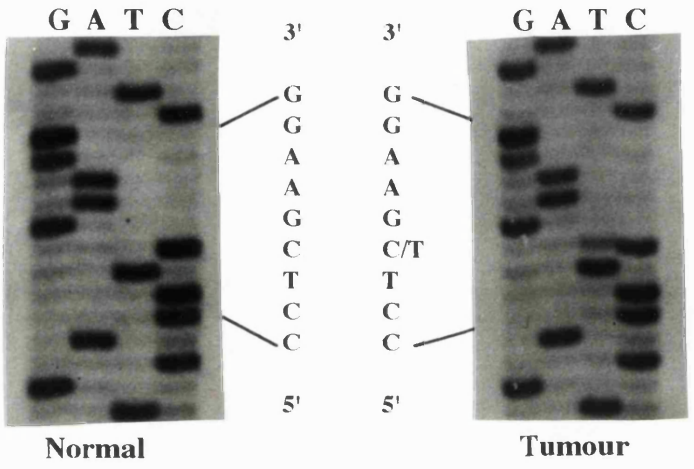
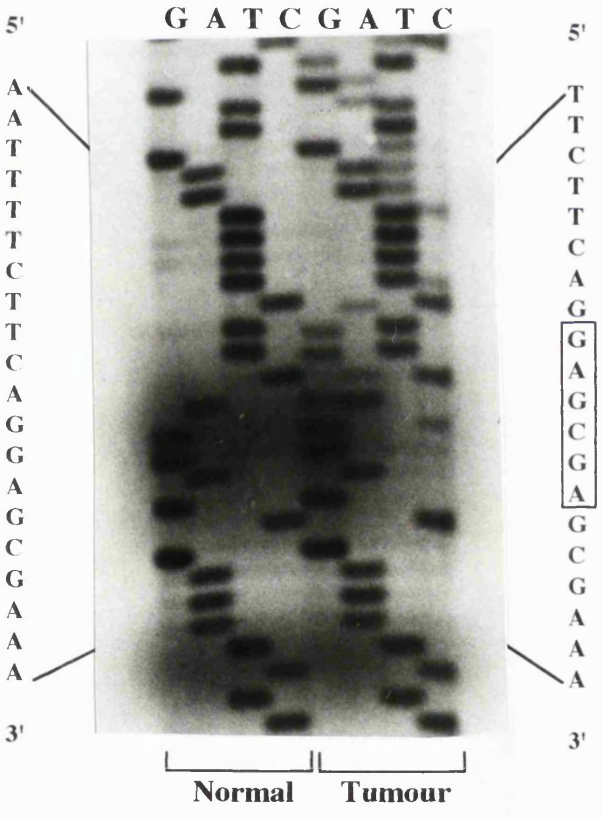


Figure 3.3. SSCP and heteroduplex analysis of the *APC* exon 8 and exon 15G amplicons in tumour and normal DNA from case COCA19. Novel single-stranded DNA conformers (SS) were detected in amplicon exon 8 in the tumour as indicated by the arrows (T=tumour DNA; N=corresponding normal DNA). No single strand variation was detected in amplicon 15G but heteroduplex bands (Het) were observed.

Figure 3.4.



(a)



(b)

Figure 3.4. Sequence analysis of exon 8 and 15G amplicons of *APC* in tumour and normal DNA from case COCA19. (**a**) Sequencing of exon 8 amplicon reveals the single strand variation observed in the tumour DNA to be due to a single base change of C to T at codon 302 resulting in a change from Arg (CGA) to a stop codon (TGA). The sequence of the sense strand is shown. (**b**) Sequencing of exon 15G amplicon (antisense strand). A 4 bp duplication at codon 1357-1359 of either GAGC or AGCG or GCGA (highlighted by box) results in a frameshift in the tumour DNA sequence and the formation of a premature stop codon downstream of the mutation.

Figure 3.5.

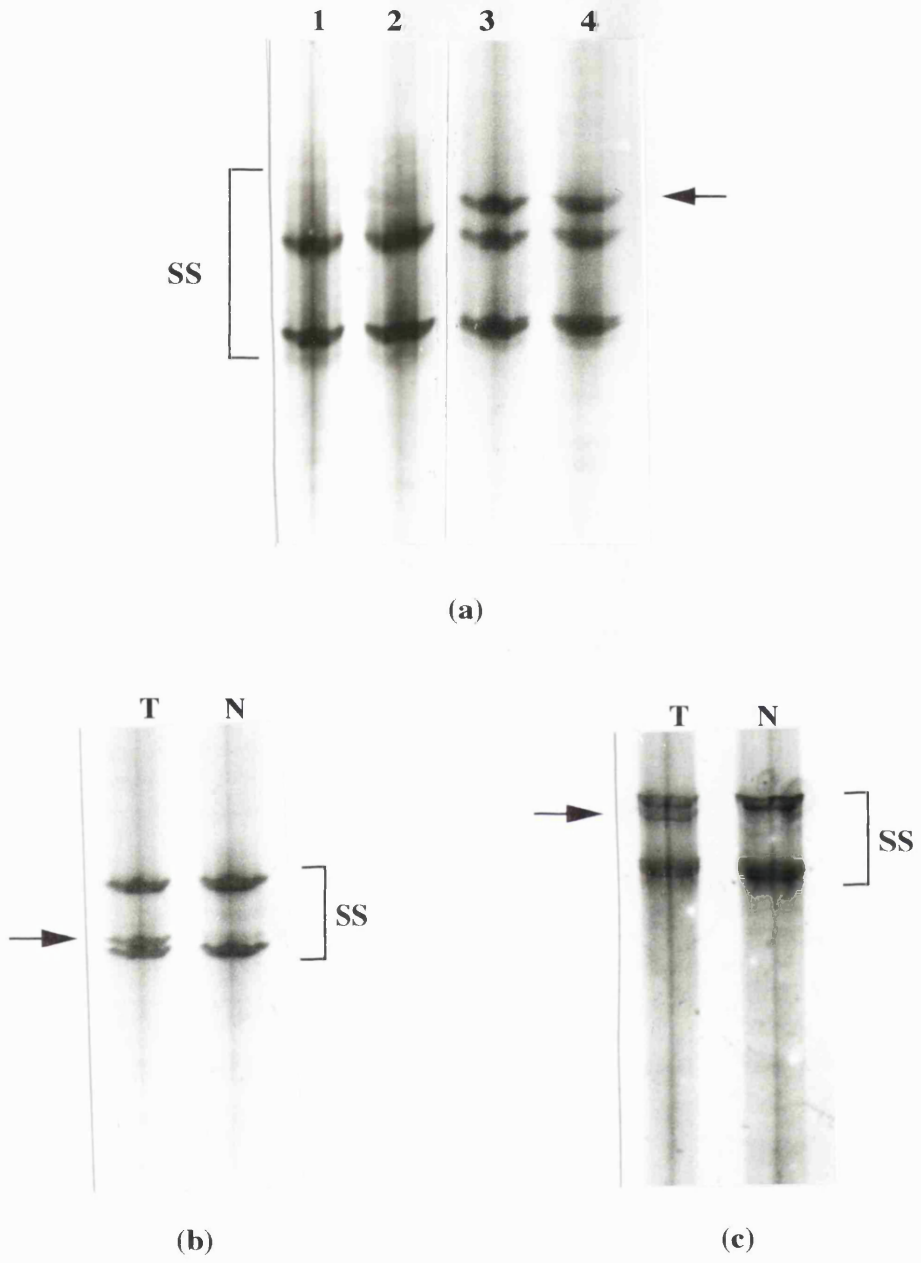


Figure 3.5. Detection of SSCP variants in amplicons 15A, 15B, and 15H of *APC* in case COCA33. (**a**) SSCP analysis of amplicon 15A. Lanes 1 and 2 show SSCP of tumour and normal DNA respectively from case COCA32. Both samples showed a normal single-stranded (SS) DNA pattern for amplicon 15A. Lanes 3 and 4 show SSCP of tumour and normal DNA respectively from case COCA33. Both samples demonstrated additional single-stranded DNA bands to the wild-type band pattern (indicated by arrow). (**b**) and (**c**) show SSCP analysis of amplicons 15B and 15H respectively for case COCA33. Unique single-stranded DNA conformers were detected in the tumour DNA sample only in both amplicons, as indicated by the arrows (T=tumour DNA; N=normal DNA).

Figure 3.6.

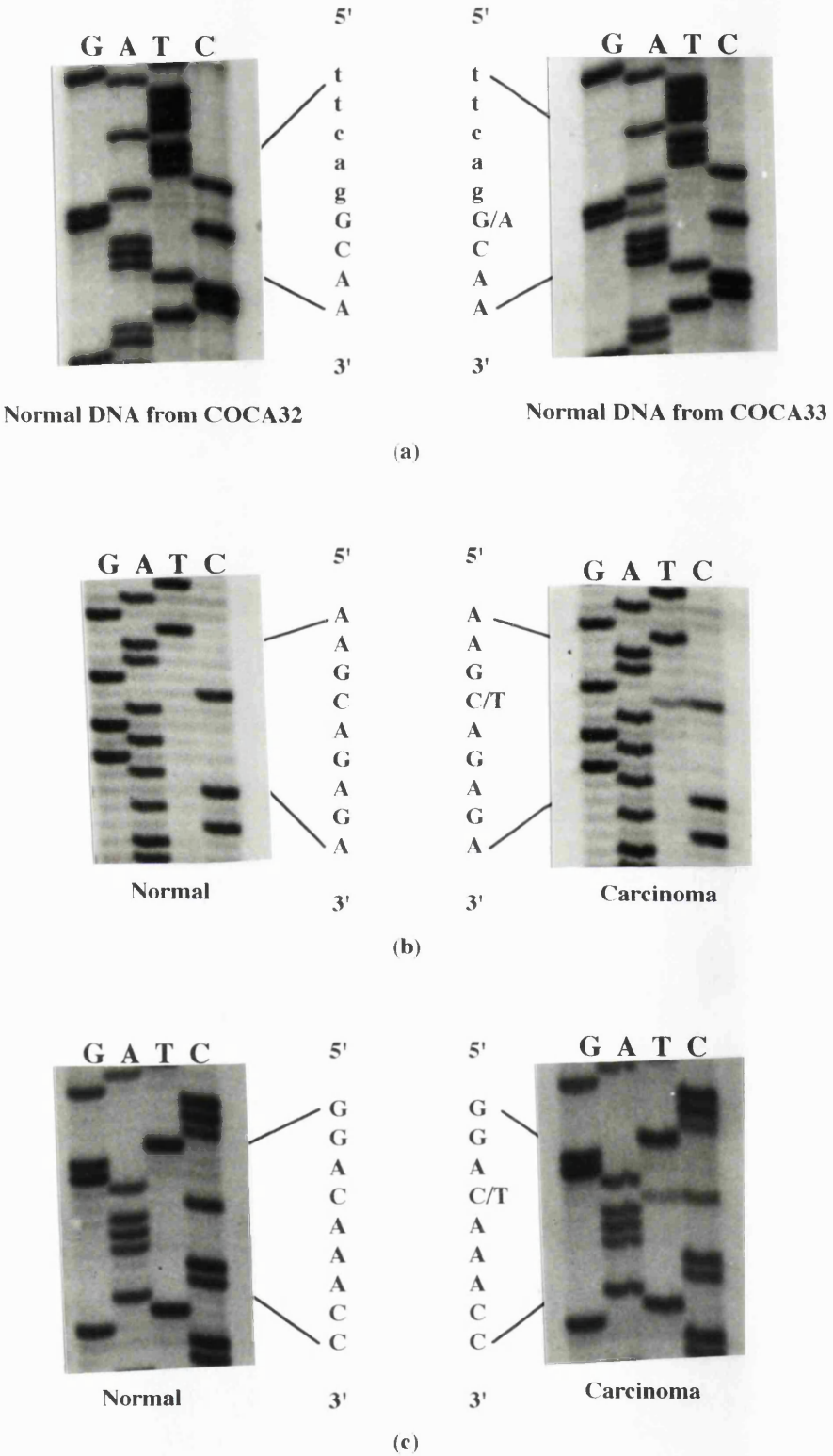


Figure 3.6. Sequencing of variants detected in amplicons 15A, 15B, and 15H of *APC* in case COCA33. **(a)** Sequence of exon 15A amplicon (antisense strand). Upper and lower case letters represent exonic and intronic sequences respectively. Normal tissue DNA from case COCA32 contains the wild-type *APC* sequence whereas normal DNA from case COCA33 contains a single base change of G to A at the third nucleotide of codon 653, which does not result in an amino acid change. **(b)** Sequencing of 15B amplicon (antisense strand) in carcinoma and normal DNA from case COCA33. A C to T transition was identified in the tumour DNA which results in a change from Gln (CAG) to a stop codon (TAG) at codon 789. **(c)** Sequence of 15H amplicon (antisense strand). A single base substitution of T for C in the tumour DNA sequence at codon 1429 results in a change from Gln (CAA) to a stop codon (TAA).

analysis of amplicons 15B and 15H in the tumour DNA demonstrated nonsense mutations (figures 3.6b and c). A C to T transition resulting in a change from glutamine to a stop codon was identified in both amplicons; at codon 789 in amplicon 15B and at codon 1429 in amplicon 15H. Thus both mutations would be predicted to lead to a truncated APC protein.

Another colorectal carcinoma, from case COCA32, was found to harbour two separate somatic mutations, illustrated in figures 3.7 and 3.8. Figure 3.7a shows SSCP and heteroduplex analysis of amplicon 15B. The wild-type single strand DNA pattern was seen in the tumour DNA, but a heteroduplex DNA band was also present. Homoduplex DNA was retained on the gel too. Subsequent seqencing analysis revealed the variation to result from a 2 bp deletion at codon 793-794 (figure 3.7b), causing frameshift and the formation of a premature stop codon, 11 nucleotides downstream. SSCP and heteroduplex analysis of amplicon 15H in tumour and normal DNA from COCA32 is shown in figure 3.8. A novel single-stranded DNA conformer as well as heteroduplex DNA bands were observed in the tumour DNA. The heteroduplexes had not migrated very much further than the single-stranded DNA through the gel, as compared with those in amplicon 15B for example. On sequencing a 29 bp deletion was identified in the tumour DNA, affecting codons 1395-1405. This alteration created a new termination codon immediately downstream due to frameshift. Both somatic mutations in tumour COCA32 were thus predicted to result in truncation.

Three somatic variants in total were detected in *APC* amplicon 15B by SSCP and heteroduplex analysis, the third in tumour DNA from case COCA34, as depicted in figure 3.9a. Heteroduplex DNA formation manifest as two separate bands was observed. The variant was subsequently characterized as a deletion of two T residues in the sequence ACTTTTGAC at codon 772-773 and is predicted to be inactivating due to the immediate creation of a stop codon (TGA) with one of the T residues (figure 3.9b).

Figure 3.10 shows SSCP and heteroduplex analysis of *APC* amplicons 15E and 15F in tumour and normal DNA from case COCA39. In the exon 15E amplicon a heteroduplex shift was detected in the tumour DNA in addition to the wild-type single-stranded DNA pattern consisting of four bands. In the adjacent 15F amplicon, again single-stranded DNA variation was not observed in the tumour DNA but two bands of heteroduplex DNA were detected. Sequencing analysis of the exon 15E and 15F amplicons was carried out in tumour and normal DNA from case COCA39.

Figure 3.7..

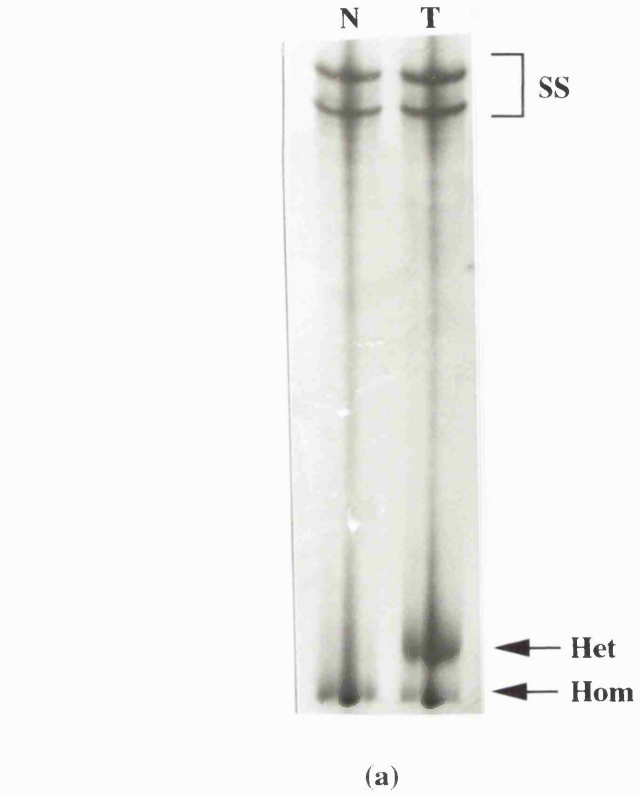


Figure 3.7. Detection of somatic mutation in exon 15B amplicon of *APC* in case COCA32. (**a**) SSCP and heteroduplex analysis. N and T denote DNA samples from normal and tumour tissues respectively. Although no single strand variation is seen, a heteroduplex band is observed in the tumour DNA (SS=single strands; Het=heteroduplex; Hom=homoduplex). (**b**) Sequence analysis (antisense strand). The above variant is the result of a 2 bp deletion (AA) at codon 793-794 which is predicted to lead to a truncated APC protein product.

Figure 3.8.

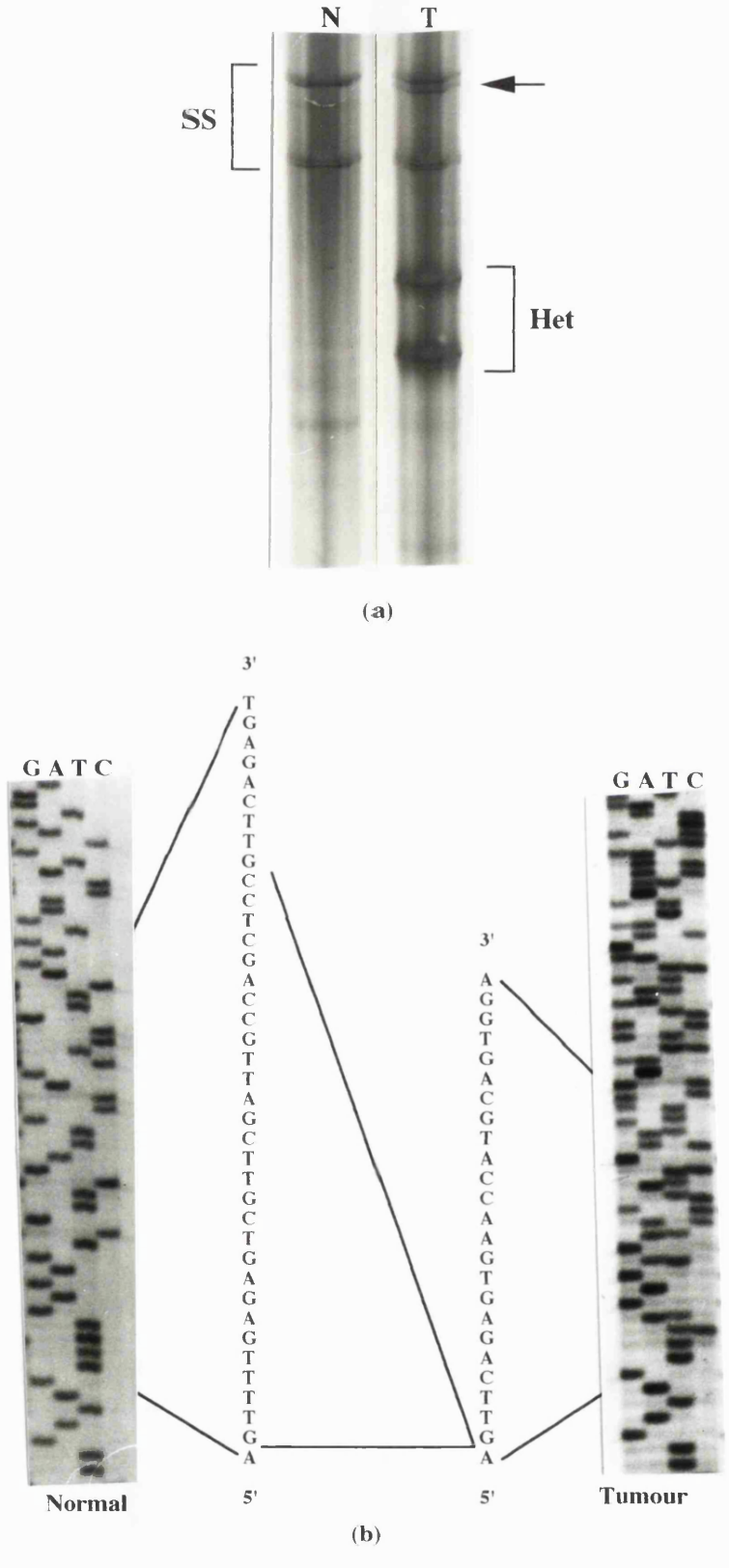
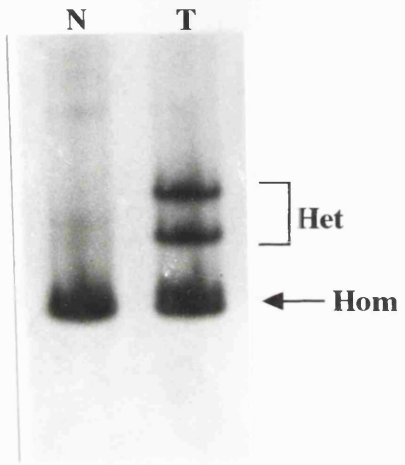
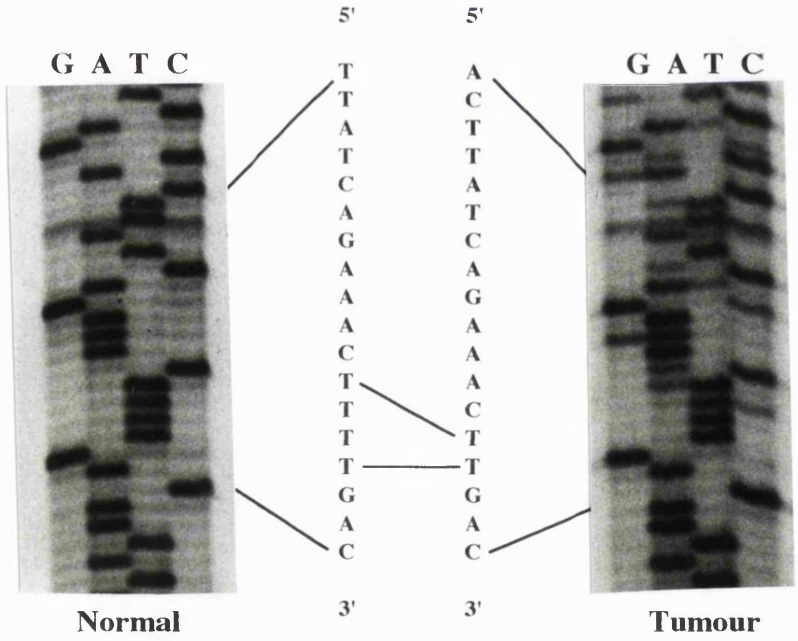


Figure 3.8. Somatic mutation of the *APC* gene identified in the exon 15H amplicon in case COCA32. (**a**) SSCP and heteroduplex analysis. N and T denote DNA samples from normal and corresponding tumour tissues respectively. Both single strand variation (SS) and heteroduplexes (Het) were detected in the tumour. (**b**) Sequence analysis revealed a 29 bp deletion in the tumour DNA involving codons 1395-1405, which creates a stop codon immediately downstream. The sequence of the sense strand is shown.

Figure 3.9.



(a)



(b)

Figure 3.9. Detection of somatic mutation in exon 15B amplicon of *APC* in case COCA34. (**a**) Heteroduplex analysis only of amplicon 15B. T and N denote tumour and corresponding normal DNA samples respectively. No single-stranded DNA mobility shift was detected in the tumour but heteroduplexes (Het) were observed, with a retarded mobility compared with homoduplex DNA (Hom). (**b**) Sequence analysis of exon 15B amplicon (antisense strand). A 2 bp deletion (TT) was identified in the tumour DNA sequence at codon 772-773 which leads to frameshift and a premature stop codon.

Figure 3.10.

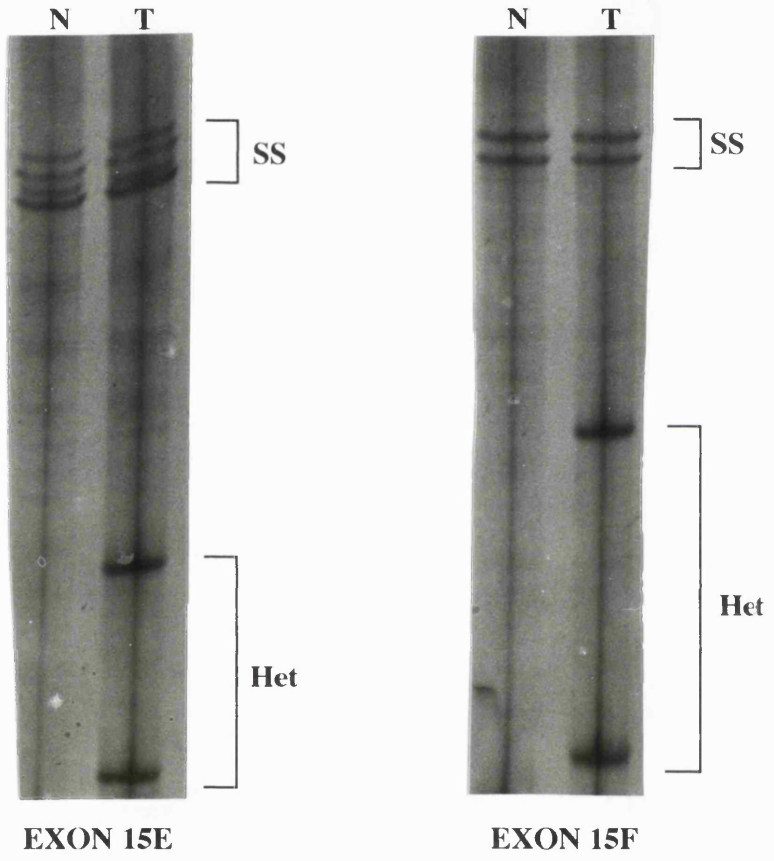


Figure 3.10. SSCP and heteroduplex analysis of amplicons 15E and 15F of *APC* in corresponding tumour and normal DNA from case COCA39. Heteroduplex shifts were detected in the tumour DNA in both amplicons 15E and 15F (Het=heteroduplexes; SS=single strands).

However no sequence alteration was identified in either amplicon in the tumour DNA.

Figures 3.11 to 3.13 illustrate somatic variants detected in amplicon 15G of *APC*. As shown in figure 3.11a, a similar variant single-stranded DNA pattern was detected in tumour DNA from cases COCA26 and COCA28. Sequence analysis (figure 3.11b) demonstrated the novel conformers to be the result of an A to T transversion resulting in a nonsense mutation of arginine (AGA) to a stop codon (TGA) at codon 1331; truncation of the APC protein would be predicted. Likewise, a similar variant single strand band pattern was detected in tumour DNA from cases COCA7 and COCA23 as illustrated in figure 3.12. The electrophoretic variation was characterized in the tumour DNA from both cases as being the result of a single base change from guanine to thymine at the first nucleotide of codon 1322, causing a nonsense mutation from glutamic acid (GAA) to a stop codon (TAA); a change also predicted to be truncating.

Figure 3.13a depicts SSCP and heteroduplex analysis of amplicon 15G in DNA from cases COCA2, COCA40, and COCA42. Tumour DNA from all three cases did not exhibit single-stranded DNA variation but a heteroduplex DNA variant of a similar mobility. Figure 3.13b shows sequence analysis of amplicon 15G in tumour and normal DNA from case COCA2. A 5 bp deletion (AAAGA) was detected at codon 1307-1311. This change caused a frameshift that generated a new stop codon at immediately downstream. An identical sequence alteration was observed in tumour DNA of cases COCA40 and COCA42.

SSCP and heteroduplex analysis identified variants in *APC* amplicon 15H in tumour DNA from three more cases as shown in figures 3.14, 3.15, and 3.16. Firstly, in case COCA9 a single-stranded DNA conformer of differing mobility was detected in the tumour DNA in addition to the wild-type single strand DNA pattern (figure 3.14a). Heteroduplexes were not detected. Subsequent sequence analysis revealed a single base substitution of an A residue for a G residue in the tumour DNA, causing a missense mutation from valine (GTT) to isoleucine (ATT) at codon 1405 (figure 3.14b).

Figure 3.15 shows heteroduplex analysis of the exon 15H amplicon in tumour and normal DNA from case COCA24. A heteroduplex variant migrating at a slightly slower rate than the homoduplex DNA molecule was detected in the tumour DNA. The variant was characterized as a 2 bp deletion, of either AG or GA in the sequence AAAAGAGAGAGAGT at codon 1462-1465, that causes a frameshift creating an

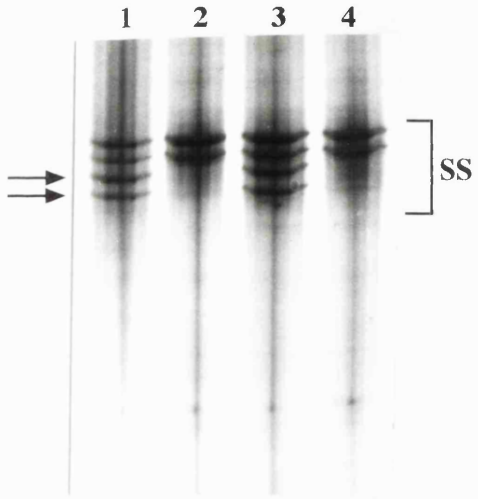
early stop codon immediately downstream. In tumour DNA from case COCA38, again a heteroduplex shift but no single strand mobility shift was observed (figure 3.16a). Sequencing of amplicon 15H in the tumour DNA revealed a 4 bp insertion at codon 1395 (figure 3.16b). This sequence change also results in frameshift and the formation of a termination codon at immediately downstream.

Finally, as shown in figure 3.17, a somatic variant was detected in case COCA8 in amplicon 15I of *APC*. Single-stranded DNA variants were not seen in the tumour DNA. Double-stranded homoduplex DNA was retained on the gel however, and a homoduplex variant of greater mobility than the wild-type homoduplex DNA molecule was detected. Sequencing demonstrated this variant to be the result of a somatic deletion of an A residue in the sequence GAAAGT at codon 1494-1495. Once again this mutation led to frameshift and created a new stop codon 33 nucleotides downstream that is predicted to cause truncation of the *APC* gene product.

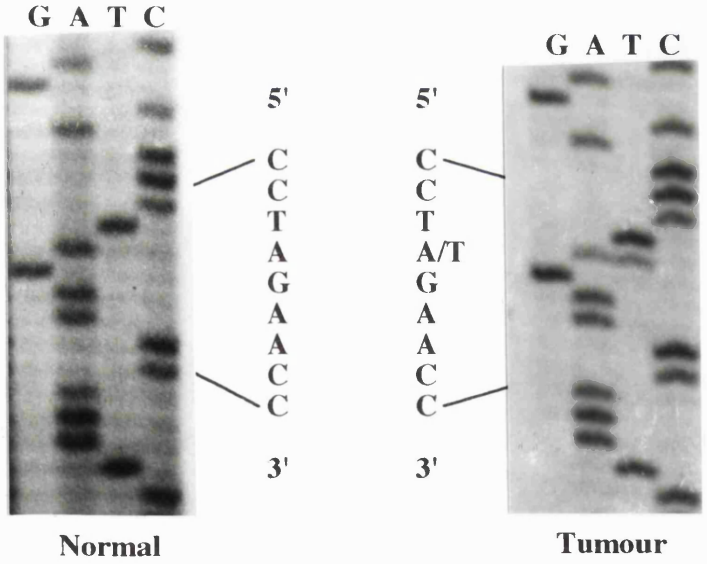
As described in table 3.2, common polymorphisms were detected in amplicons 15I and 15J, these will be more fully discussed in section 3.1.2. A total of 19 somatic *APC* mutations were identified in 43 colorectal carcinomas as described in table 3.1. Nine of them (47%) were point mutations; seven of these were nonsense mutations resulting in truncation of the gene product whereas only one was a missense mutation. The remaining point mutation occurred at a splice site and could lead to frameshift (case COCA35). Five of the nine point mutations were transitions and four were transversions. Four of the transitions were G:C to A:T changes, of which two occurred at CpG dinucleotide pairs and two occurred at CpA dinucleotide pairs. One transition was an A:T to G:C change. Of the four transversions, two were G:C to T:A changes and two were A:T to T:A changes. Eight of the nineteen somatic mutations were deletions of 1 to 29 bp and two were insertions of 4 bp. All of the deletions and insertions produced a frameshift creating new termination codons at downstream. Thus the great majority of somatic mutations observed (18 of 19, 95%) were predicted to lead to truncation of the APC product.

Three of the mutations characterized were found in more than one case: the 5 bp deletion at codon 1307-1311 was observed in three cases, the nonsense mutation at codon 1322 (Glu to stop) in two cases, and the nonsense mutation at codon 1331 (Arg to stop) in two cases. The majority of somatic mutations were located in exon 15 of *APC*. Seventy-four percent (14 of 19) fell into the region of this exon, between codons 1286 and 1513, designated as 'mutation cluster region' (Miyoshi *et al.*, 1992b).

Figure 3.11.



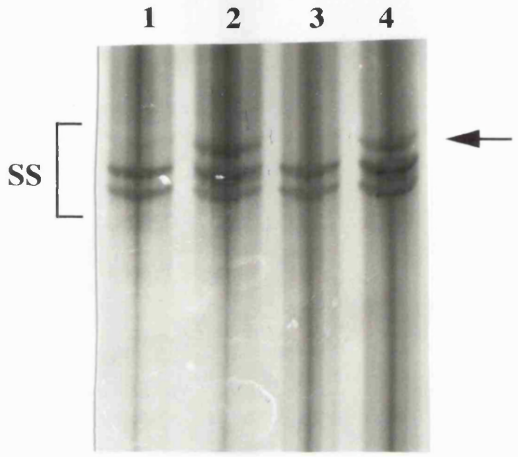
(a)



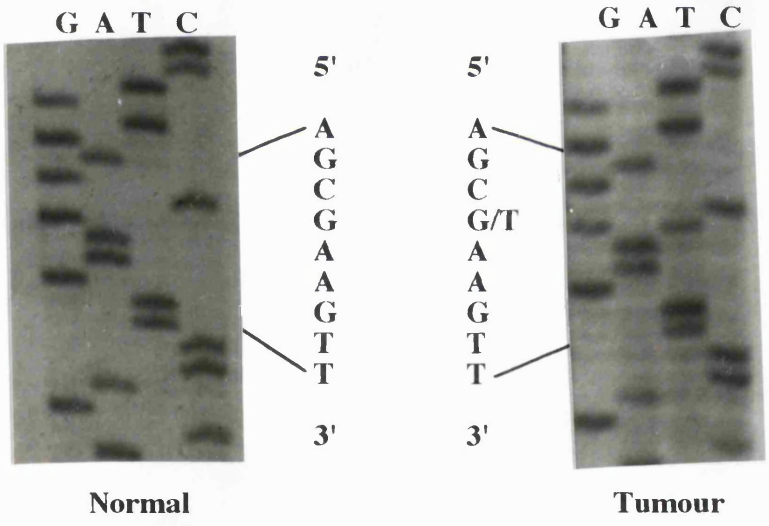
(b)

Figure 3.11. Detection of somatic mutation in exon 15G amplicon of *APC* in cases COCA26 and COCA28. (a) SSCP analysis. Lanes 1 and 2 represent SSCP of corresponding tumour and normal DNA respectively from case COCA26, lanes 3 and 4 represent tumour and normal DNA from case COCA28. In addition to the wild-type single strand (SS) band pattern, tumour DNA from both cases demonstrate a similar variant single-stranded DNA pattern, as indicated by the arrows. **(b)** Sequencing of 15G amplicon (antisense strand) in tumour and normal DNA from case COCA26. A single base substitution of T for A causes a change from Arg (AGA) to stop (TGA) at codon 1331. An identical sequence change was identified in tumour DNA from COCA28.

Figure 3.12.



(a)



(b)

Figure 3.12. Detection of somatic mutation in exon 15G amplicon of *APC* in cases COCA7 and COCA23. (**a**) SSCP analysis. Lanes 1 and 2 represent SSCP of corresponding normal and tumour DNA respectively from case COCA7, lanes 3 and 4 represent normal and tumour DNA respectively from case COCA23. Novel single-stranded (SS) DNA conformers of a similar mobility were observed in the tumour DNA from both cases (see arrow). (**b**) Sequence analysis of exon 15G amplicon in tumour and normal DNA from case COCA23. A G to T transversion was identified in the tumour resulting in a change from glutamic acid (GAA) to a stop codon (TAA) at codon 1322. The sequence of the antisense strand is shown.

Figure 3.13.

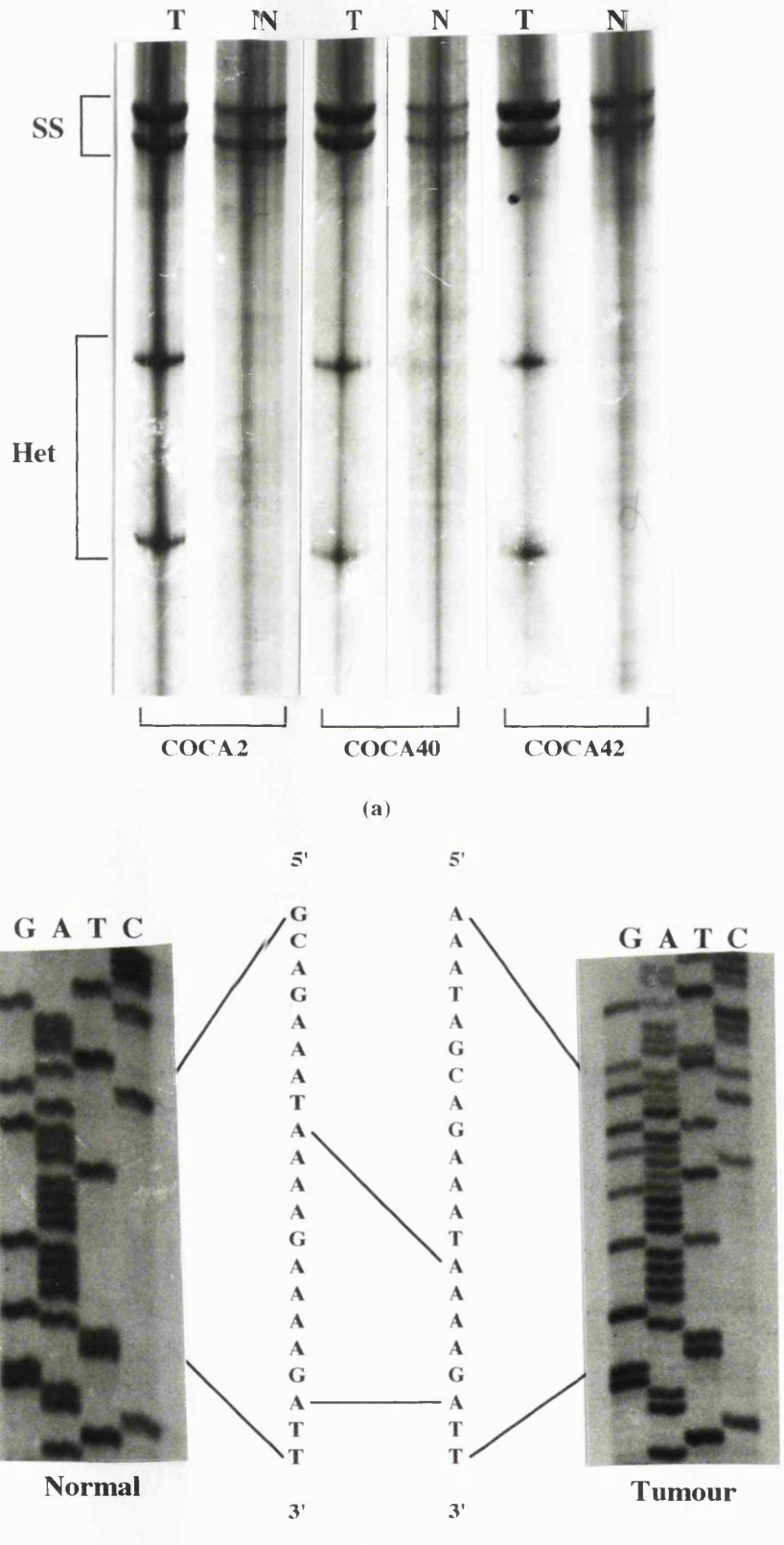
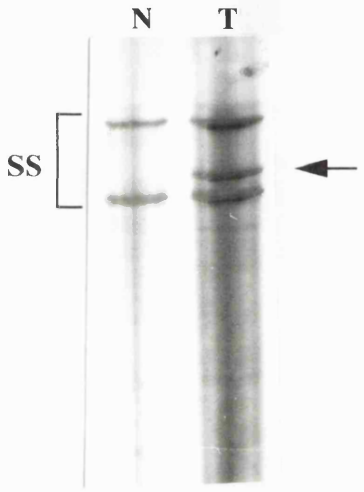
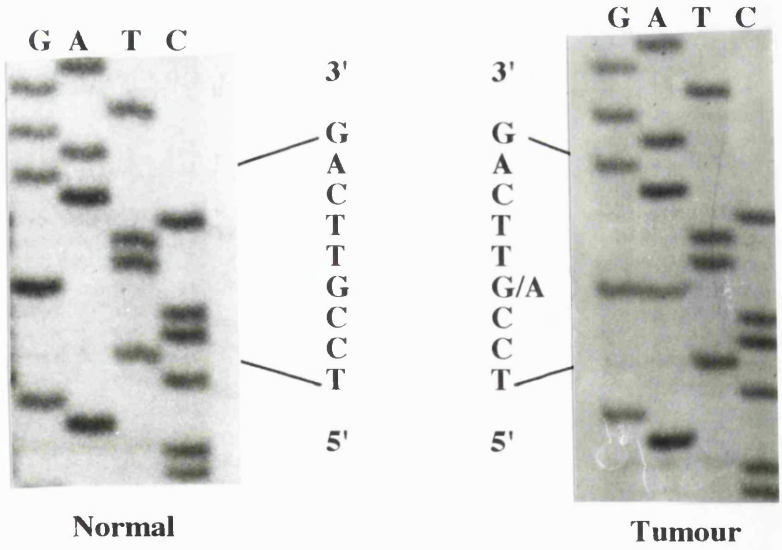


Figure 3.13. Somatic mutation observed in exon 15G amplicon of *APC* in cases COCA2, COCA40, and COCA42. **(a)** SSCP and heteroduplex analysis. T and N denote DNA samples from tumour and normal tissues respectively. A similar heteroduplex variant is seen in tumour DNA from all three cases although no single-stranded variants are observed (Het=heteroduplex DNA; SS=single-stranded DNA). **(b)** Sequence analysis of 15G amplicon in tumour and normal DNA from COCA2 (antisense strand). A 5 bp deletion was identified in the tumour DNA, in the tandemly repeated sequence AAAAGAAAAGA at codon 1307-1311, resulting in a frameshift and the creation of a premature stop codon immediately downstream of the mutation.

Figure 3.14.



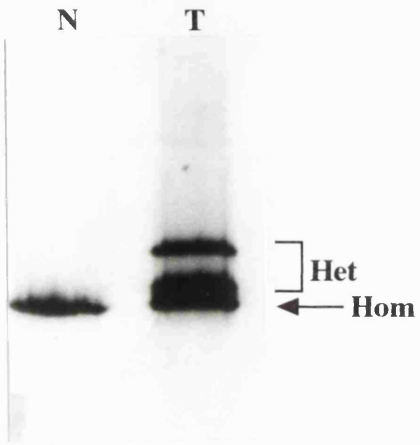
(a)



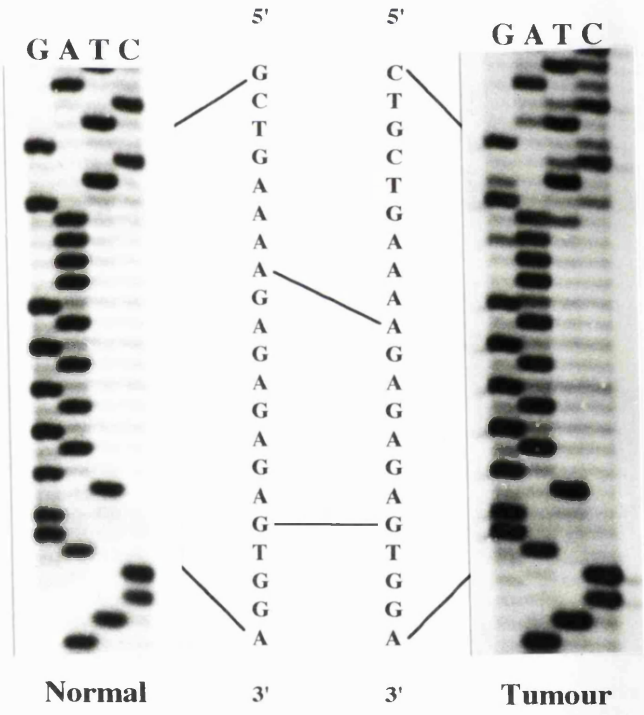
(b)

Figure 3.14. Detection of somatic mutation in exon 15H amplicon of *APC* in case COCA9. (**a**) SSCP analysis. A variant single-stranded DNA conformer was detected in the tumour DNA (T) as compared with corresponding normal DNA (N) as indicated by the arrow (SS=single-stranded DNA). (**b**) Sequence analysis of amplicon 15H in corresponding tumour and normal DNA (sense strand). A single base substitution (G to A) was detected in tumour DNA from COCA9, which results in Val (GTT) to Ile (ATT) at codon 1405.

Figure 3.15.



(a)



(b)

Figure 3.15. Detection of somatic mutation in exon 15H amplicon of *APC* in case COCA24. (**a**) A heteroduplex shift was detected in tumour DNA (T) but not in normal DNA (N) from case COCA24 (Het=heteroduplex DNA; Hom=honoduplex DNA). (**b**) Sequencing of exon 15H amplicon in tumour and normal DNA from COCA24 (antisense strand). The tumour shows a 2 bp deletion of either AG or GA in GAAAAGAGAGAGAGT at codon 1462-1465 of *APC* resulting in a frameshift followed by a stop codon immediately downstream.

Figure 3.16.

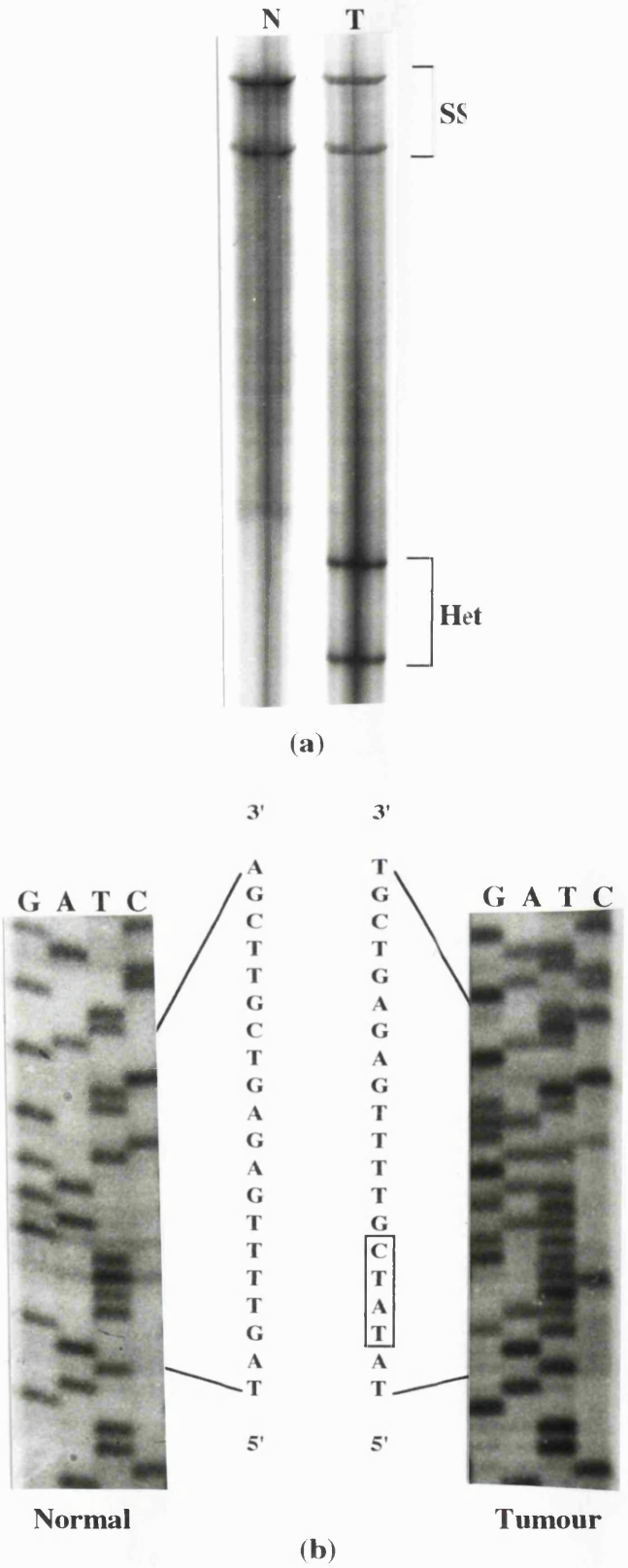


Figure 3.16. Somatic mutation of the *APC* gene observed in case COCA38 in the exon 15H amplicon. (**a**) SSCP and heteroduplex analysis. N and T denote corresponding normal and tumour DNA samples. Although no single strand (SS) variants are observed in the tumour DNA, two heteroduplex bands (Het) are seen. (**b**) Sequencing of amplicon 15H in tumour and normal DNA from case COCA38 (sense strand). The above variant is the result of a 4 bp insertion (TATC) at codon 1395 which creates a new stop codon immediately downstream due to frameshift.

Figure 3.17.

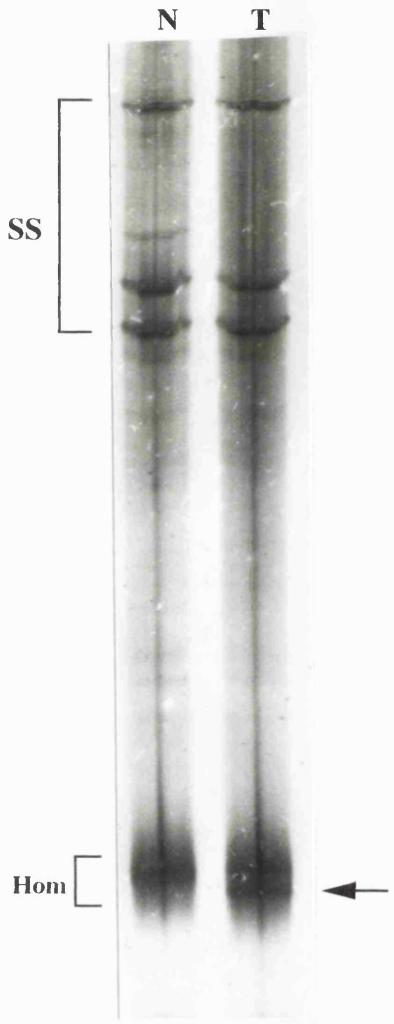


Figure 3.17. Detection of somatic homoduplex variant in exon 15I amplicon of *APC* in case COCA8. N and T denote DNA samples from corresponding normal and tumour tissues respectively. No variation was detected in the single strand DNA pattern in the tumour but an additional homoduplex band was observed as indicated by the arrow (SS=single-stranded DNA; Hom=homoduplex DNA). This variant was subsequently characterized as a 1 bp deletion (A) at codon 1494-1495 of *APC*.

In three carcinomas, two independent somatic alterations of the *APC* gene were identified (COCA19, COCA32, and COCA33), all were inactivating. However it could not be determined whether the changes occurred on one or on both alleles, as in each case the mutations were too distant to be analysed in one PCR product.

3.1.2. Loss of heterozygosity at the *APC* gene locus

During the course of screening the 5' half of exon 15 of the *APC* gene for mutations in our laboratory, two common polymorphisms were detected by SSCP analysis and characterized by sequence analysis. The first is in the exon 15I amplicon and is due to a single base substitution of G for A at the third nucleotide of codon 1493. This sequence change does not result in an amino acid change. The second polymorphism, in amplicon 15J, is due to a substitution of G for A at the third nucleotide of codon 1678 and again does not result in amino acid substitution. Both polymorphisms have been previously described (Nagase *et al.*, 1992; Kraus and Ballhausen, 1992). Figures 3.18a and 3.19a illustrate detection of the two polymorphisms by SSCP analysis. The two alleles at each polymorphic locus could be clearly distinguished as two well separated bands, thus both polymorphisms were useful for the analysis of loss of heterozygosity (LOH) in tumours.

In our laboratory the polymorphism detected in amplicon 15I (codon 1493) of *APC* was characterized in 155 unrelated people: 38 patients with familial adenomatous polyposis, 46 sporadic colorectal cancer patients including the 43 patients being investigated in the present study, and 71 people unaffected with either disorder (Gayther *et al.*, 1995). An allele frequency of 0.58 was calculated for the allele with G at the third nucleotide of codon 1493 and an allele frequency of 0.42 was calculated for the allele with A at this position. In figure 3.18a the bands denoted 1 and 2 respectively represent the alleles with G and A at the third nucleotide of codon 1493.

Furthermore, both the exon 15I (codon 1493) and exon 15J (codon 1678) polymorphisms were analysed in DNA from 87 unrelated individuals, inclusive of the 43 patients being investigated in the present study. The two polymorphisms were found to be in complete linkage disequilibrium. Additionally, DNA from 50 unrelated people that had been analysed for these two polymorphisms, were also typed by PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) analysis for the previously described *Msp* I polymorphism that occurs in *APC* exon 15 region N (codon 1960) (Cottrell *et al.*, 1992). With the exception of

Figure 3.18.

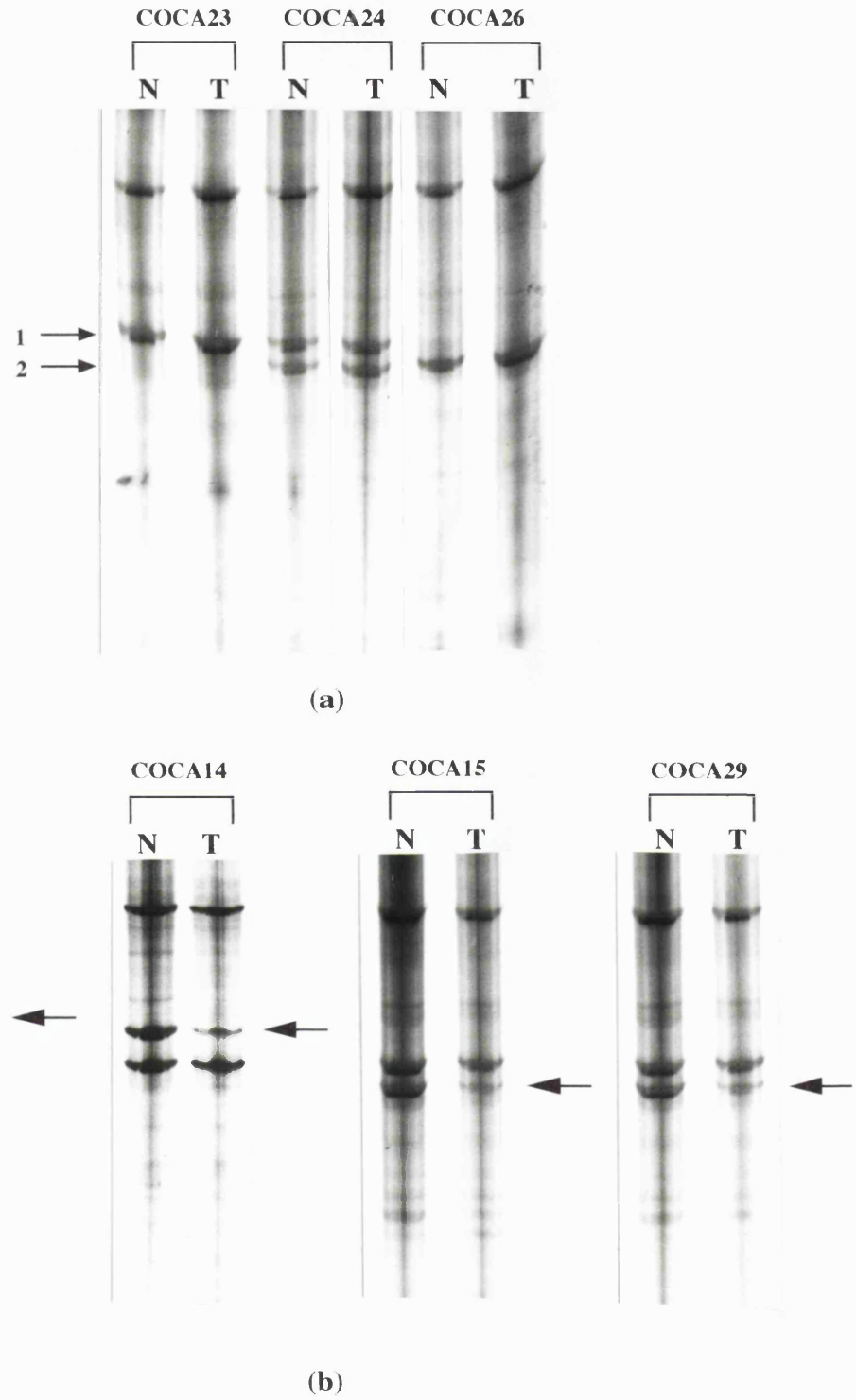
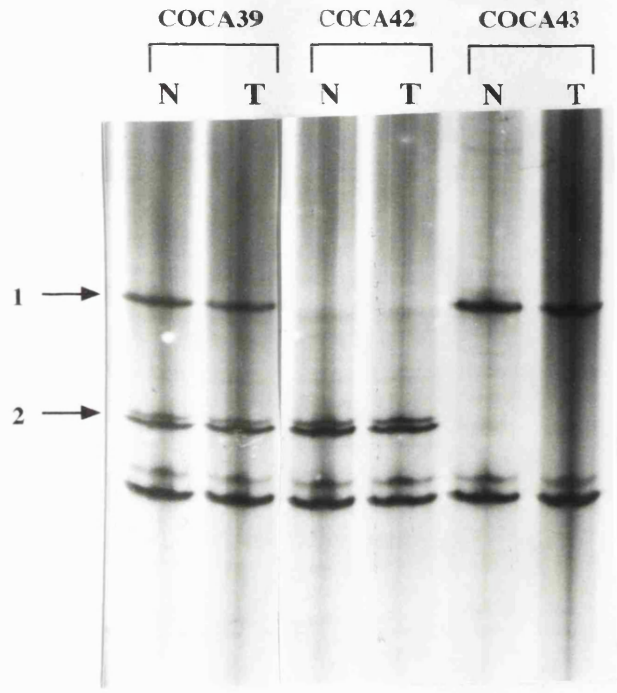
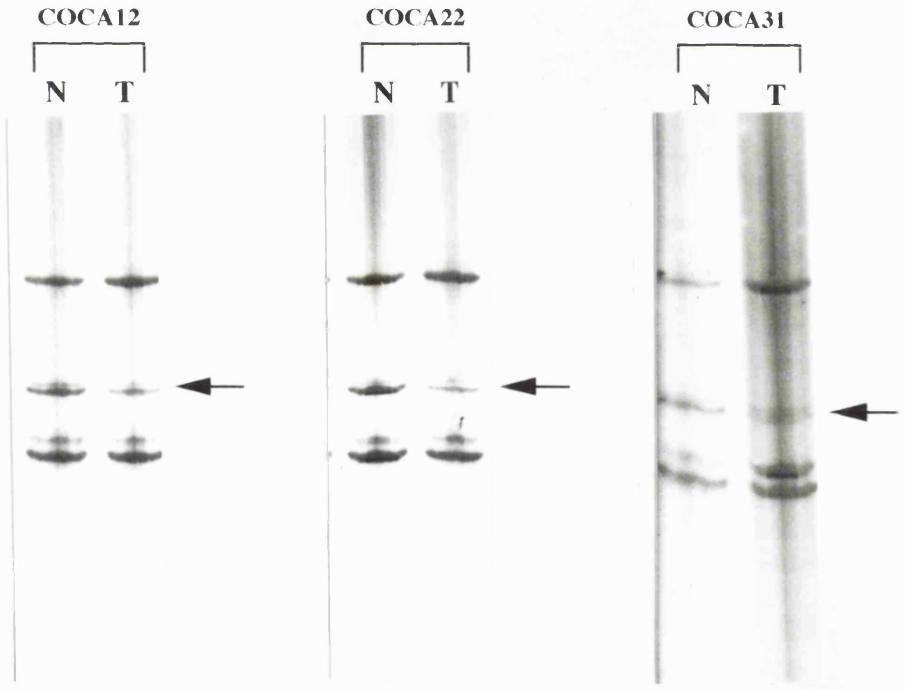


Figure 3.18. Detection of loss of heterozygosity in colorectal carcinomas at the codon 1493 polymorphic locus in the exon 15I amplicon of *APC* . (**a**) Lanes: N, normal DNA; T, tumour DNA. The two alleles of the polymorphism are distinguished by SSCP analysis as two bands of distinct mobilities, denoted 1 and 2. Cases COCA23 and COCA26 are constitutionally homozygous for alternative alleles and case COCA24 is constitutionally heterozygous but does not demonstrate loss of heterozygosity (LOH) in the tumour. (**b**) Cases COCA6, COCA14, COCA15, and COCA29 are constitutionally heterozygous and therefore informative. A reduction in intensity of a band representing one allele in tumour DNA relative to corresponding normal DNA indicates LOH (see arrows).

Figure 3.19.



(a)



(b)

Figure 3.19. Detection of loss of heterozygosity in colorectal carcinomas at the codon 1678 polymorphic locus in amplicon 15J of *APC*. (**a**) Lanes: N, normal DNA; T, tumour DNA. The two alleles of the codon 1678 polymorphism are represented by single-stranded DNA bands of distinct mobilities, denoted 1 and 2. Case COCA39 is constitutionally heterozygous but negative for LOH in the tumour, and cases COCA42 and COCA43 are constitutionally homozygous for alternative alleles. (**b**) Cases COCA12, COCA22, and COCA31 are constitutionaly heterozygous. In each case, LOH is observed as a reduction in intensity of one band in tumour DNA relative to corresponding normal DNA, as highlighted by the arrows.

two colorectal cancer patients, linkage disequilibrium was complete in all samples (Gayther *et al.*, 1995).

In the present study, for the detection of LOH in tumours, five intragenic polymorphic loci of the *APC* gene were analysed in tumour and corresponding normal DNA from the 43 colorectal carcinoma cases. These included the polymorphisms detected in the exon 15I, exon 15J, and exon 15N amplicons as described above. Two further polymorphisms, an *Rsa* I RFLP located within exon 11 of *APC* (Kraus and Ballhausen, 1992) and an *Ssp* I RFLP located in the 3' untranslated region (3' UTR) of *APC* (Heighway *et al.*, 1991) were also studied. Furthermore, the *D5S346* microsatellite repeat marker was analysed for replication error and loss of heterozygosity in the colorectal carcinomas (see section 3.1.3). The LOH results will be described in the present section as *D5S346* lies only 30-70kb away from the *APC* gene (Spirio *et al.*, 1991). The results of LOH analysis at the intragenic polymorphic loci and at *D5S346* are summarized in table 3.3.

Analysis of normal tissue DNA revealed that 29 of the 43 colorectal carcinoma cases were constitutionally heterozygous for at least one intragenic polymorphism and therefore considered to be informative for LOH analysis. LOH was observed in 7 of 29 (24.1%) tumours. Detection of LOH at the exon 15I (codon 1493) and exon 15J (codon 1678) polymorphic loci is shown in figures 3.18b and 3.19b. LOH was assessed visually and defined as a change in allele:allele ratio in tumour relative to corresponding normal DNA. A reduction in intensity of a band representing one allele was seen in the tumours rather than complete loss and was scored as LOH. The residual signal can be interpreted as either reflecting the presence of contaminating normal stromal cells in the tumour specimen, or the presence of more than one neoplastic clone, or amplification of a mutant allele without loss of the wild-type allele, or preferential duplication of one chromosome.

Thirty-one of the forty-three colorectal carcinoma cases were constitutionally heterozygous and therefore informative at the *D5S346* locus (table 3.3). One tumour (from case COCA9) exhibited RER (replication error) at *D5S346* and was thus considered to be uninformative for LOH analysis at this locus. LOH was observed in 8 of 31 (25.8%) informative tumours. Analysis of the *D5S346* marker is shown in figure 3.20. All microsatellite repeat markers were analysed by SSCP analysis (see section 3.1.3). A case that is constitutionally heterozygous at *D5S346* (case COCA4) and a case that is constitutionally homozygous at this locus (case COCA19) is shown in figure 3.20. Each allele is represented by two bands. Extra bands, known as 'stutter bands' are seen in addition to the main bands and are a problem common to all

Table 3.3. Results of LOH analysis at intragenic polymorphic loci of the *APC* gene and at the *D5S346* microsatellite repeat locus in forty-three colorectal carcinoma cases.

Case	*APC* exon 11	*APC* exon 15I	*APC* exon 15J	*APC* exon 15N	*APC* 3' UTR	*D5S346*
COCA1	hom	hom	hom	hom	hom	**LOH**
COCA2	HET	HET	HET	HET	HET	HET
COCA3	HET	HET	HET	HET	HET	HET
COCA4	hom	HET	HET	HET	hom	HET
COCA5	hom	hom	hom	hom	hom	HET
COCA6	hom	**LOH**	**LOH**	**LOH**	**LOH**	**LOH**
COCA7	hom	hom	hom	hom	hom	**LOH**
COCA8	HET	HET	HET	HET	hom	HET
COCA9	HET	HET	HET	HET	HET	**RER**
COCA10	hom	hom	hom	hom	HET	HET
COCA11	hom	hom	hom	hom	hom	HET
COCA12	**LOH**	**LOH**	**LOH**	**LOH**	**LOH**	**LOH**
COCA13	hom	HET	HET	hom	hom	hom
COCA14	**LOH**	**LOH**	**LOH**	**LOH**	**LOH**	**LOH**
COCA15	**LOH**	**LOH**	**LOH**	**LOH**	**LOH**	**LOH**
COCA16	hom	hom	hom	hom	HET	HET
COCA17	hom	hom	hom	HET	HET	hom
COCA18	HET	HET	HET	HET	HET	HET
COCA19	hom	hom	hom	hom	hom	hom
COCA20	hom	hom	hom	hom	hom	HET
COCA21	HET	HET	HET	HET	hom	hom
COCA22	**LOH**	**LOH**	**LOH**	**LOH**	**LOH**	hom
COCA23	hom	hom	hom	hom	hom	HET
COCA24	HET	HET	HET	HET	hom	HET
COCA25	HET	hom	hom	hom	HET	HET
COCA26	hom	hom	hom	hom	hom	hom
COCA27	hom	hom	hom	hom	hom	HET
COCA28	hom	hom	hom	hom	hom	hom
COCA29	**LOH**	**LOH**	**LOH**	**LOH**	**LOH**	**LOH**
COCA30	HET	HET	HET	HET	HET	HET
COCA31	**LOH**	**LOH**	**LOH**	**LOH**	**LOH**	**LOH**
COCA32	HET	HET	HET	HET	HET	HET
COCA33	HET	HET	HET	HET	HET	HET
COCA34	HET	HET	HET	HET	HET	HET
COCA35	HET	hom	hom	hom	HET	HET
COCA36	hom	hom	hom	hom	hom	hom
COCA37	HET	HET	HET	HET	HET	HET
COCA38	hom	hom	hom	hom	hom	hom
COCA39	HET	HET	HET	HET	HET	HET
COCA40	HET	HET	HET	HET	HET	hom
COCA41	HET	HET	HET	HET	HET	HET
COCA42	hom	hom	hom	hom	hom	hom
COCA43	hom	hom	hom	hom	hom	HET

HET, constitutional heterozygosity retained in tumour; **LOH**, loss of heterozygosity observed in tumour; hom, constitutionally homozygous therefore uninformative; **RER**, replication error observed in tumour.

Figure 3.20.

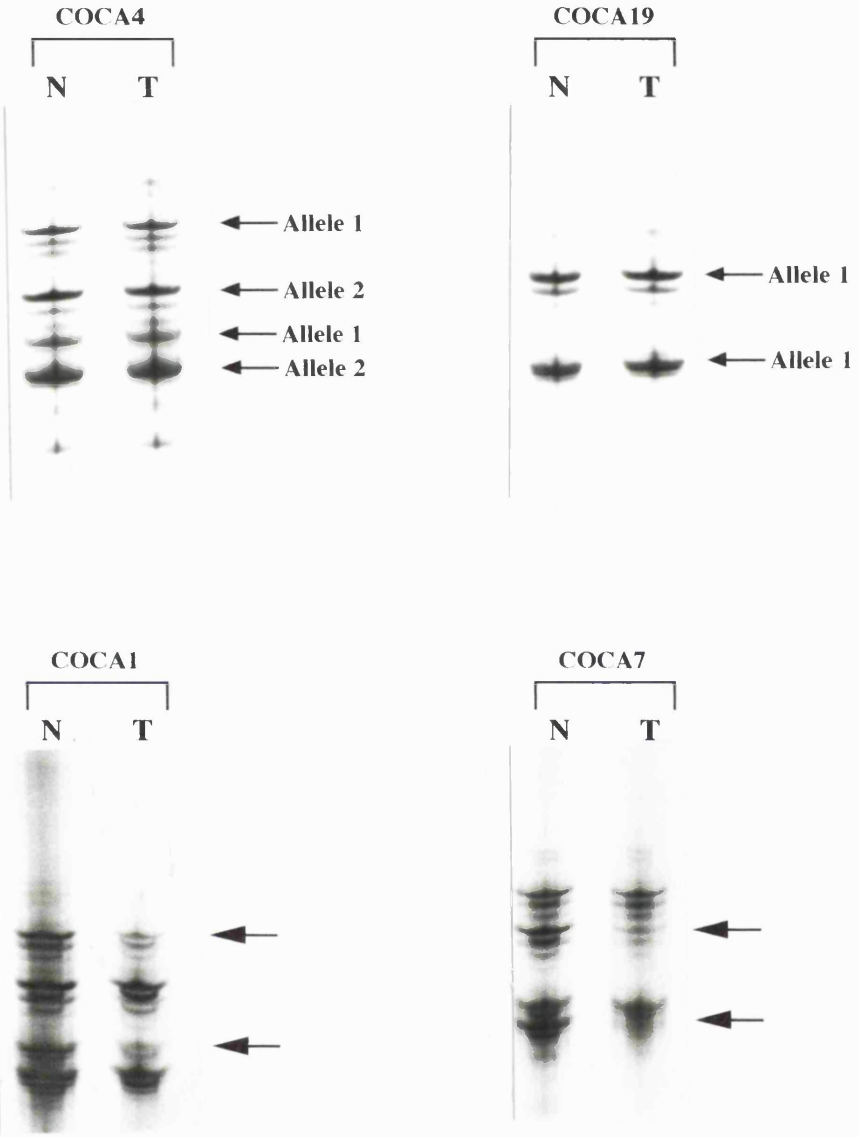


Figure 3.20. Detection of loss of heterozygosity in colorectal carcinomas at *D5S346*. N, normal DNA; T, tumour DNA. The *D5S346* marker was analysed by the SSCP method. Analysis of normal DNA from case COCA4 shows that this case is constitutionally heterozygous at *D5S346*, each allele being represented by two bands. No LOH is observed in the tumour DNA. Case COCA19 is constitutionally homozygous. Cases COCA1 and COCA7 are constitutionally heterozygous, each allele being represented by two bands in the normal tissue DNA. In both these cases, a reduction in intensity of bands representing one allele is observed in tumour DNA relative to normal DNA indicating LOH, as highlighted by the arrows.

detection methods for microsatellite repeat markers. Stutter bands are thought to be PCR artefacts. The major mechanism postulated for this is 'slipped strand mispairing', the multiple repeats permitting slippage of the copied strand on the template, producing fragments which differ in size by units of 2 nucleotides. Other postulated mechanisms include the addition of nucleotides by the polymerase to the 3' end of the product and the failure of the polymerase to read through the repeats (Koreth *et al.*, 1996). Detection of LOH at *D5S346* in two tumours (from cases COCA1 and COCA7) is also shown in figure 3.20.

In total 37 of the 43 colorectal carcinoma cases were informative (heterozygous) at an intragenic polymorphic locus or at *D5S346* (table 3.3). Nine of 37 (24.3%) tumours exhibited LOH. Six tumours demonstrated LOH at *D5S346* and at intragenic polymorphic loci. One tumour (from case COCA22) exhibited LOH at intragenic polymorphic loci but was uninfomative at *D5S346*, while two tumours (COCA1 and COCA7) showed LOH at *D5S346* but were uninformative for the intragenic polymorphisms (table 3.3). It is probable that cases COCA1 and COCA7 have lost an allele of the *APC* gene in their tumour tissues as *D5S346* is located close to *APC* on chromosome 5q. In addition, a somatic mutation of glutamic acid to a stop codon at codon 1322 was identified in case COCA7 (see table 3.1). Thus the tumour from this case probably has complete loss of *APC*, with one allele mutated by point mutation and the other by loss.

In total 4 carcinomas were found to contain two independent somatic alterations of the *APC* gene. In 3 carcinomas the alterations consisted of nonsense and/or frameshift mutations, and in 1 carcinoma they were a nonsense mutation and allele loss. In 20 carcinomas, one somatic alteration of the *APC* gene was identified: a point mutation or insertion or deletion in 12 tumours and allele loss in 8 tumours (tables 3.1 and 3.3).

3.1.3. Alterations at microsatellite repeat loci

Twelve microsatellite repeat loci located on ten different chromosomes were analysed for replication error (RER) and loss of heterozygosity (LOH) in the colorectal carcinoma cases. The loci analysed were *D2S119*, *D2S391*, *D2S123* (chromosome 2p), *D4S175* (chromosome 4q), *D5S346* (chromosome 5q), *D9S156* (chromosome 9p), *D14S50* (chromosome 14q), *D16S266* (chromosome 16q), *p53CA* (chromosome 17p), *D18S61* (chromosome 18q), *D19S49* (chromosome 19q), and *D22S351* (chromosome 22q). All repeat sequences were of the dinucleotide $(CA)_n$ type. Further details on each marker are given in appendix 3.1.6.

Conventionally, microsatellite repeat markers are analysed by denaturing gel electrophoresis of radiolabelled PCR products followed by autoradiography. Fluorescent detection of microsatellite PCR products following denaturing electrophoresis is now widely used. Detection of microsatellite PCR products by ethidium bromide or silver staining after nondenaturing acrylamide gel electrophoresis has also been described (Todd *et al.*, 1991). In this study, microsatellites were detected by SSCP analysis. PCR products were denatured, electrophoresed in nondenaturing polyacrylamide gels (PhastGels) and detected by silver staining using the PhastSystem as described in section 2.2.4. This approach was chosen because when the microsatellite PCR products were run undenatured on the PhastGels, it was not possible to distinguish the alleles at each microsatellite locus whereas this was possible when the PCR products were previously denatured.

Figure 3.21 illustrates analysis of undenatured and denatured PCR products from four different tumour and normal pairs at four different microsatellite repeat loci. When the undenatured microsatellite PCR products are run on the PhastGels, two bands would be expected to be observed for a heterozygote and 1 band would be expected to be observed for a homozygote. However this was not found to be the case for any of the markers. Figure 3.21 shows that at *D14S50* many bands were observed in both the tumour and normal undenatured PCR products. SSCP analysis of denatured PCR product from the same tumour and normal pair showed that the individual was constitutionally heterozygous at *D14S50*. Each allele was represented by two bands in the normal DNA, which were probably the positive and negative strands of DNA, although this cannot be confirmed. In the tumour DNA from this individual, a reduction in intensity of the bands denoted allele 1 indicated LOH. The LOH was not detected in the undenatured tumour PCR product.

At *D2S123*, again multiple bands were seen in both the tumour and normal undenatured PCR products. SSCP analysis of denatured PCR product from this same tumour and normal pair demonstrated that the individual was heterozygous at *D2S123*, each allele being represented by two bands. At the *p53CA* and *D22S351* loci, again the alleles could not be interpreted in the tumour and normal undenatured PCR products. For both the *p53CA* and *D22S351* markers, SSCP analysis of the denatured PCR products showed that individuals were constitutionally heterozygous, each allele being represented by two bands in the normal DNAs. LOH was detected at both loci in the tumour DNA.

It should be noted that of the 8 colorectal tumours in which LOH was observed at the *D5S346* locus, 6 tumours had also exhibited LOH at intragenic

Figure 3.21.

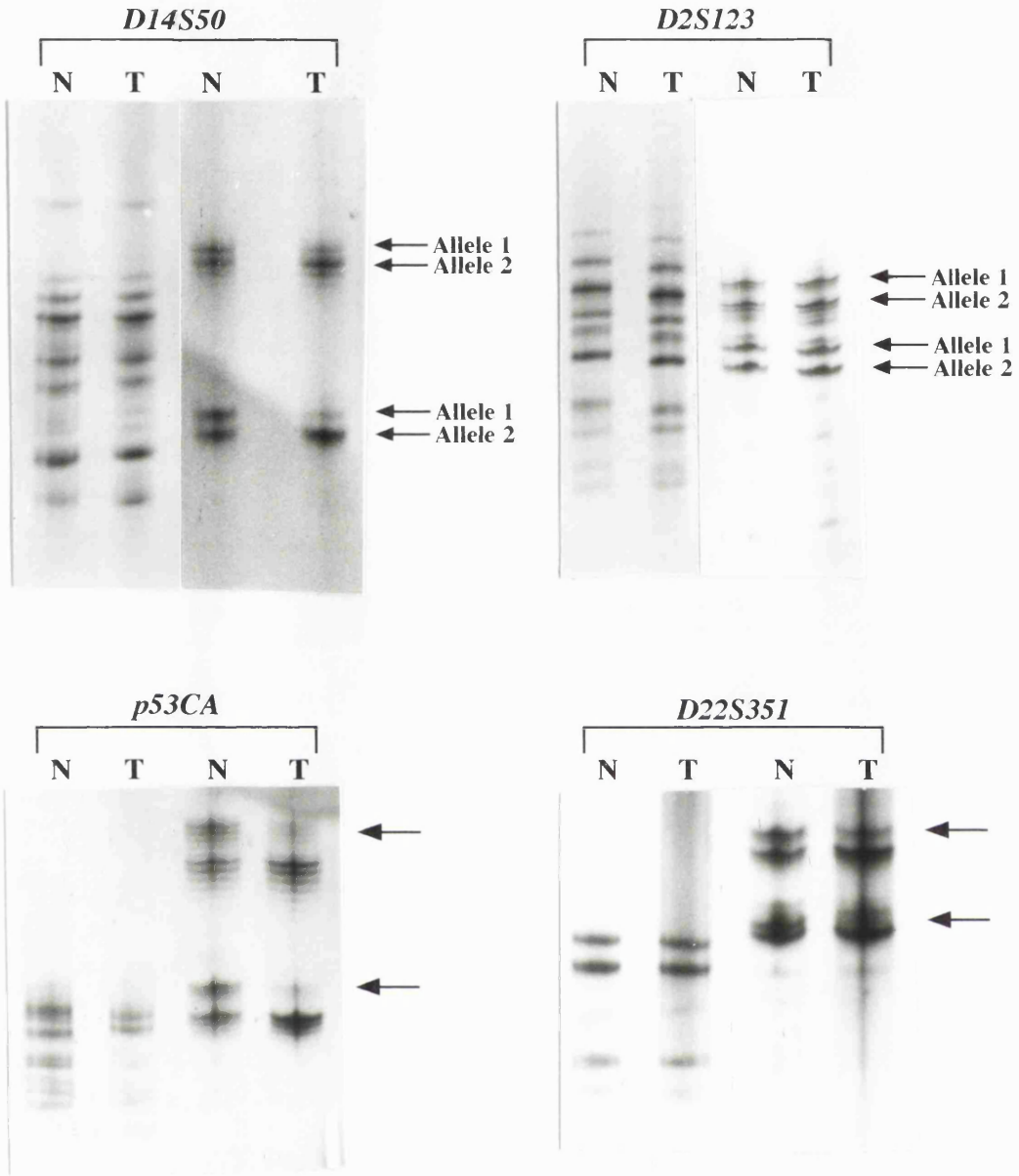


Figure 3.21. Double-stranded DNA analysis and SSCP analysis of microsatellite repeat markers. T=tumour DNA; N=corresponding normal DNA. At each locus , the first two lanes are undenatured PCR products, the last two are denatured PCR products from the same tumour and normal pair. At the *D14S50* locus, multiple bands are seen in both the tumour and normal undenatured PCR products. However SSCP analysis of tumour and normal denatured PCR products from the same case shows that the individual is constitutionally heterozygous at *D14S50*. Each allele is represented by two bands as highlighted by the arrows. A reduction in intensity of bands representing one allele ('allele 1') is observed in the tumour from this case, indicating LOH. At the *D2S123* locus, again multiple bands are seen in the tumour and normal undenatured PCR products. SSCP analysis of dentaured PCR products from the same tumour and normal pair shows that the individual is heterozygous at *D2S123*. At the *p53CA* locus the alleles cannot be distinguished in the undenatured PCR products. SSCP analysis of the denatured PCR products shows that the individual is constitutionally heterozygous at *p53CA*, each allele is represented by two bands in the normal DNA. Loss of bands representing one allele is observed in the tumour DNA from this case as highlighted by the arrows. Similarly at *D22S351*, SSCP analysis of denatured normal and tumour PCR products shows that the individual is constitutionally heterozygous, and a reduction in intensity of bands representing one allele is observed in the tumour DNA, suggesting LOH (highlighted by arrows). This LOH is not observed in the undenatured tumour PCR product.

polymorphic loci of the *APC* gene (table 3.3). The other 2 tumours were not informative at the intragenic polymorphic loci. Thus the interpretation of the results for the *D5S346* marker was verified by the results for the intragenic polymorphisms. As will be discussed later in section 3.2, LOH was observed at microsatellite repeat loci closely linked to the *hMSH2* gene on chromosome 2 and at the *p53CA* locus in gastric tumours. Again the results were verified in some tumours by the observation of LOH at polymorphisms within the *hMSH2* and *p53* genes.

In order to directly compare the detection method used for microsatellite repeat sequences in this study with another technique, the *D22S351* marker was also analysed under fully denaturing conditions by capillary electrophoresis of fluorescent PCR products in the colorectal carcinoma and matching normal DNAs, as described in section 2.2.11.

3.1.3 (a) Replication error

Table 2.2 described clinicopathological and family history data on the colorectal carcinoma cases thus it is also included in this section for reference (see next page).

As described in section 1.4.4 replication error (RER) at microsatellite repeat loci is observed in tumours from patients with hereditary nonpolyposis colorectal cancer (HNPCC) and in sporadic tumours as additional new alleles in tumour DNA compared to constitutional DNA. These insertions and deletions at repeat sequences are thought to be the result of a defect in the DNA mismatch repair system. RER was investigated in 43 colorectal carcinoma and normal DNA pairs - at all twelve microsatellite repeat loci in 41 cases but at six loci in 2 cases (COCA17 and COCA20), due to a lack of DNA. RER was detected in 6 of 43 (14%) colorectal carcinomas, at one or more loci. The highest frequency of alterations was found in the tumour from case COCA9: RER was observed at 10 of 12 (83.3%) loci analysed (figure 3.22). RER was also highly prevalent in the carcinoma from case COCA26, in which it was observed at 8 of 12 (66.7%) loci analysed (figure 3.23). The tumour from case COCA37, however, demonstrated RER at 5 (41.7%) loci (figure 3.24). Cases COCA38 and COCA43 both manifested RER at two loci each (16.7%) (figure 3.25a and b), and case COCA3 at just one locus (8.3%) (figure 3.25c). Table 3.4 summarizes the results of the RER analysis at each locus. RER was most frequently observed at the *D2S123* and *D19S49* microsatellite loci.

Table 2.2. Clinicopathological data and family history from forty-three colorectal carcinoma cases.

Case	Age	Sex	Tumour site	Dukes' stage	Family history
COCA1	48	M	Sigmoid colon	C2	NK
COCA2	72	M	Recto-sigmoid	C1	None
COCA3	63	M	Rectum	B	None
COCA4	85	F	Splenic flexure	B	Sister-cancer
COCA5	71	M	Asc. colon	C2	Father-CRC
COCA6	53	M	Rectum	B	Mother-CRC
COCA7	73	F	Rectum	B	None
COCA8	61	F	Sigmoid colon	C2	None
COCA9	74	F	Recto-sigmoid	B	HNPCC-type
COCA10	48	M	Sigmoid colon	B	None
COCA11	72	M	Sigmoid colon	C1	None
COCA12	72	M	Caecum	C2	None
COCA13	66	F	Des. colon	B	NK
COCA14	56	M	Rectum	A	NK
COCA15	80	M	Rectum	C1	NK
COCA16	70	M	Sigmoid colon	B	Both parents-cancer
COCA17	76	M	Rectum	C1	None
COCA18	60	M	Caecum	C2	NK
COCA19	83	F	Rectum	C1	NK
COCA20	43	F	Sigmoid colon	B	Grandfather-CRC
COCA21	65	M	Trans colon	B	None
COCA22	65	M	Rectum	B	None
COCA23	75	M	Rectum	B	None
COCA24	53	F	Splenic flexure	B	NK
COCA25	83	M	Rectum	B	NK
COCA26	49	M	Sigmoid colon	B	HNPCC-type
COCA27	49	M	Rectum	B	NK
COCA28	60	M	Rectum	C1	None
COCA29	66	M	Rectum	C1	None
COCA30	58	F	Ileocaecal valve	C1	NK
COCA31	42	F	Rectum	B	None
COCA32	78	M	Rectum	A	NK
COCA33	67	F	Rectum	A	None
COCA34	56	M	Caecum	C1	None
COCA35	60	M	Rectum	B	NK
COCA36	48	M	Caecum	C2	None
COCA37	74	F	Hepatic flexure	B	None
COCA38	70	M	Rectum	B	None
COCA39	71	F	Sigmoid colon	C2	NK
COCA40	52	M	Rectum	C2	NK
COCA41	43	M	Rectum	C1	None
COCA42	69	M	Rectum	B	Brother-CRC
COCA43	67	M	Rectum	C1	None

Asc. colon, Ascending colon; Des. colon, Descending colon; Trans. colon, Transverse colon; NK, Not known; CRC, Colorectal cancer.

Figure 3.22.

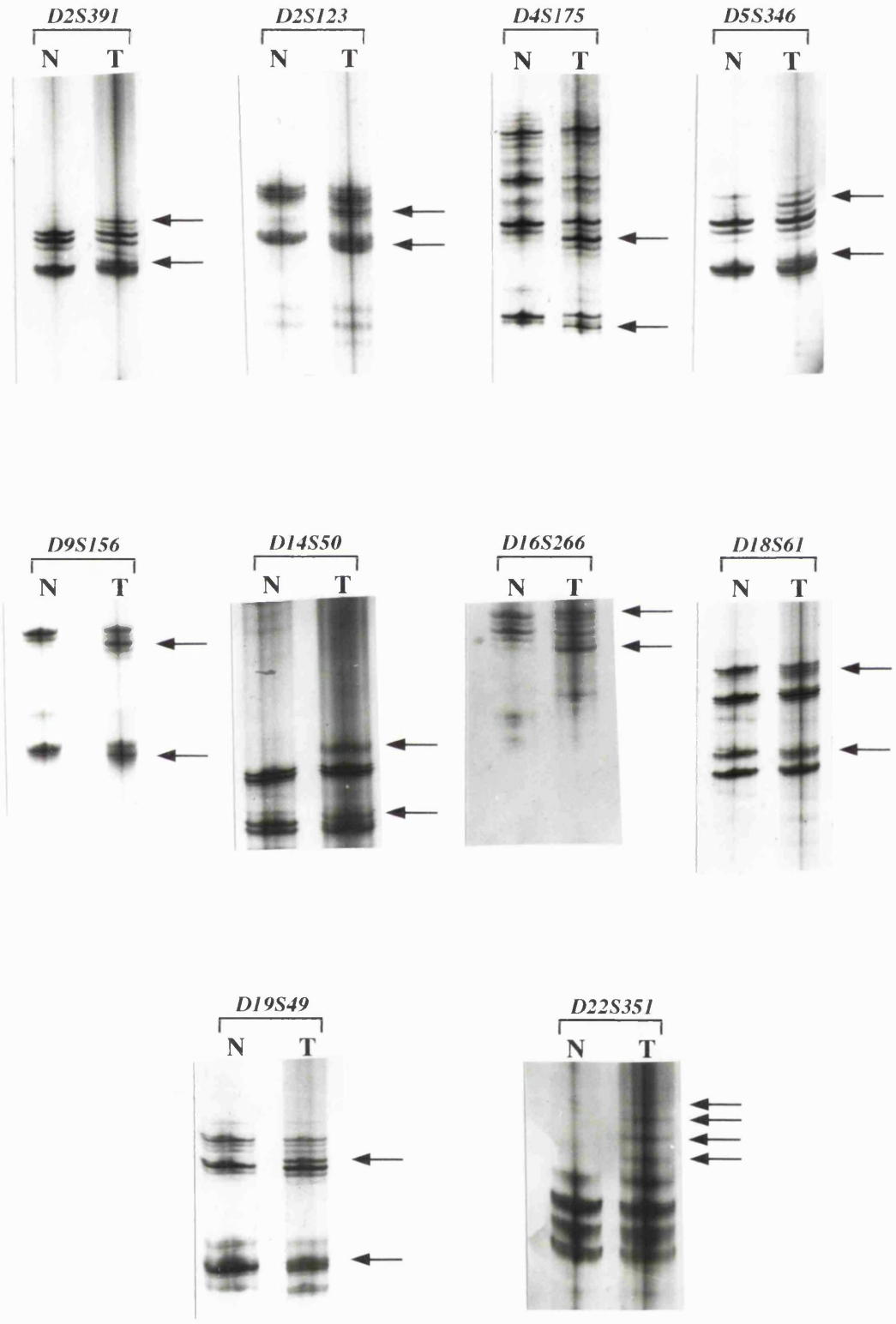


Figure 3.22. Detection of RER at microsatellite repeat loci in case COCA9. RER was observed as new bands in tumour DNA (T) not present in corresponding normal DNA (N) from case COCA9, as indicated by the arrows.

Figure 3.23.

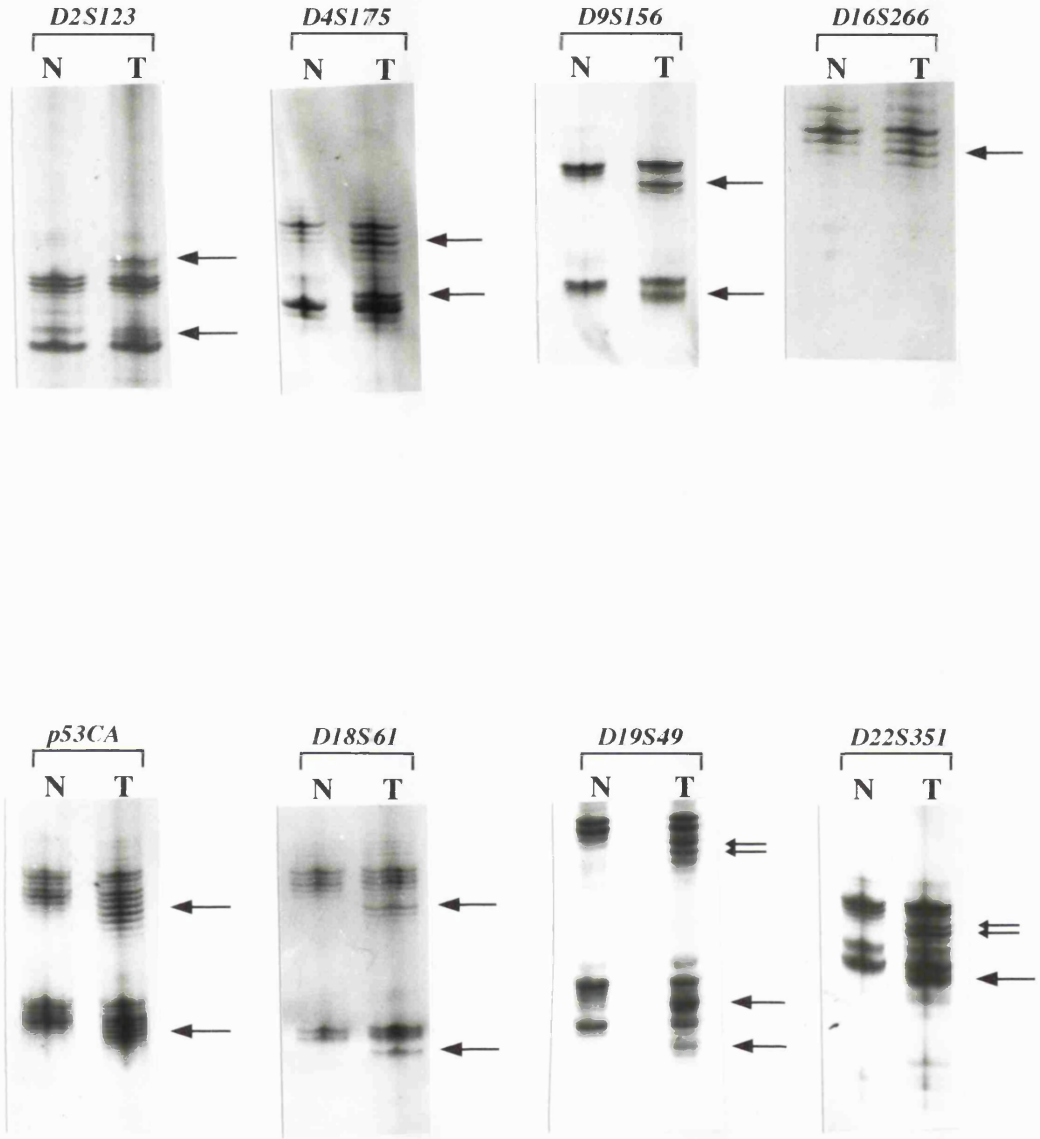


Figure 3.23. Analysis of RER at microsatellite repeat loci in case COCA26. N and T denote DNA samples from matching normal and tumour issues respectively. RER was detected in the tumour DNA of case COCA26 at each ocus shown as new fragments not present in matching normal DNA, as highlighted by the arrows.

Figure 3.24.

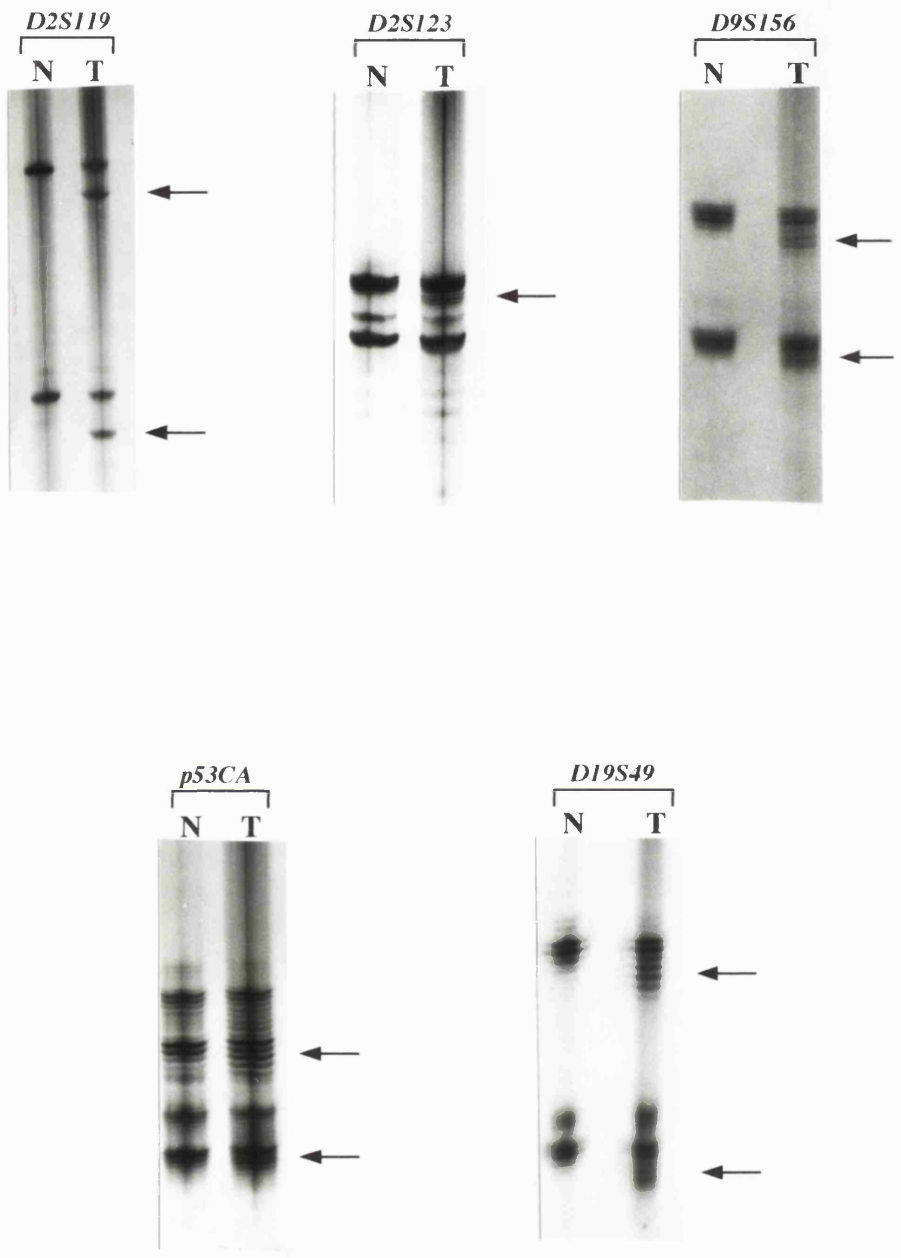


Figure 3.24. Detection of RER at microsatellite repeat sequences in case COCA37. T, tumour DNA; N, normal DNA. RER was observed as additional bands in the tumour DNA compared with corresponding normal DNA (see arrows). RER was detected at a total of five microsatellite loci in this carcinoma from case COCA37.

Figure 3.25.

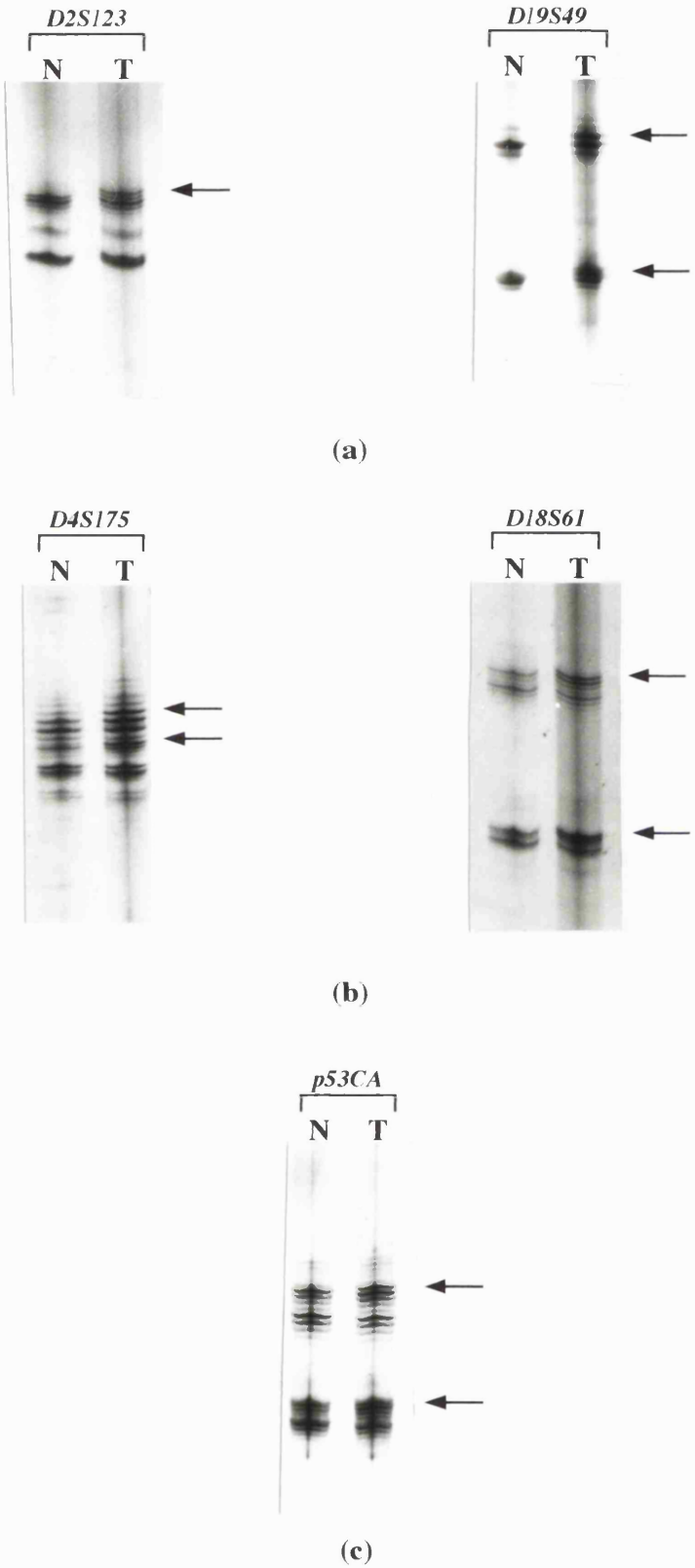


Figure 3.25. Detection of RER in tumours from cases COCA3, COCA38, and COCA43. T=tumour DNA; N=corresponding normal DNA. (**a**) and (**b**) show the detection of RER in tumour DNA from cases COCA38 and COCA43 respectively. RER was observed in both tumours at two microsatellite repeat loci each as extra bands not present in corresponding normal tissues, as indicated by the arrows. (**c**) Detection of RER in the carcinoma from case COCA3. RER was observed at one locus (*p53CA*) in this tumour.

Table 3.4. Detection of RER in colorectal carcinomas.

Locus	COCA3	COCA9	COCA26	COCA37	COCA38	COCA43	Total
D2S119	-	-	-	RER	-	-	1 / 7
D2S391	-	RER	-	-	-	-	1 / 7
D2S123	-	RER	RER	RER	RER	-	4 / 7
D4S175	-	RER	RER	-	-	RER	3 / 7
D5S346	-	RER	-		-	-	1 / 7
D9S156	-	RER	RER	RER	-	-	3 / 7
D14S50	-	RER	-	-	-	-	1 / 7
D16S266	-	RER	RER	-	-	-	2 / 7
p53CA	RER	-	RER	RER	-	-	3 / 7
D18S61	-	RER	RER	-	-	RER	3 / 7
D19S49	-	RER	RER	RER	RER	-	4 / 7
D22S351	-	RER	RER	-	-	-	2 / 7

The *D22S351* marker was also analysed under fully denaturing conditions by capillary electrophoresis of fluorescent PCR products, in 41 colorectal carcinoma and matching normal DNAs. Using this technique, RER was detected in tumour DNA from cases COCA9 and COCA26 at *D22S351*. As was the case with SSCP analysis, RER was not observed in the rest of the colorectal tumours at this locus.

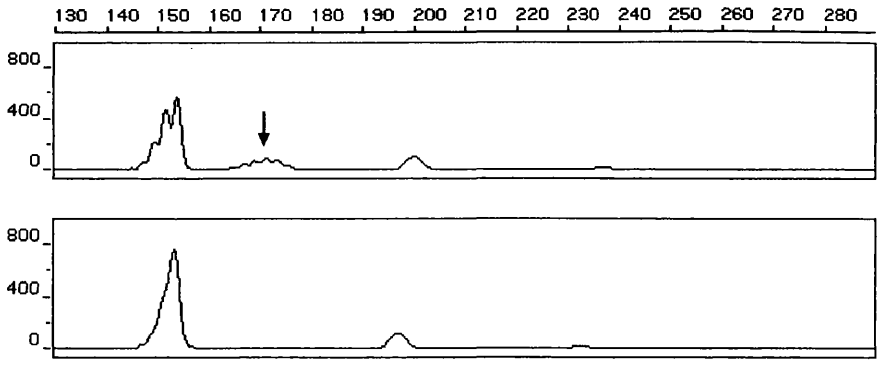
SSCP analysis of the *D22S351* marker in normal DNA from cases COCA9 and COCA26 (figures 3.22 and 3.23) showed that both were constitutionally homozygous. Each allele is represented by 3 bands. As more than 2 bands were observed for each allele with this marker it was chosen for analysis by capillary electrophoresis. Figure 3.26 shows electropherograms of tumour and normal fluorescent PCR products from cases COCA9 and COCA26. The capillary electrophoresis results confirmed that cases COCA9 and COCA26 were constitutionally homozygous at *D22S351*. A single peak of size within the published allele size range (145-163 bp) was observed in normal DNA from both cases. The electropherogram of tumour DNA from case COCA9 shows that it contains the constitutional allele peak. This has an additional smaller size peak which could represent a stutter peak or a novel allele (RER) peak but this cannot be distinguished. As previously explained, stutters are PCR artefacts. Stutter peaks are usually distinguished from alleles because of their relative low height and area and smaller size than the allele. As indicated by the arrow, novel peaks of greater size than the constitutional allele were seen in the tumour DNA from case COCA9 (figure 3.26a). These correspond to the faint RER bands observed in this tumour by SSCP analysis (figure 3.22).

The electropherograms of tumour and normal DNA from case COCA26 show there is a clear difference between the two samples (figure 3.26b). In the tumour DNA RER was observed as novel alleles denoted n1 and n2. Two novel bands were detected in tumour DNA from case COCA26 by SSCP analysis (figure 3.23).

Table 3.5 compares the clinicopathological features of the 6 RER+ and 37 RER- colorectal carcinomas. Possible associations were investigated between the RER types and age (using Student's *t* test) and sex and tumour side (using Fisher's exact test). However no significant associations were found between the RER types and these clinicopathological characteristics. Due to the small number of RER+ tumours, an association between RER type and Dukes' stage could not be calculated.

Figure 3.26.

(a)



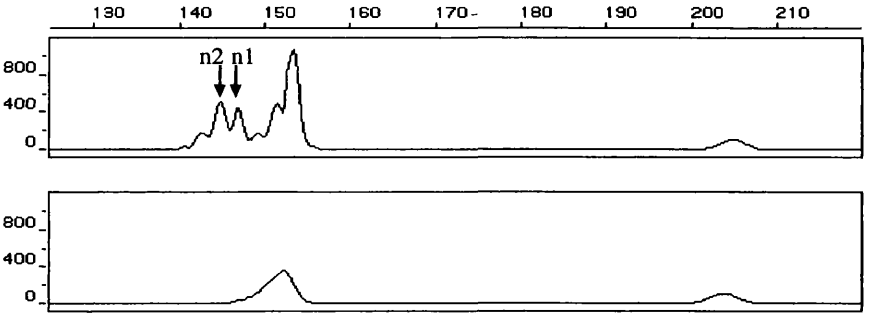
Tumour DNA (top) :

Peak type	Peak size	Peak height	Peak area
Stutter	148.61	218	2219
Stutter	151.60	481	5134
Constitutional allele	154.82	575	5661
Novel peak 1	168.43	27	237
Novel peak 2	170.91	49	505
Novel peak 3	172.60	72	630
Novel peak 4	174.82	99	1046
Novel peak 5	176.82	74	886
Novel peak 6	178.96	39	313

Normal DNA (bottom) :

Constitutional allele	154.17	770	14987

(b)



Tumour DNA (top) :

Peak type	Peak size	Peak height	Peak area
Stutter	140.65	185	1547
Novel peak 2 (n2)	143	525	4859
Novel peak 1 (n1)	145.46	456	3769
Stutter	148.28	171	1433
Stutter	151.41	497	4182
Constitutional allele	154.24	1080	10688

Normal DNA (bottom) :

Constitutional allele	153.87	364	7850

Figure 3.26. Electropherogram results for the *D22S351* marker in tumour and normal DNA from cases COCA9 and COCA26. (a) COCA9. The x axes show size in base pairs and the y axes show peak height units. The type, size, height, and area of the peaks are shown below. In the tumour DNA, the constitutional allele peak has an additional smaller size peak which could represent a stutter or a RER peak, however this cannot be distinguished. Novel peaks of greater size than the constitutional allele are present in the tumour DNA but not corresponding normal DNA, as highlighted by the arrow. (b) COCA26. In the tumour DNA, the constitutional allele has two stutter peaks of lower height, area, and size. Novel allele peaks are observed in the tumour DNA that are not present in the normal DNA and are denoted n1 and n2. Peak n1 can be distinguished as an allele peak and not a stutter as its peak height and area is greater than that of the stutter peak of greater size beside it. Similarly peak n2 can be distinguished as an allele as its peak height and area is greater than that of n1.

Table 3.5. Comparison of clinicopathological features of RER+ and RER- colorectal carcinomas.

Clinicopathological characteristics	RER+ cases (n = 6)	RER- cases (n = 37)	Total (n = 43)
Mean age ± SD	66.1 ± 9.1	63.5 ± 12.1	63.9 ± 12.0
Sex			
Female	2	11	13
Male	4	26	30
Tumour side*			
Right	1	7	8
Left	5	30	35
Dukes' stage			
A	0	3	3
B	5	16	21
C1	1	10	11
C2	0	8	8

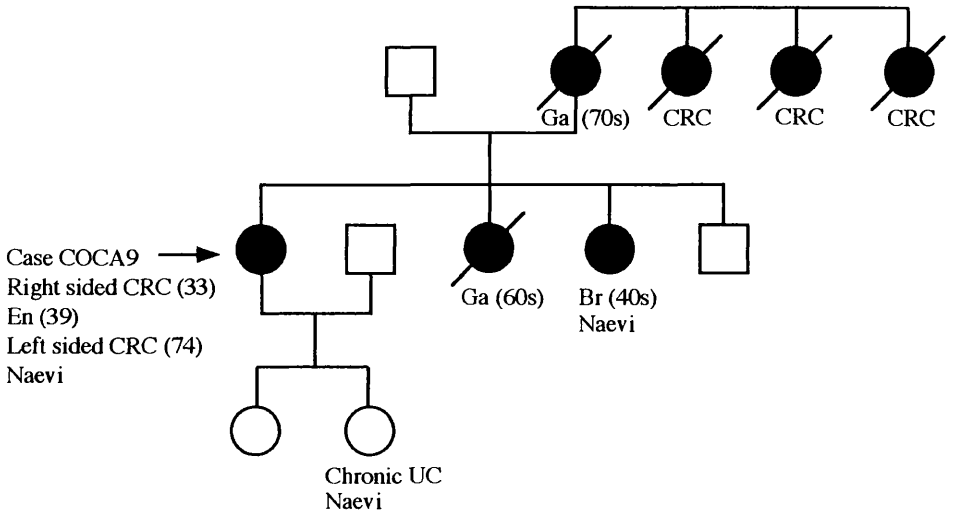
* Carcinomas in the caecum, ileocaecal valve, ascending colon, hepatic flexure and transverse colon are classified as right sided and carcinomas in the splenic flexure, descending colon, sigmoid colon and rectum are classified as left sided.

Information on a family history of cancer was available for all 6 RER+ colorectal carcinoma cases but for only 23 of the 37 RER- cases (table 2.2). None of the RER- cases had a family history indicative of HNPCC. However 2 of the RER+ cases, COCA9 and COCA26, did appear to belong to HNPCC-type families. Of all the RER+ cases, RER was most prevalent in the tumours from these two cases, being observed at 10 microsatellite repeat loci in tumour COCA9 and at 8 microsatellite repeat loci in tumour COCA26. Pedigrees of cases COCA9 and COCA26 are shown in figure 3.27 and indicate the family history information that was available. Both families meet the Amsterdam criteria for diagnosis of HNPCC which are that at least three relatives should have colorectal cancer, one of whom is a first degree relative of the other two, at least two generations should be affected, and one colorectal cancer case should be diagnosed before age 50 (Vasen *et al.*, 1991).

It was ascertained that although case COCA9 had a colectomy for a carcinoma located in the left side of the colorectum at the age of 74, which is being analysed in the present study, she previously had a right hemicolectomy for a carcinoma in her right colon at age 33 years. Furthermore, she developed an endometrial carcinoma shortly after the first colon carcinoma. In addition, the patient has naevi as do two other family members. Muir-Torre syndrome is a variant of the HNPCC syndrome

Figure 3.27.

Case COCA9 pedigree :



Case COCA26 pedigree :

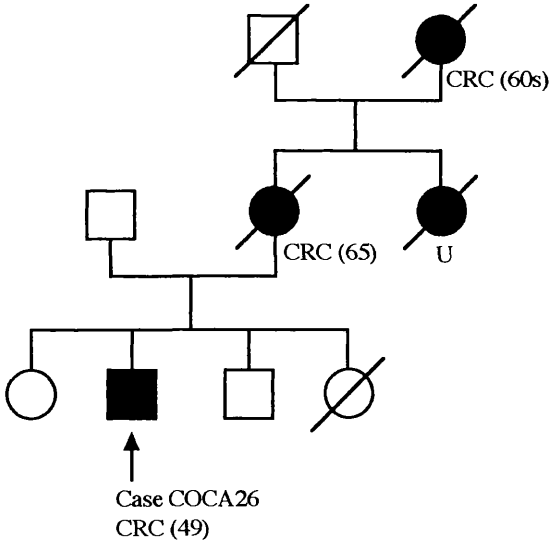


Figure 3.27. Family pedigrees of cases COCA9 and COCA26. Blackened circles and squares indicate cancer patients, ( ) indicates age with cancer, and diagonal lines indicate deceased. Cancer sites: CRC, colorectal; Ga, gastric; En, endometrial; Br, breast; U, unknown. Chronic UC indicates chronic ulcerative colitis.

and is characterized by the occurrence of multiple skin lesions as well as a tumour spectrum similar to that in HNPCC (Lynch *et al.*, 1985a). Thus there is a possibility that case COCA9 belongs to a Muir-Torre family. The second daughter of case COCA9 has chronic ulcerative colitis which is a disease associated with a high risk of colorectal cancer (Devroed and Taylor, 1976).

The widely accepted model of colorectal tumourigenesis involves an accumulation of mutations in tumour suppressor genes and oncogenes which include *APC*, *p53*, and K-*ras* (Fearon and Vogelstein, 1990). A negative correlation between the presence of RER and mutations in *p53* and K-*ras* has been reported in colorectal carcinomas (Ionov *et al.*, 1993). Aaltonen *et al.* (1993) however reported that alterations of the *APC*, *p53*, and K-*ras* genes were frequently observed in HNPCC tumours. In the present study the 43 colorectal carcinomas that were analysed for RER were also investigated for mutations of the *APC* gene as described in section 3.1.1. Three of the 6 (50%) RER+ colorectal carcinomas harboured somatic *APC* mutations (table 3.6). Two of these tumours are from the patients belonging to families with histories indicative of HNPCC (COCA9 and COCA26). Thirteen of the 37 (35%) RER- colorectal carcinomas contained somatic mutations of the *APC* gene.

Table 3.6. *APC* gene mutations in RER+ colorectal tumours.

RER+ tumour	*APC* mutation
COCA3	None
COCA9	GTT → ATT
COCA26	AGA → TGA
COCA37	None
COCA38	AGT → ATATCGT
COCA43	None

3.1.3 (b) Loss of heterozygosity

Loss of heterozygosity (LOH) was also investigated at the microsatellite repeat loci in the colorectal carcinomas, the results are summarized in table 3.7. Table 3.8 shows the percentage informativeness and percentage LOH at each locus. Cases exhibiting RER at a given locus were excluded from LOH analysis at that locus and deemed uninformative. The frequency of LOH ranged from 0-58%, being most frequently observed at a microsatellite repeat marker at the *p53* locus (*p53CA*). Representative examples of LOH observed at this locus are shown in figure 3.28. A reduction in intensity of bands representing one allele rather than complete loss was typically seen. The residual signal could be due to contamination of the tumour sample with normal tissue, clonal variation, duplication or amplification of an allele.

Table 3.7. Results of analysis of twelve microsatellite repeat loci in forty-three colorectal carcinoma cases.

Case	*D2S119*	*D2S391*	*D2S123*	*D4S175*	*D5S346*	*D9S156*	*D14S50*	*D16S266*	*p53CA*	*D18S61*	*D19S49*	*D22S351*
COCA1	HET	HET	hom	HET	**LOH**	HET	HET	hom	**LOH**	hom	HET	hom
COCA2	HET	hom	HET	**LOH**	HET	HET	HET	HET	HET	HET	HET	hom
COCA3	hom	HET	HET	HET	HET	hom	HET	hom	**RER**	**LOH**	HET	HET
COCA4	HET	hom	HET	HET	HET	HET	HET	hom	HET	HET	HET	HET
COCA5	hom	HET	HET	HET	HET	HET	HET	HET	**LOH**	HET	HET	hom
COCA6	HET	HET	HET	hom	**LOH**	hom	HET	hom	HET	HET	HET	**LOH**
COCA7	hom	HET	HET	HET	**LOH**	HET	**LOH**	hom	**LOH**	hom	HET	**LOH**
COCA8	HET	HET	HET	HET	HET	HET	**LOH**	HET	HET	HET	hom	HET
COCA9	HET	**RER**	**RER**	**RER**	**RER**	**RER**	**RER**	**RER**	HET	**RER**	**RER**	**RER**
COCA10	HET	hom	HET	HET	HET	HET	HET	hom	**LOH**	HET	HET	hom
COCA11	HET	HET	HET	HET	HET	hom	hom	hom	**LOH**	HET	HET	HET
COCA12	HET	HET	HET	HET	**LOH**	hom	HET	HET	**LOH**	HET	hom	HET
COCA13	HET	HET	HET	HET	hom	hom	HET	HET	HET	**LOH**	hom	hom
COCA14	hom	hom	HET	HET	**LOH**	HET	HET	HET	hom	**LOH**	HET	HET
COCA15	hom	hom	hom	HET	**LOH**	HET	HET	HET	**LOH**	**LOH**	hom	HET
COCA16	HET	HET	hom	hom	HET	HET	**LOH**	HET	hom	**LOH**	HET	HET
COCA17	hom	hom	HET	ND	hom	ND	HET	ND	ND	ND	HET	ND
COCA18	HET	HET	HET	hom	HET	HET	hom	HET	HET	**LOH**	hom	HET
COCA19	HET	HET	hom	HET	hom	HET	hom	hom	**LOH**	HET	HET	HET
COCA20	HET	HET	HET	ND	HET	ND	hom	ND	ND	ND	HET	ND
COCA21	HET	HET	HET	HET	hom	hom	HET	HET	hom	HET	HET	HET
COCA22	HET	HET	HET	hom	hom	HET	HET	HET	**LOH**	HET	HET	HET
COCA23	HET	hom	HET	HET	HET	hom	HET	HET	hom	HET	HET	HET
COCA24	hom	HET	hom	**LOH**	HET	HET	HET	hom	**LOH**	hom	HET	hom
COCA25	hom	HET	HET	HET	HET	HET	HET	hom	**LOH**	hom	hom	**LOH**
COCA26	hom	HET	**RER**	**RER**	hom	**RER**	HET	**RER**	**RER**	**RER**	**RER**	**RER**
COCA27	HET	HET	HET	HET	HET	**LOH**	**LOH**	HET	**LOH**	hom	hom	HET
COCA28	hom	**LOH**	hom	HET	hom	HET	HET	HET	HET	HET	HET	HET

Table continued overleaf.

Table 3.7. (continued)

Case	*D2S119*	*D2S391*	*D2S123*	*D4S175*	*D5S346*	*D9S156*	*D14S50*	*D16S266*	*p53CA*	*D18S61*	*D19S49*	*D22S351*
COCA29	HET	hom	HET	HET	**LOH**	HET	**LOH**	**LOH**	hom	hom	HET	**LOH**
COCA30	hom	HET	HET	HET	HET	hom	HET	HET	HET	HET	HET	hom
COCA31	HET	HET	hom	HET	**LOH**	hom	HET	hom	**LOH**	**LOH**	HET	HET
COCA32	HET	HET	HET	HET	HET	**LOH**	HET	hom	HET	HET	hom	HET
COCA33	hom	hom	HET	HET	HET	HET	HET	hom	HET	HET	HET	HET
COCA34	hom	HET	HET	HET	HET	HET	hom	HET	**LOH**	**LOH**	HET	HET
COCA35	HET	HET	hom	HET	HET	hom	hom	HET	**LOH**	HET	hom	HET
COCA36	HET	HET	HET	**LOH**	hom	HET	HET	HET	hom	hom	HET	HET
COCA37	**RER**	HET	**RER**	HET	HET	**RER**	hom	hom	**RER**	hom	**RER**	HET
COCA38	HET	HET	**RER**	HET	hom	HET	HET	hom	**LOH**	**LOH**	**RER**	HET
COCA39	HET	HET	HET	hom	HET	HET	HET	HET	HET	HET	hom	hom
COCA40	HET	HET	HET	hom	hom	HET	hom	HET	**LOH**	HET	HET	HET
COCA41	HET	HET	HET	HET	HET	HET	hom	hom	HET	HET	HET	HET
COCA42	HET	hom	HET	HET	hom	hom	hom	HET	**LOH**	**LOH**	hom	HET
COCA43	HET	HET	hom	**RER**	HET	HET	HET	HET	hom	**RER**	HET	HET

HET, constitutional heterozygosity retained in tumour; **LOH**, loss of heterozygosity observed in tumour; hom, constitutionally homozygous therefore uninformative; **RER**, replication error; ND, not detemined.

Thus some authors now prefer to use the term allelic imbalance rather than LOH when detecting such alterations. In addition, it cannot be excluded that a RER has been detected and the extra band has migrated to the same position as one of the wild-type allele bands thus indicating that the other allele is lost.

Table 3.8. Frequency of informativity and LOH observed at microsatellite repeat loci in 43 colorectal carcinoma cases.

Locus	No. of informative cases / Total No. of cases* (%)	Cases with LOH (%)
D2S119	29 / 43 (67.4)	0
D2S391	32 / 43 (74.4)	1 (3.1)
D2S123	30 / 43 (69.8)	0
D4S175	32 / 41 (78.1)	3 (9.4)
D5S346	31 / 43 (72.1)	8 (25.8)
D9S156	27 / 41 (65.9)	2 (7.4)
D14S50	32 / 43 (74.4)	5 (15.6)
D16S266	23 / 41 (56.1)	1 (4.4)
p53CA	31 / 41 (75.6)	18 (58.1)
D18S61	30 / 41 (73.2)	10 (33.3)
D19S49	28 / 43 (65.1)	0
D22S351	31 / 41 (75.6)	4 (13)

* The *D4S175*, *D9S156*, *D16S266*, *p53CA*, *D18S61*, and *D22S351* markers were analysed in 41 of the 43 colorectal carcinoma cases.

LOH was also frequent at *D18S61*, with 10 of 30 (33.3%) informative tumours exhibiting loss, examples are depicted in figure 3.29. Figure 3.30 illustrates detection of LOH at the *D2S391*, *D16S266*, *D9S156*, and *D4S175* microsatellite loci. A low rate of LOH was observed with these markers which demonstrated frequencies of 3.1%, 4.4%, 7.4%, and 9.4% LOH respectively. A higher frequency of LOH was detected at *D14S50* (15.6%), examples are shown in figure 3.31a. Finally, of 31 cases that were informative for the *D22S351* marker, LOH was found in 4 tumours (13%) (figure 3.31b).

The *D22S351* marker was also analysed under denaturing conditions by capillary electrophoresis of fluorescent PCR products. No difference in results was found between this method and the SSCP method. SSCP analysis of the *D22S351* marker had shown that LOH observed in the tumours from cases COCA6 and COCA25 was not as extensive as that observed in the other two tumours (figure

Figure 3.28.

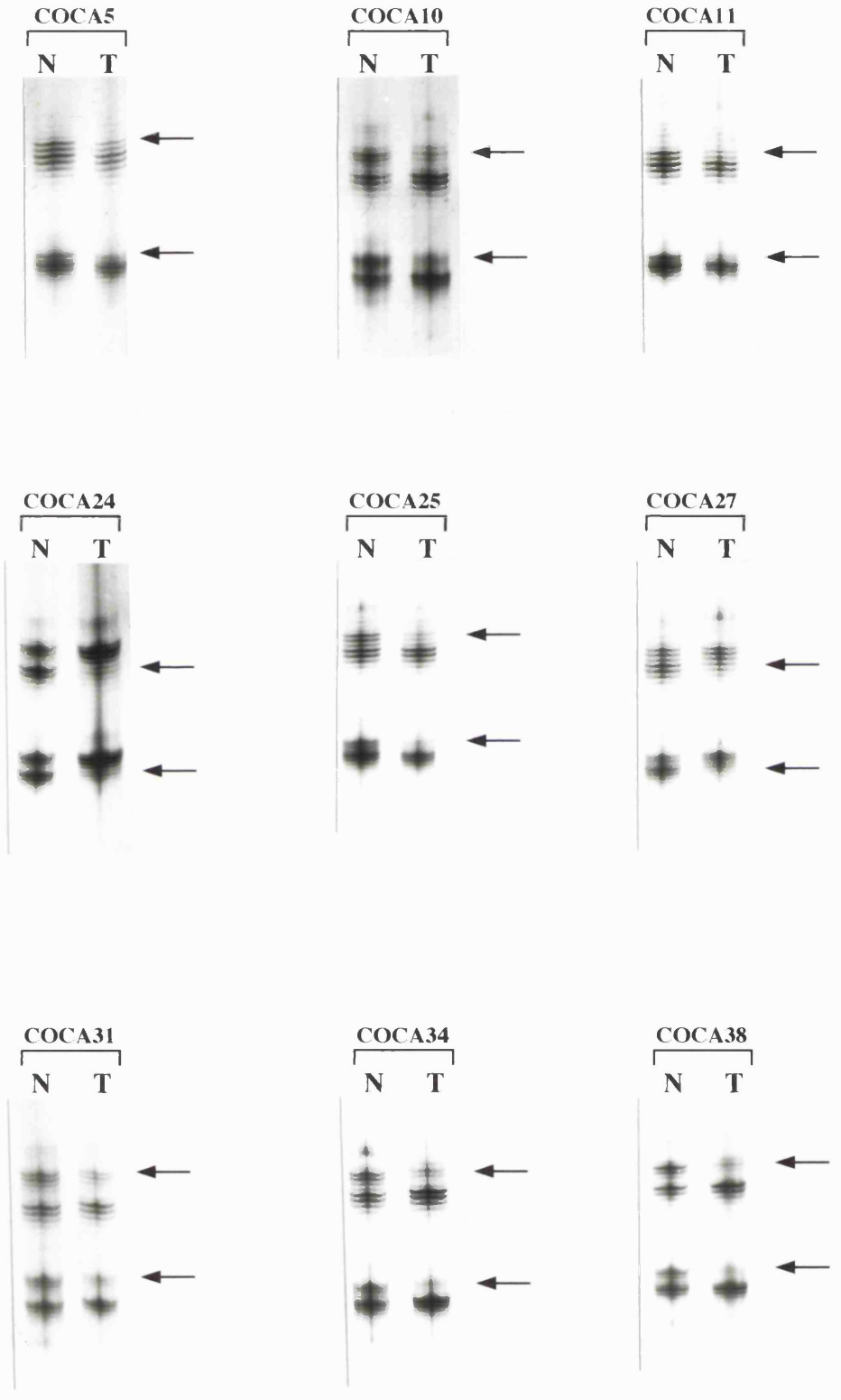


Figure 3.28. Analysis of LOH at the *p53CA* locus in colorectal carcinomas. T and N denote DNA samples derived from tumour and matching normal tissues. All cases shown are constitutionally heterozygous, each allele being represented by two bands in the normal DNAs. A reduction in intensity of bands representing one allele in tumour DNA relative to matching normal DNA indicates LOH, as highlighted by the arrows.

Figure 3.29.

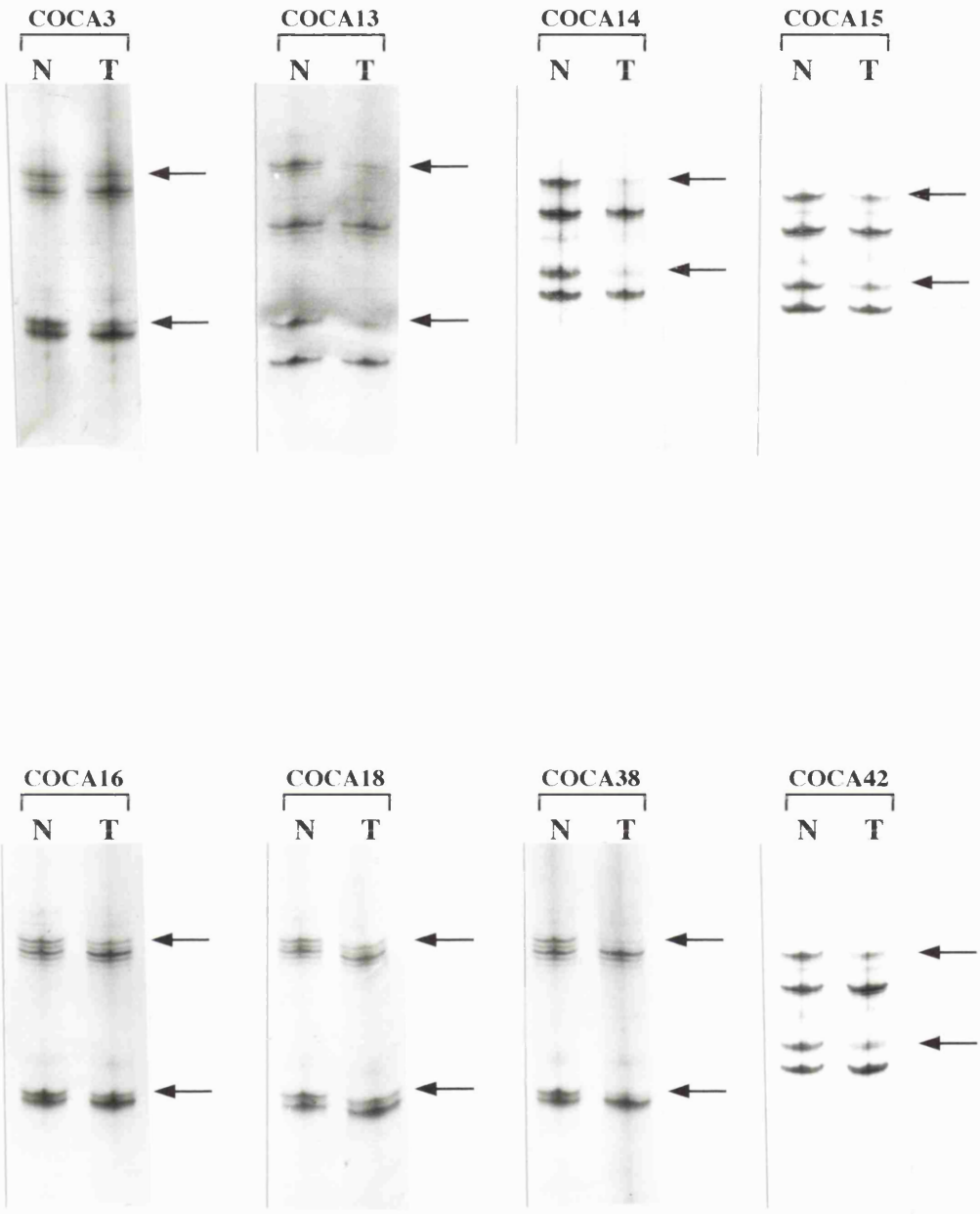


Figure 3.29. LOH analysis at the *D18S61* microsatellite repeat locus in colorectal carcinomas. N=normal DNA; T=tumour DNA. Each case shown is constitutionally heterozygous at *D18S61*, each allele being represented by two bands in the normal DNA. A reduction in intensity of two bands in the corresponding tumour DNAs indicated LOH, as highlighted by the arrows.

Figure 3.30.

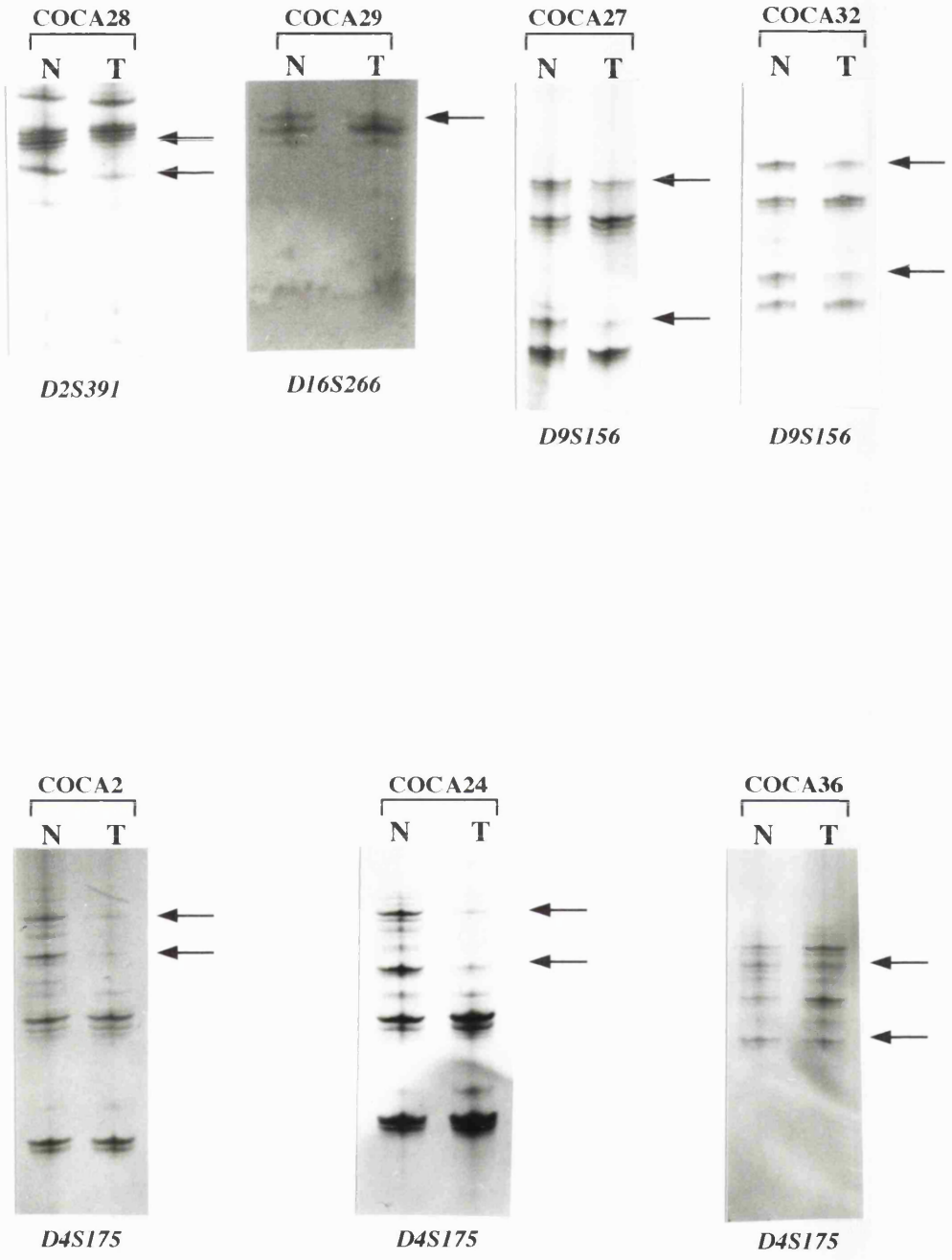
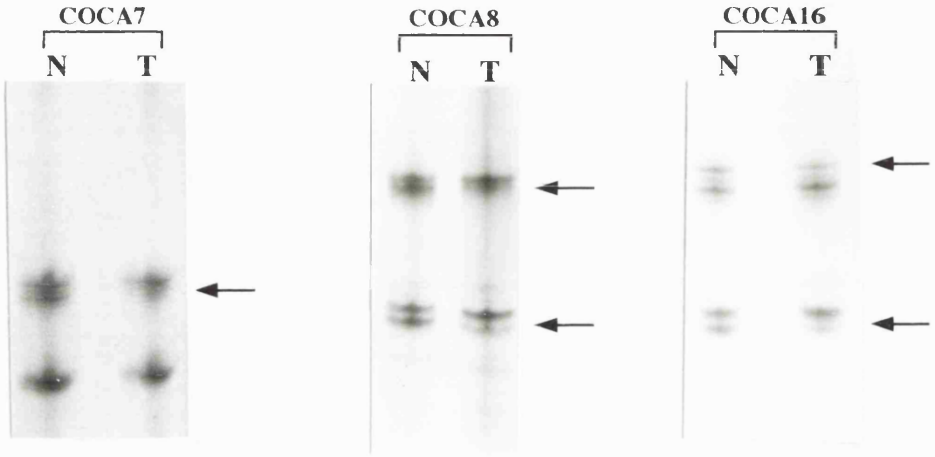
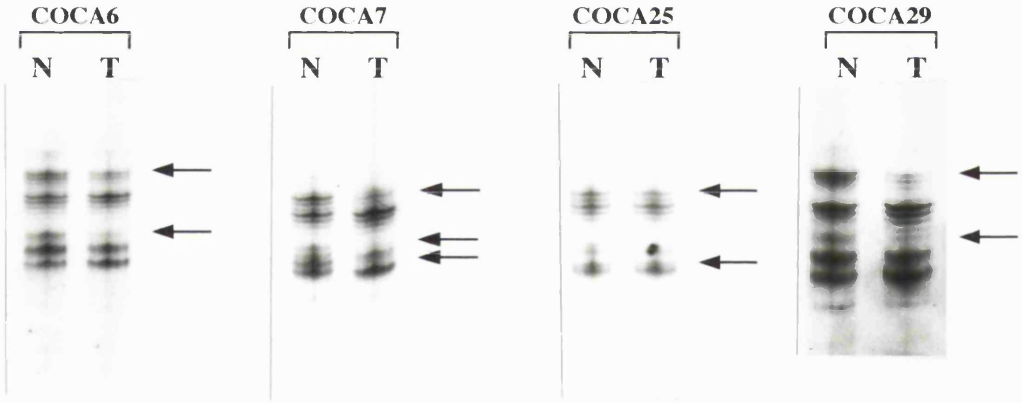


Figure 3.30. Detection of LOH at the *D2S391*, *D4S175*, *D9S156*, and *D16S266* microsatellite repeat loci in colorectal carcinomas. T and N denote DNA samples derived from corresponding tumour and normal tissues. Each colorectal caecinoma case shown is constitutionally heterozygous at the locus analysed. A reduction in intensity of bands representing one allele in tumour relative to corresponding normal DNA indicated LOH, as highlighted by the arrows.

Figure 3.31.



(a)

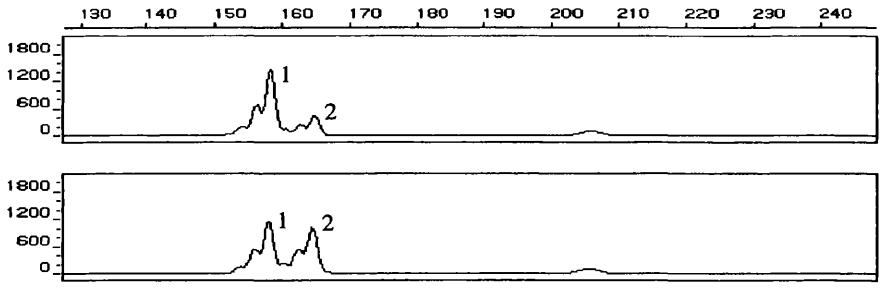


(b)

Figure 3.31. Detection of LOH at the *D14S50* and *D22S351* microsatellite loci in colorectal carcinomas. T, tumour DNA; N, normal DNA. **(a)** Examples of LOH observed at *D14S50*. Analysis of normal DNA from each case shown demonstrated constitutional heterozygosity, each allele being represented by two bands. In case COCA7 two of the bands have migrated close together. In the tumour DNA from each case a reduction in intensity of bands representing one allele suggested LOH (highlighted by arrows). **(b)** LOH analysis at *D22S351*. For the *D22S351* marker, each allele is usually represented by 3 bands. Each case shown is constitutionally heterozygous. In the normal DNA from case COCA7 each allele is represented by three bands. In the normal DNA from cases COCA6 and COCA29 two of the bands have migrated to the same position, thus three bands are not seen for each allele. In the normal DNA from case COCA25 two bands are seen for each allele. A reduction in intensity of bands representing one allele in the tumour DNA from each case relative to corresponding normal DNA indicates LOH, as highlighted by the arrows.

Figure 3.32.

(a)



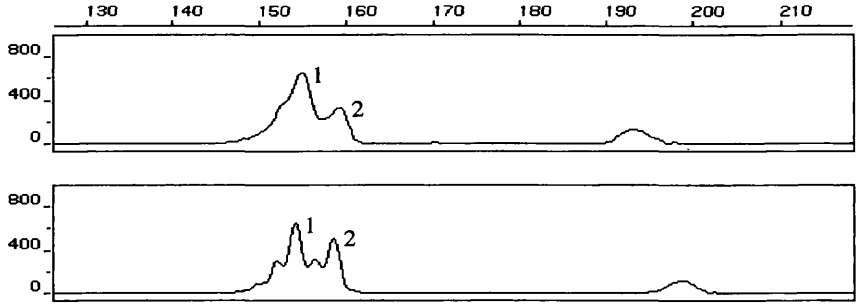
Tumour DNA (top) :

Peak type	Peak size	Peak height	Peak area
Stutter	157.96	700	5785
Allele 1	160.88	1479	14079
Stutter	165.83	252	2186
Allele 2	168.12	453	4057

Normal DNA (bottom) :

Stutter	157.68	539	5141
Allele 1	160.45	1177	10860
Stutter	165.45	560	4948
Allele 2	167.56	1028	9755

(b)



Tumour DNA (top) :

Peak type	Peak size	Peak height	Peak area
Allele 1	155.98	663	14678
Allele 2	161.77	331	4702

Normal DNA (bottom) :

Stutter	152.26	308	3589
Allele 1	155.10	661	6731
Stutter	158.55	315	2769
Allele 2	161.33	510	4877

Figure 3.32. Electropherogram results for the *D22S351* marker in tumour and normal DNA from cases COCA6 and COCA25. (**a**) COCA6. The x axes show size in base pairs and the y axes show peak height units. The constitutional allele peaks are denoted 1 and 2. Loss of the allele denoted 2 is observed in the tumour DNA from this case. (**b**) COCA25. Analysis of the normal DNA shows that this case is constitutionally heterozygous at *D22S351*. Loss of the allele denoted 2 is observed in the tumour DNA.

3.31b). Figure 3.32 shows detection of LOH in tumour COCA6 and tumour COCA25 using the capillary electrophoresis technique. The results confirmed that case COCA6 is constitutionally heterozygous at the *D22S351* locus, the alleles are denoted 1 and 2. In the tumour DNA from this case loss of the larger sized allele (denoted 2) was observed. The ratio of allele 1: allele 2, calculated using the values for peak area, is 1.1:1 in the normal DNA and 3.5:1 in the tumour DNA. The electropherogram of normal DNA from COCA25 also shows that this case is constitutionally heterozygous at *D22S351*. Loss of the allele denoted 2 was observed in the tumour DNA. The ratio of allele 1:allele 2 is 1.4:1 in the normal DNA, but is 3:1 in the tumour DNA.

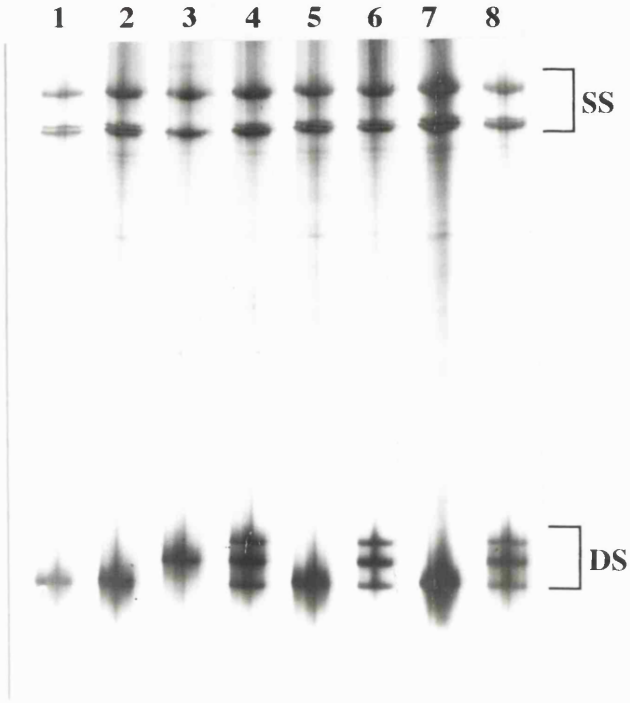
3.1.4. Mutational analysis of the *hMSH2* gene

The *hMSH2* DNA mismatch repair gene consists of 16 exons. Exons 12-14 of the *hMSH2* gene, which encode the most conserved region of the hMSH2 protein, were screened for mutations by SSCP and heteroduplex analysis in DNA derived from the 43 colorectal carcinoma and corresponding normal tissues.

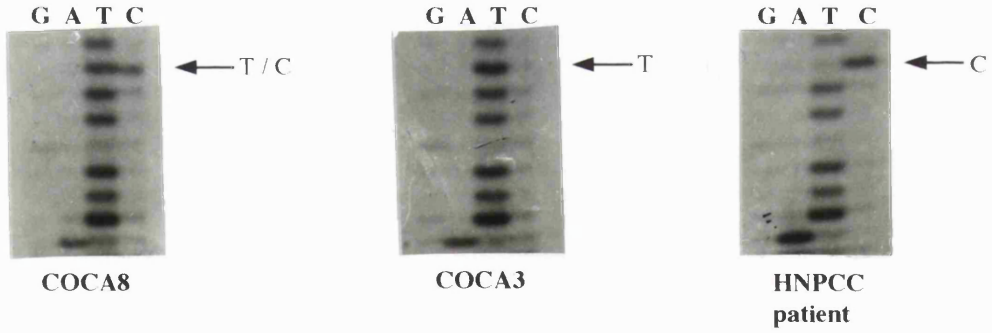
During mutational analysis of exon 13, double-stranded DNA variation was detected in several carcinomas, which was also present in the corresponding normal DNA. Figure 3.33a shows SSCP and heteroduplex analysis of the exon 13 amplicon in DNA derived from the normal tissues of seven colorectal carcinoma cases and in constitutional DNA from an individual belonging to a small HNPCC-type kindred being investigated in our laboratory. Double-stranded DNA was retained on the gel. In normal tissue samples from four cases (COCA1, COCA2, COCA3, and COCA4) a single double-stranded DNA band was observed. However in the other three cases (COCA8, COCA13, and COCA25), two additional double-stranded DNA bands of distinctly slower mobilities were seen. In DNA derived from the individual from a HNPCC-type family, a single band corresponding to the DNA band of intermediate mobility observed in the three previously described cases is seen. Some variation was detected in the single-stranded DNA pattern but this was not clearly resolved.

The exon 13 amplicon was then sequenced in normal tissue DNA from cases COCA3 and COCA8, and in genomic DNA from the HNPCC patient, thus all three types of double-stranded DNA pattern observed were repesented (figure 3.33b). In the normal tissue DNA sequence from case COCA8, a single base substitution of a C for a T residue was identified, in the intronic splice acceptor site, at a position six base pairs upstream of the start of exon 13. In the normal DNA from case COCA3, a T residue was observed at this position, the wild-type sequence. However in DNA

Figure 3.33.



(a)



(b)

Figure 3.33. Detection of variants in exon 13 amplicon of *hMSH2*. (**a**) SSCP and heteroduplex analysis. Lanes 1 and 2 represent normal tissue DNA from cases COCA1 and COCA2 respectively, lane 3 is a HNPCC patient, and lanes 4-8 represent normal DNA from cases COCA8, COCA3, COCA13, COCA4, and COCA25 respectively. A single double-stranded DNA (DS) band was observed in cases COCA1, COCA2, COCA3, and COCA4. A similar variant double-stranded DNA pattern was observed in cases COCA8, COCA13, and COCA25. The double-stranded DNA band in the HNPCC patient migrated to the same position as the band of intermediate mobility observed in these three cases. The variation seen in the double-stranded DNA pattern was not clearly detected in the single-stranded DNA (SS) pattern. (**b**) Sequence analysis of exon 13 amplicon. A T to C transition was identified in the normal tissue DNA sequence from case COCA8, at the -6 position of the splice acceptor site of exon 13. The wild-type sequence (T) was identified in normal DNA from case COCA3 at this position, while homozygosity for the C residue was detected in genomic DNA from the HNPCC patient.

from the HNPCC patient a C residue was observed. The T to C transition was previously identified by the two groups that cloned the *hMSH2* gene. Fishel *et al.* (1993) observed it in two sporadic colon tumours but not in corresponding normal tissues. In addition, affected individuals from two HNPCC kindreds were found to be constitutionally heterozygous for the T to C transition. Leach *et al.* (1993), however, identified this single base substitution in two of twenty unrelated normal individuals as well as in genomic DNA from an individual from a HNPCC kindred. It was thus unclear whether the T to C transition represented a polymorphism or a colorectal cancer-predisposing mutation.

The sequencing results therefore revealed that the fastest migrating double-stranded DNA band observed on the PhastGel represented the wild-type 'T' allele and the band of intermediate mobility represented the variant 'C' allele. The slowest migrating band was probably heteroduplex DNA. Thus cases COCA8, COCA13, and COCA25 were constitutionally heterozygous for the T to C transition. Cases COCA1, COCA2, COCA3, and COCA4 were homozygous for the 'T' allele, and the individual belonging to the HNPCC-type kindred was homozygous for the 'C' allele.

SSCP and heteroduplex analysis of the exon 13 amplicon was carried out in tumour and normal DNA from all forty-three colorectal cancer cases. In each case, no difference in the double-stranded DNA pattern was observed betweeen corresponding tumour and normal tissues. Sequence analysis of normal DNA from six further cases confirmed that the DNAbands of fastest and intermediate mobilities represented the 'T' and 'C' alleles respectively. In addition, normal tissue DNA from twenty-six gastric carcinoma cases and DNA from fifty unrelated normal individuals, who were spouses of FAP patients being investigated for *APC* mutations in our laboratory, were also analysed for the T to C sequence variation using the SSCP and heteroduplex assay. These 50 individuals had no known family history of HNPCC. The results are shown in table 3.9. None of the 119 individuals analysed were found to be homozygous for the 'C' allele. The T to C transition was identified in 28% of the group of normal unrelated individuals, and in 21% and 23% of colorectal and gastric carcinoma cases respectively, there was no significant difference between the three groups ($\chi^2 = 0.66$; $p > 0.1$). Thus the T to C transition appears to be a polymorphism that is common in the general population. From analysis of the 119 unrelated individuals, allele frequencies of 0.88 and 0.12 are calculated for the alleles with T and C respectively at the -6 position of the splice acceptor site of exon 13.

Information on family history was available for 4 of the 9 colorectal carcinoma cases with the T to C transition. Only one case (COCA42) had a history, a

first degree relative with colorectal cancer. Similarly among 6 gastric carcinoma cases heterozygous for the T to C transition, just one case (GACA17) had a family history - a sister with breast cancer. No other variants were identified in exons 12, 13, or 14 of *hMSH2* in the colorectal carcinoma cases.

Table 3.9 Genotypes of 119 individuals at the -6 position of the splice acceptor site of exon 13 of *hMSH2*.

Genotype at -6 position of splice acceptor site	Colorectal carcinoma cases	Gastric carcinoma cases	Group of unrelated normal individuals	Total
wild-type / wild-type (TT)	34 (79%)	20 (77%)	36 (72%)	90 (75.6%)
wild-type / variant (TC)	9 (21%)	6 (23%)	14 (28%)	29 (24.4%)
Total	43	26	50	119 (100%)

3.1.5. Summary of genetic alterations in colorectal carcinomas

Table 3.10 summarizes the somatic genetic changes identified in the forty-three colorectal cancers, and illustrates the multi-step nature of this malignancy. At least two somatic alterations were observed in 27 (63%) carcinomas. Three to five alterations were detected in 11 carcinomas. One alteration was observed in 9 tumours, while no alterations were found in 7 tumours. Genetic alterations were detected at all stages of malignancy. Of note, all 3 tumours of the earliest stage (Dukes' stage A) were found to contain alterations of the *APC* tumour suppressor gene: 2 tumours (COCA32 and COCA33) harboured two somatic *APC* gene mutations each and 1 tumour (COCA14) demonstrated allele loss at polymorphic loci within the *APC* gene. The most frequent alterations observed in colorectal carcinomas were mutations of the *APC* gene (37.2%) tumours and LOH at a microsatellite repeat locus on chromosome 17p (*p53CA*) (58% tumours). LOH on chromosome 18q was also frequent (33% informative tumours).

Table 3.10. Summary of somatic alterations identified in 43 colorectal carcinomas.

Case	Dukes' stage	No. of *APC* mutations	RER	LOH	Total No. of alterations
COCA1	C2	0	-	5q, 17p	2
COCA2	C1	1	-	4q	2
COCA3	B	0	+	18q	2
COCA4	B	0	-	-	0
COCA5	C2	0	-	17p	1
COCA6	B	0	-	5q, 22q	2
COCA7	B	1	-	5q, 14q, 17p, 22q	5
COCA8	C2	1	-	14q	2
COCA9	B	1	+	-	2
COCA10	B	0	-	17p	1
COCA11	C1	0	-	17p	1
COCA12	C2	0	-	5q, 17p	2
COCA13	B	0	-	18q	1
COCA14	A	0	-	5q, 18q	2
COCA15	C1	0	-	5q, 17p, 18q	3
COCA16	B	0	-	14q, 18q	2
COCA17	C1	0	-	-	0
COCA18	C2	0	-	18q	1
COCA19	C1	2	-	17p	3
COCA20	B	0	-	-	0
COCA21	B	0	-	-	0
COCA22	B	0	-	5q, 17p	2
COCA23	B	1	-	-	1
COCA24	B	1	-	4q, 17p	3
COCA25	B	0	-	17p, 22q	2
COCA26	B	1	+	-	2
COCA27	B	0	-	9p, 14q, 17p	3
COCA28	C1	1	-	2p	2
COCA29	C1	0	-	5q, 14q, 16q, 22q	4
COCA30	C1	0	-	-	0
COCA31	B	0	-	5q, 17p, 18q	3
COCA32	A	2	-	9p	3
COCA33	A	2	-	-	2
COCA34	C1	1	-	17p, 18q	3
COCA35	B	1	-	17p	2
COCA36	C2	0	-	4q	1
COCA37	B	0	+	-	1
COCA38	B	1	+	17p, 18q	4
COCA39	C2	0	-	-	0
COCA40	C2	1	-	17p	2
COCA41	C1	0	-	-	0
COCA42	B	1	-	17p, 18q	3
COCA43	C1	0	+	-	1

3.2. Genetic alterations in gastric cancer

Twenty-six gastric carcinoma and corresponding normal tissues were analysed for genetic alterations. Table 2.1 described useful clinicopathological and family history data on the gastric carcinoma cases and so is also included in this section (see next page).

3.2.1. Mutational analysis of the *APC* gene

APC gene mutations were investigated in the 26 gastric carcinoma cases using three different methods of mutation detection: SSCP and heteroduplex analysis, protein truncation test, and restriction digest analysis for the detection of specific translation-terminating *APC* mutations.

3.2.1 (a) SSCP and heteroduplex analysis

As described for the colorectal carcinoma cases, exons 6, 8, 11, 14, and the 5' half of exon 15 (codons 653-1700) of *APC* were analysed for mutations with a SSCP and heteroduplex assay, in DNA derived from 26 gastric carcinoma and corresponding normal tissues. Somatic mutation was detected in 1 of 26 (4%) gastric carcinomas. As figure 3.34a illustrates, in the tumour from case GACA17 both a single-stranded DNA variant conformer and heteroduplex DNA bands were observed which were not detected in corresponding normal DNA. Sequence analysis (figure 3.34b) demonstrated the variation to be the result of a 4 bp deletion of either AGAG or GAGA in the sequence GAAAAGAGAGAGAGT at codon 1462-1465. This sequence change caused frameshift and the formation of a premature stop codon at immediately downstream, thus it was predicted to result in truncation of the *APC* gene product. The mutation is located within the mutation cluster region (MCR) in exon 15 of *APC* which includes codons 1286-1513. The tumour of case GACA17 was of the intestinal histological type.

3.2.1 (b) Protein truncation test

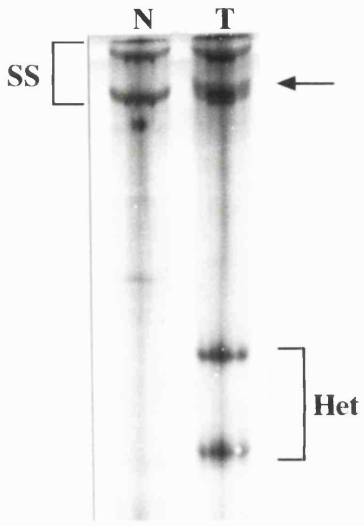
The 26 gastric carcinoma and normal DNA pairs were also investigated for mutations in exon 15 of *APC* by the protein truncation test (PTT), a method that is specific for the detection of translation-terminating mutations. The part of this exon that was analysed included the mutation cluster region. A 2 kb product, encompassing codons 1028-1700, was amplified from genomic DNA and *in vitro*

Table 2.1. Clinicopathological data and family history from twenty-six gastric carcinoma cases.

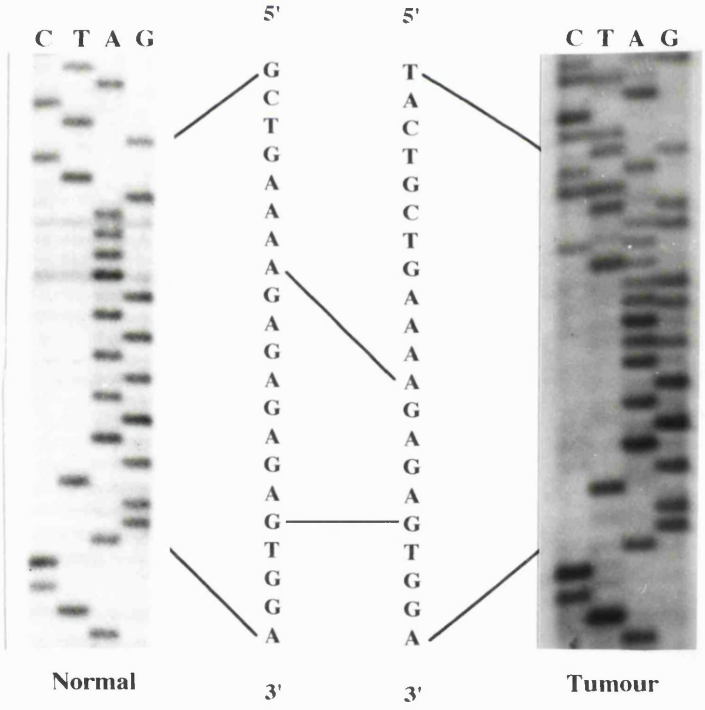
Case	Age	Sex	Tumour site	Histological type	Family history
GACA1	72	F	Cardia	Diffuse	Sister-breast cancer
GACA2	54	M	Antrum	Diffuse	None
GACA3	42	M	NK	Intestinal	NK
GACA4	79	F	NK	Intestinal	None
GACA5	67	M	Body	Diffuse	None
GACA6	35	F	Cardia	Diffuse	None
GACA7	65	M	NK	Diffuse	NK
GACA8	72	M	NK	Intestinal	None
GACA9	83	F	Antrum	Diffuse	None
GACA10	60	M	Cardia	Intestinal	None
GACA11	74	F	Cardia	Intestinal	None
GACA12	65	M	NK	Intestinal	None
GACA13	47	M	Cardia	Intestinal	None
GACA14	77	M	Cardia	Intestinal	NK
GACA15	71	F	Cardia	Intestinal	None
GACA16	75	M	Body	Intestinal	None
GACA17	67	F	NK	Intestinal	Sister-breast cancer
GACA18	70	F	Body	Diffuse	None
GACA19	49	M	Fundus	Diffuse	Father-gastric cancer
GACA20	55	M	Antrum	Intestinal	None
GACA21	85	F	Cardia	Intestinal	Brother-cancer
GACA22	84	F	Pylorus	Intestinal	None
GACA23	60	M	Cardia	Intestinal	Father-cancer; brother-CRC
GACA24	66	M	Body	Diffuse	None
GACA25	81	F	Cardia	Intestinal	None
GACA26	61	M	NK	Intestinal	None

NK, not known; CRC, colorectal cancer.

Figure 3.34.



(a)



(b)

Figure 3.34. Detection of somatic mutation in exon 15H amplicon of *APC* in case GACA17. **(a)** SSCP and heteroduplex analysis. T and N denote DNA samples from matching tumour and normal tissues. Both single-stranded DNA (SS) variation and heteroduplex DNA (Het) were detected in the tumour. **(b)** Sequence analysis of exon 15H amplicon (antisense strand). A 4 bp deletion of AGAG or GAGA in GAAAAGAGAGAGAGT at codon 1462-1465 was identified in the tumour DNA sequence resulting in frameshift and the creation of a premature stop codon immediately downstream.

Figure 3.35.

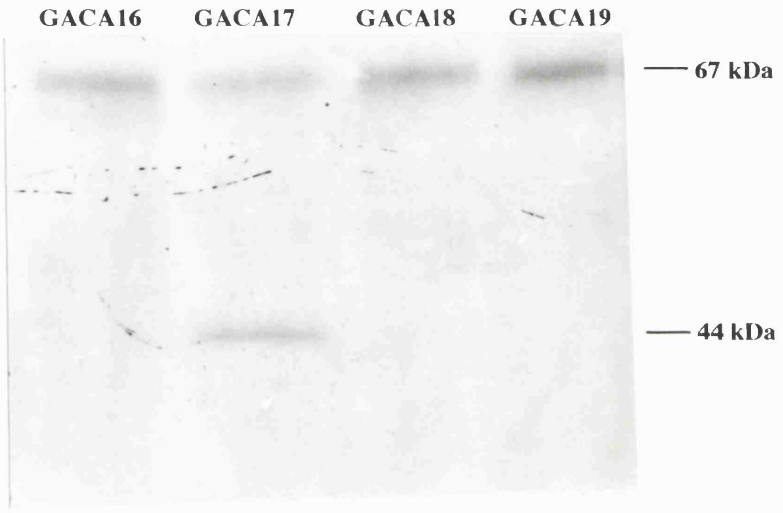


Figure 3.35. PTT analysis of a 2 kb fragment of *APC* exon 15 in gastric carcinomas. PTT of tumour DNAs from four gastric carcinoma cases is shown. The normal protein product of 67 kDa was detected in gastric tumours from all cases. A protein truncating mutation was detected only in the tumour from case GACA17 as a shorter peptide of 44 kDa.

transcription and translation of this fragment was performed. The resultant labelled translation products were separated in SDS-PAGE gels which were then autoradiographed.

A translation-terminating mutation is identified as a smaller protein product compared to the wild-type product. As illustrated in figure 3.35, a truncating mutation was detected in the carcinoma from case GACA17. A translation product of 67 kDa corresponding to the wild-type *APC* allele was observed in all gastric tumours. A shorter product of 44 kDa was observed in the tumour of case GACA17, which corresponded in size to the truncated protein expected to be produced as a result of the 4 bp deletion at codon 1462-1465, that was previously detected in this tumour by SSCP and heteroduplex analysis. Thus GACA17 could serve as a positive control. However truncated proteins were not detected in the other gastric carcinomas.

3.2.1 (c) Restriction digest analysis for mutation detection

Some relatively common translation-terminating mutations in exons 6, 8, and 14 of *APC* alter the recognition site of restriction enzymes as described by Ando *et al.*, (1993). The specific mutation detected and the restriction enzyme used have been described in section 2.2.8. However none of the mutations were detected in the 26 gastric carcinomas.

3.2.2. Loss of heterozygosity at the *APC* and *MCC* loci

Loss of heterozygosity (LOH) was investigated in 26 gastric carcinoma cases at four intragenic polymorphic loci of the *APC* gene: the *Rsa* I RFLP in exon 11, the exon 15I and exon 15J polymorphisms detected by SSCP, and the *Ssp* I RFLP in the 3' untranslated region. The *Msp* I RFLP within exon 15N of *APC* was not analysed in the gastric carcinomas as the colorectal carcinoma data had indicated that this locus was in almost complete linkage disequilibrium with the polymorphisms in exons 15I and 15J (section 3.1.2). The *MCC* (mutated in colorectal cancer) tumour suppressor gene lies in close proximity to *APC* at chromosome 5q21-22. The two genes are transcribed in opposite directions and their 3' ends are separated by approximately 150 kb (Kinzler *et al.*, 1991b). LOH was investigated at two intragenic polymorphic loci of the *MCC* gene, located in exon 10 and in the 3' untranslated region. In addition the *D5S346* microsatellite repeat marker was analysed for LOH. This marker was also analysed for replication error (RER) in the gastric carcinomas. The RER results will be described in a section 3.2.6. The LOH results will be described

in the present section as *D5S346* is located between the *APC* and *MCC* genes, lying only 30-70 kb from *APC*.

Table 3.11 summarizes the results of LOH analysis at the *APC* and *MCC* intragenic polymorphic loci and at *D5S346*. Of 26 gastric carcinoma cases 12 were constitutionally heterozygous for at least one locus within the *APC* gene and were therefore informative for LOH analysis. LOH was observed in 1 of 12 (8%) informative cases. Detection of LOH in this case (GACA23) at the *Rsa* I polymorphic site in *APC* exon 11 is shown in figure 3.36. LOH was assessed visually and defined as a change in the allele:allele ratio in tumour relative to corresponding normal DNA. Case GACA23 was informative at all four *APC* intragenic polymorphic investigated and as table 3.11 shows, LOH was detected in the tumour at each locus.

Twenty of the 26 gastric carcinoma cases were constitutionally heterozygous, and therefore informative, for at least one intragenic polymorphism of the *MCC* gene. LOH was observed in 2 of 20 (10%) informative carcinomas. These were tumours from cases GACA15 and GACA26. Detection of LOH in GACA15 at the polymorphic locus in the 3' untranslated region of *MCC* is shown in figure 3.37. This polymorphism is the result of a single base substitution of C for T in sequence encoding the 3' untranslated region of *MCC* and was originally described as a *Mae* III polymorphic site and was detected by PCR-RFLP analysis (Curtis *et al.*, 1994). Detection of LOH at the exon 10 polymorphic locus in GACA26 is illustrated in figure 3.38. Both cases were constitutionally heterozygous for just one *MCC* intragenic polymorphism.

There are 9 diffuse type gastric carcinomas and 17 intestinal type gastric carcinomas. Analysis of the *APC* intragenic polymorphisms according to histological type found that only 2 diffuse type carcinoma cases were constitutionally heterozygous and thus informative but LOH was detected in neither (table 3.11). Ten of 17 intestinal type carcinomas were informative for at least one locus, LOH was observed in one case (10%). At *MCC*, LOH was detected in 2 of 12 (16.7%) informative intestinal type gastric tumours but in none of 8 informative diffuse type tumours.

At *D5S346*, 20 of the 26 gastric carcinoma cases were constitutionally heterozygous and therefore informative. LOH was observed in 2 of 20 (10%) informative tumours as shown in figure 3.39. According to histological type, although 8 of the 9 diffuse type tumours were informative at *D5S346*, none exhibited

Table 3.11. Results of LOH analysis at intragenic polymorphic loci of the *APC* and *MCC* genes and at the *D5S346* microsatellite repeat locus in twenty-six gastric carcinoma cases.

Case	Histological type[a]	*APC* exon 11	*APC* exon 15I	*APC* exon 15J	*APC* 3' UTR	*D5S346*	*MCC* 3' UTR	*MCC* exon 10
GACA1	D	hom	hom	hom	hom	HET	HET	HET
GACA2	D	hom	hom	hom	hom	hom	hom	HET
GACA3	I	HET	hom	hom	HET	HET	hom	hom
GACA4	I	HET	HET	HET	HET	HET	HET	HET
GACA5	D	hom	hom	hom	hom	HET	hom	hom
GACA6	D	HET	HET	HET	hom	HET	HET	hom
GACA7	D	hom	hom	hom	hom	HET	HET	hom
GACA8	I	hom	hom	hom	hom	HET	hom	hom
GACA9	D	hom	hom	hom	hom	HET	HET	HET
GACA10	I	HET	hom	hom	HET	hom	hom	hom
GACA11	I	hom	hom	hom	hom	HET	HET	HET
GACA12	I	HET	HET	HET	HET	hom	HET	HET
GACA13	I	hom	hom	hom	hom	HET	HET	hom
GACA14	I	hom	hom	hom	hom	hom	hom	HET
GACA15	I	hom	hom	hom	hom	**LOH**	**LOH**	hom
GACA16	I	hom	hom	hom	hom	hom	HET	HET
GACA17	I	hom	HET	HET	hom	HET	hom	HET
GACA18	D	HET	HET	HET	hom	HET	hom	HET
GACA19	D	hom	hom	hom	hom	HET	HET	hom
GACA20	I	HET	hom	hom	hom	HET	HET	hom
GACA21	I	HET	HET	HET	HET	hom	HET	hom
GACA22	I	HET	HET	HET	HET	HET	hom	hom
GACA23	I	**LOH**	**LOH**	**LOH**	**LOH**	**LOH**	hom	hom
GACA24	D	hom	hom	hom	hom	HET	HET	HET
GACA25	I	hom	hom	hom	hom	HET	HET	hom
GACA26	I	HET	HET	HET	HET	HET	hom	**LOH**

[a] I, intestinal type; D, diffuse type. HET, constitutional heterozygosity retained in tumour; **LOH**, loss of heterozygosity observed in tumour; hom, constitutionally homozygous therefore uninformative.

Figure 3.36.

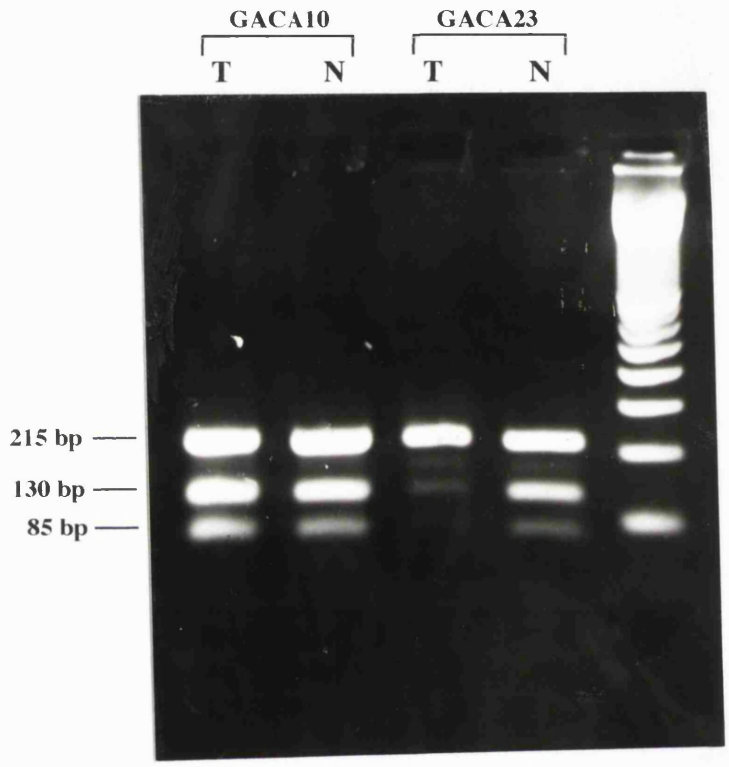


Figure 3.36. LOH analysis at the *Rsa* I polymorphic site in exon 11 of the *APC* gene. *APC* exon 11 was amplified in tumour and corresponding normal DNA from gastric carcinoma cases. The PCR products were digested with *Rsa* I and then electrophoresed in 3% agarose gels. The tumour DNA lane (T) from each case shown above is followed by its corresponding normal DNA lane (N). Lane 5 contains a 100 bp ladder. Cases GACA10 and GACA23 were constitutionally heterozygous and thus informative, as normal DNA from both cases showed three bands: the 215 bp band represents the allele lacking the *Rsa* I restriction site and the 130 bp and 85 bp bands represent the allele containing the site. Loss of the allele cleaved by *Rsa* I was seen in tumour DNA relative to normal DNA from case GACA23. A significant reduction in intensity of the allele rather than complete loss was seen. The residual signal could reflect contamination of the tumour specimen with normal stromal cells, the presence of more than one neoplastic clone, amplification of a mutant allele without loss of the wild-type allele, or preferential duplication of one chromosome. The tumour from case GACA10 did not exhibit LOH.

Figure 3.37.

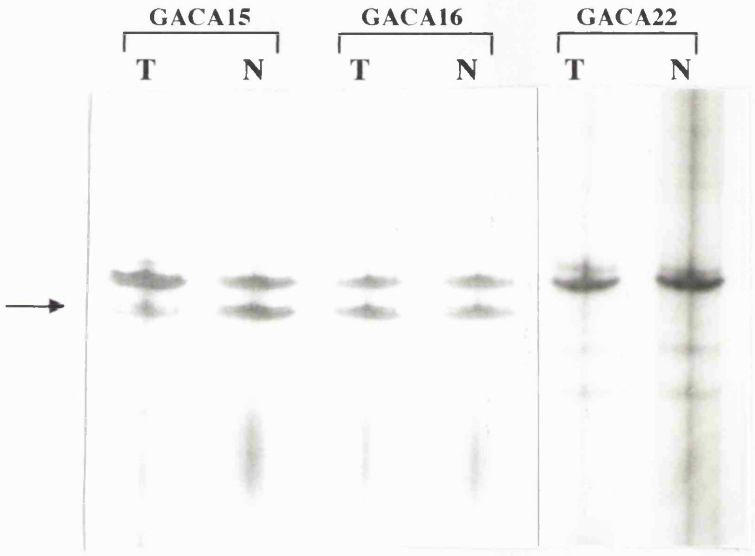


Figure 3.37. Detection of LOH at the polymorphic site in the 3' untranslated region of the *MCC* gene in case GACA15. T, tumour DNA; N, corresponding normal DNA. A polymorphism in sequence encoding the 3' untranslated region of *MCC*, the result of a single base substitution of C for T, was detected by PCR-SSCP. Analysis of normal DNA from cases GACA15 and GACA16 demonstrated that both were constitutionally heterozygous and thus informative, the two bands representing the two alleles. Case GACA22 was constitutionally homozygous and therefore uninformative. A reduction in intensity of one band in tumour DNA from case GACA15 relative to corresponding normal DNA indicated LOH (see arrow). LOH was not observed in the tumour from case GACA16.

Figure 3.38.

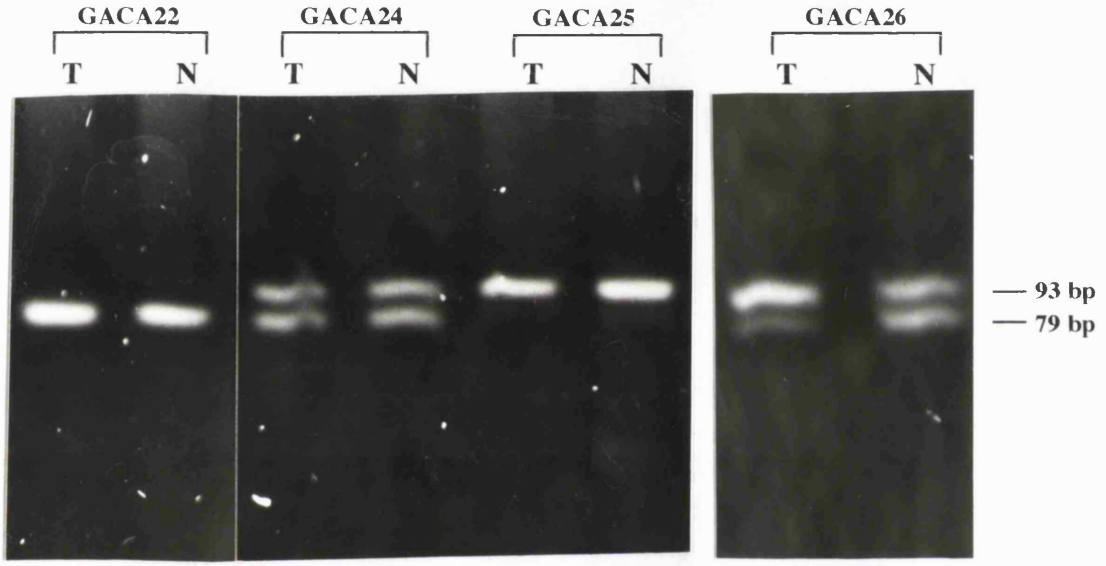


Figure 3.38. PCR-LOH analysis of the *MCC* gene at the exon 10 polymorphic site in gastric carcinoma cases. A 14 bp variable insertion region in *MCC* exon 10 was amplified in tumour (T) and normal (N) DNA from gastric carcinoma cases, generating PCR products of either 79 bp or 93 bp long depending upon the absence or presence of the insertion. PCR products were electrophoresed directly in 3% agarose gels. Case GACA22 was constitutionally homozygous for the 79 bp allele, cases GACA24 and GACA26 were constitutionally heterozygous, and case GACA25 was homozygous for the 93 bp allele. A reduction in intensity of the 79 bp allele was seen in tumour DNA from case GACA26 leading to a change in the allele:allele ratio relative to that in matching normal DNA and thus indicating LOH. The tumour from case GACA24 did not show LOH.

Figure 3.39.

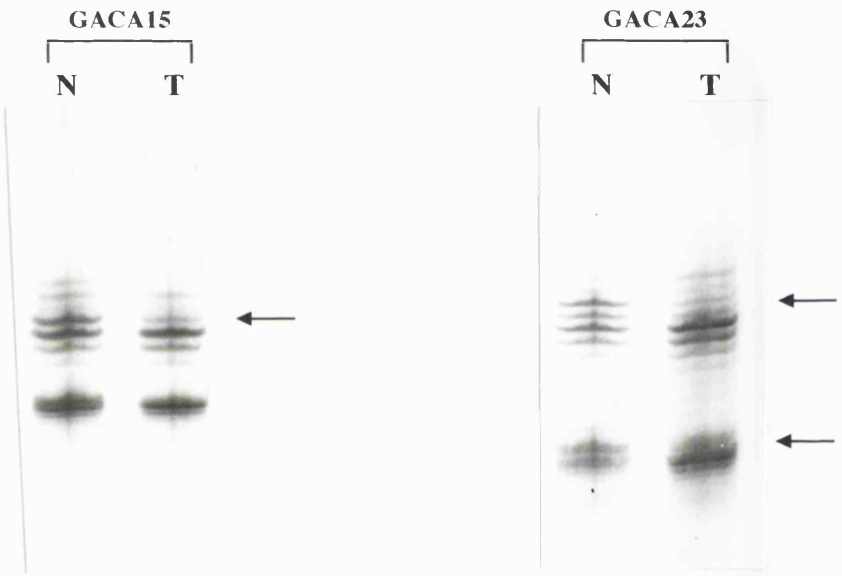


Figure 3.39. Analysis of LOH at the *D5S346* microsatellite repeat locus in cases GACA15 and GACA23. T and N denote DNA derived from tumour and corresponding normal tissues. The *D5S346* marker was analysed by the SSCP method. Analysis of normal DNA from gastric carcinoma cases GACA15 and GACA23 shows that both are constitutionally heterozygous and therefore informative. In both GACA15 and GACA23, a significant reduction in intensity of bands representing one allele is observed in tumour relative to corresponding normal DNA indicating LOH, as highlighted by the arrows. Both tumours are intestinal type gastric carcinomas.

LOH. The LOH was detected in 2 of 12 (16.7%) informative intestinal type carcinomas. One of these tumours, from case GACA23, had also shown LOH at the *APC* intragenic polymorphic loci. Case GACA23 was constitutionally homozygous for the *MCC* intragenic polymorphisms (table 3.11). On the other hand, the other tumour exhibiting *D5S346* LOH, from case GACA15, had shown LOH at an *MCC* intragenic polymorphic locus but was constitutionally homozygous for the *APC* intragenic polymorphisms. As previously described, two gastric carcinomas had shown LOH of the *MCC* gene, the second tumour being from case GACA26. Table 3.11 shows that case GACA26 was informative at the *APC* intragenic polymorphic loci and at *D5S346* too but did not exhibit LOH at any of these loci.

3.2.3. Loss of heterozygosity at the *DCC* locus

The *DCC* (deleted in colorectal cancer) tumour suppressor gene has been localized to chromosome 18q21 (Fearon *et al.*, 1990). LOH of *DCC* was investigated by PCR-based detection of two *Msp* I polymorphic sites, termed M2 and M3, at the *D18S8* locus which lies within the *DCC* gene (Parry *et al.*, 1991). The polymorphic *Msp* I sites have previously been detected by Southern analysis with the probes 15-65 and OLVIIE10 which mark the *D18S8* locus (Marlhens *et al.*, 1987; Fearon *et al.*, 1990). *D18S8* is located in the intron between *DCC* exons 5 and 6 (Cho *et al.*, 1994). The results of the LOH analysis are presented in table 3.12. Twenty of twenty-six gastric carcinoma cases were constitutionally heterozygous for at least one polymorphic locus and therefore informative for LOH analysis. LOH was detected in 1 of 20 (5%) informative cases. The LOH was observed in the tumour from case GACA17, an intestinal type gastric carcinoma, at the M2 polymorphic site as depicted in figure 3.40. Analysis of the results according to histological type finds that 14 of 17 intestinal type gastric carcinomas were heterozygous for at least one locus and were thus informative. LOH was observed in 1 of 14 (7.1%) informative intestinal type tumours. Six of the nine diffuse type gastric carcinomas were informative.

3.2.4. Mutational analysis of the *p53* gene

The *p53* tumour suppressor gene is located at chromosome 17p13 (Harlow *et al.*, 1985; McBride *et al.*, 1986; Miller *et al.*, 1986). The entire coding region of the gene (exons 2-11) was screened for mutations by SSCP and heteroduplex analysis in DNA derived from 26 gastric carcinoma and corresponding normal tissues. The first exon of *p53* is a non-coding exon. The exact nature of any variant found was then determined by sequencing. Mutations of the *p53* gene were identified in 8 of 26

Table 3.12. Results of LOH analysis at two *Msp* I polymorphic sites within the *DCC* gene in twenty-six gastric carcinoma cases.

Case	Histological type[a]	Polymorphic site	
		M2	M3
GACA1	D	hom	HET
GACA2	D	HET	HET
GACA3	I	HET	hom
GACA4	I	HET	HET
GACA5	D	HET	hom
GACA6	D	hom	hom
GACA7	D	hom	hom
GACA8	I	HET	hom
GACA9	D	HET	HET
GACA10	I	HET	HET
GACA11	I	HET	HET
GACA12	I	hom	hom
GACA13	I	HET	HET
GACA14	I	hom	hom
GACA15	I	HET	HET
GACA16	I	hom	hom
GACA17	I	**LOH**	hom
GACA18	D	HET	hom
GACA19	D	hom	hom
GACA20	I	hom	HET
GACA21	I	hom	HET
GACA22	I	HET	HET
GACA23	I	HET	HET
GACA24	D	hom	HET
GACA25	I	HET	hom
GACA26	I	HET	HET

[a] I, intestinal type; D, diffuse type. HET, constitutional heterozygosity retained in tumour; **LOH**, loss of heterozygosity observed in tumour; hom, constitutionally homozygous therefore uninformative.

Figure 3.40.

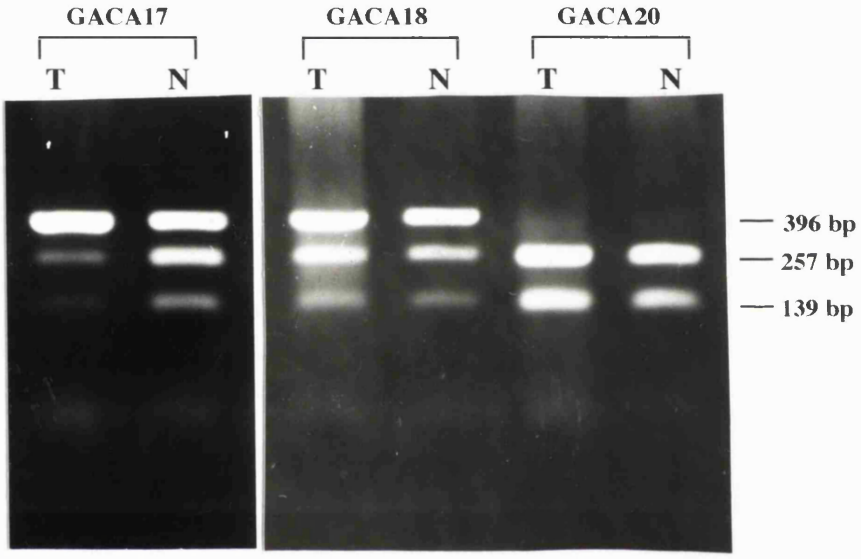


Figure 3.40. PCR-RFLP analysis at the M2 polymorphic site within the *DCC* gene for the detection of LOH. PCR amplification of tumour and matching normal DNA from gastric carcinoma cases was performed using primers flanking the *Msp* I polymorphic site to generate fragments of 396 bp. The PCR products were treated with *Msp* I and then electrophoresed in 2% agarose gels. If the restriction site was present, cleavage with *Msp* I yielded two bands of 257bp and 139bp. For each case shown above, the tumour DNA lane (T) is followed by its corresponding normal DNA lane (N). Cases GACA17 and GACA18 were constitutionally heterozygous and therefore informative, as normal DNA from both cases contained both uncleaved and cleaved fragments. Case GACA20 was constitutionally homozygous for the cleaved allele and thus uninformative. A reduction in intensity of the cleaved allele (257 bp and 139 bp bands) in tumour DNA from case GACA17 indicated LOH. The tumour from case GACA18 did not demonstrate LOH.

(30.8%) gastric carcinomas (table 3.13). Each mutation was detected in tumour but not corresponding normal tissue thus all were of somatic origin.

Table 3.13. Somatic mutations of the *p53* gene detected in gastric carcinomas.

Case	Histological type *	Exon	Codon	Nucleotide change	Nature of mutation
GACA15	I	4	103	TAC → TA<u>A</u>	Tyr → Stop
		4	104	CAG → <u>T</u>AG	Gln → Stop
GACA14	I	5	161	ATG<u>GC</u>C → ATGC	2 bp deletion
GACA4	I	6	193	CAT → C<u>G</u>T	His → Arg
GACA20	I	6	195	ATC → A<u>C</u>C	Ile → Thr
GACA17	I	7	237	ATG → AT<u>A</u>	Met → Ile
GACA26	I	8	266	GGA → <u>A</u>GA	Gly → Arg
GACA2	D	8	282	CGG → <u>T</u>GG	Arg → Trp
GACA23	I	10	342	CGA → <u>T</u>GA	Arg → Stop

* I, intestinal type carcinoma; D, diffuse type carcinoma.

SSCP analysis of exon 4 of *p53* in matching tumour and normal DNA from case GACA15 is shown in figure 3.41a. A novel single-stranded DNA conformer was detected only in the tumour DNA. Furthermore it was observed that in the tumour DNA, the band that corresponded to the band of slowest mobility in the normal DNA, was weaker. On sequencing of the exon 4 amplicon in tumour DNA from case GACA15 (figure 3.41b), single base substitutions of two adjacent C residues were identified. These belonged to adjacent codons, one C residue being the third nucleotide of codon 103, and the other being the first nucleotide of codon 104. At codon 103, substitution of the C residue with an A residue causes a nonsense mutation from tyrosine (TAC) to a stop codon (TAA). The base change from C to T at codon 104 also results in a nonsense mutation, of glutamine (CAG) to a stop codon (TAG). Thus both substitutions were predicted to result in truncation of the *p53* gene product. As figure 3.41b shows, sequencing of the exon 4 amplicon in corresponding normal DNA confirmed the mutations to be somatic events. In the tumour DNA sequence the two wild-type C residues were hardly visible. This indicated evidence that both of the single base substitutions were located on the same *p53* allele and that loss of the wild-type *p53* allele had occurred.

Figure 3.42 illustrates the detection of somatic variants in cases GACA14 and GACA17. Heteroduplex analysis only of the exon 5 amplicon in tumour and normal DNA from case GACA14 is shown in figure 3.42a. No variation in the single strand DNA band pattern was seen in the tumour but a heteroduplex DNA band was observed. Sequence analysis of the exon 5 amplicon in the tumour DNA identified a 2 bp deletion at codon 161. This sequence change resulted in frameshift and the creation of a new termination codon at 18 codons downstream. Thus the mutation was predicted to result in truncation of the *p53* gene product. Figure 3.42b shows SSCP analysis of the exon 7 amplicon in DNA derived from tumour and normal tissues of case GACA17. In the tumour from this case a single-stranded DNA variant but no double-stranded DNA variation was observed. The single-stranded DNA band migrating immediately below this variant band was weaker than the corresponding band in the normal DNA. Sequencing revealed the variant to be the result of a single base substitution of adenine for guanine at the third nucleotide of codon 237, causing a predicted amino acid change from methionine (ATG) to isoleucine (ATA).

SSCP analysis of the *p53* exon 6 amplicon is illustrated in figure 3.43. Unique single-stranded DNA conformers were detected in tumour DNA from cases GACA4 and GACA20 which were not present in corresponding normal DNA. The variant pattern was different in the two tumours. In the tumour DNA from case GACA4, the single-stranded DNA band migrating immediately above this variant band was weaker than the corresponding band in the normal DNA. Sequence analysis of the exon 6 amplicon identified single base pair substitutions in tumour DNA from both cases (figure 3.44a and b). A substitution of a G for an A residue in the tumour DNA from GACA4 results in amino acid substitution of arginine for histidine at codon 193. The wild-type sequence was very faint in the tumour DNA, suggesting evidence for allele loss. At two codons downstream, a single base change from T to C was detected in the tumour DNA from case GACA20, resulting in an amino acid change from isoleucine to threonine.

Exons 8 and 9 of the *p53* gene were amplified together generating a product of 330 bp. SSCP analysis of the exons 8-9 amplicon in tumour DNA from two gastric carcinoma cases, GACA2 and GACA26, showed single-stranded DNA mobility shifts, as depicted in figures 3.45a and 3.46a. The variant observed in tumour GACA2 was characterized by sequence analysis as a C to T transition at the first nucleotide of codon 282 (figure 3.45b). This codon is located in exon 8 of *p53*. The sequence change leads to an amino acid change from arginine (CGG) to tryptophan (TGG). As figure 3.46b illustrates, a single base substitution was also identified in the tumour DNA from case GACA26, of A for G at codon 266, resulting in a

missense mutation from glycine (GGA) to arginine (AGA). Codon 266 also lies in exon 8.

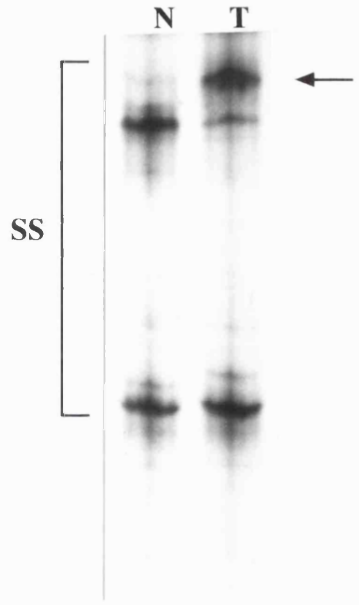
Finally, a variant was detected in the exon 10 amplicon of *p53* by SSCP analysis as shown in figure 3.47a. Two unique single-stranded DNA conformers were observed in tumour but not matching normal DNA from case GACA23. Sequence analysis demonstrated this variation to be the result of a single base substitution of T for C at the first nucleotide of codon 342, causing a nonsense mutation from arginine (CGA) to a stop codon (TGA) (figure 3.47b). The wild-type sequence (C) was faint in the tumour DNA, indicating evidence of allele loss.

Polymorphisms in the exons 2-3 amplicon and the exon 4 amplicon were detected by SSCP analysis but these will be discussed in section 3.25. A total of 9 somatic mutations of the *p53* gene were identified in 26 gastric carcinomas (table 3.13). In one case (GACA15) two somatic mutations were detected in exon 4 by direct sequencing. Eight of the nine mutations (89%) were point mutations, of which five were missense mutations resulting in amino acid substitution and three were nonsense mutations resulting in truncation of the gene product. One mutation was a 2 bp deletion that resulted in frameshift and created a new stop codon at downstream. Thus 4 of the 9 somatic mutations were predicted to result in truncation of the *p53* gene product.

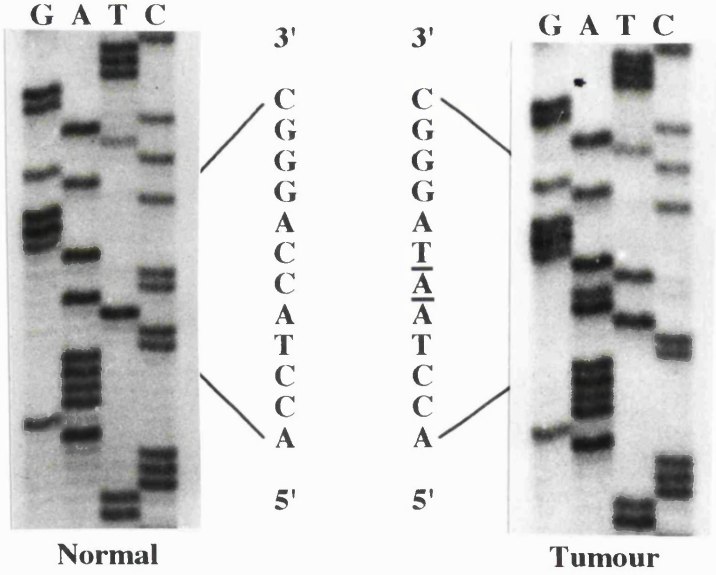
The majority of the mutations (6 of 9, 67%) were found in exons 5 to 8. This is a region of the gene where most *p53* mutations have been reported in the literature and is where most of the highly conserved amino acids are located (Hollstein *et al.*, 1991). Seven of the eight point mutations (87.5%) were transitions. One point mutation was a transversion of G:C to T:A. Of the seven base transitions, five (71.4%) were G:C to A:T changes, of which two occurred at CpG dinucleotide pairs. The other two transitions were A:T to G:C changes.

As shown in table 3.13, seven of the gastric carcinomas with *p53* mutation were of the intestinal type while only one carcinoma was of the diffuse type. However the difference between the two histological types with regard to the frequency of *p53* gene mutation was not found to be statistically significant ($p = 0.19$; Fisher's exact test).

Figure 3.41.



(a)



(b)

Figure 3.41. Detection of somatic mutation in exon 4 amplicon of *p53* in case GACA15. (**a**) SSCP analysis. T, tumour tissue; N, normal tissue. SS indicates single-stranded DNA bands. A unique single-stranded DNA conformer was observed in the tumour DNA as indicated by the arrow. In addition, in the tumour DNA the band corresponding to the band of slowest mobility in the normal DNA, was weaker. (**b**) Sequence analysis of exon 4 amplicon in tumour and normal DNA from case GACA15 (sense strand). Two single base substitutions of adjacent C residues were detected in the tumour DNA sequence, the mutant bases are underlined. The substitution of C with A results in a nonsense mutation from Tyr (TAC) to stop (TA<u>A</u>) at codon 103. The substitution of T for C also causes a nonsense mutation from Glu (CAG) to stop (<u>T</u>AG) at codon 104. Loss of the wild-type residues is observed in the tumour DNA sequence.

Figure 3.42.

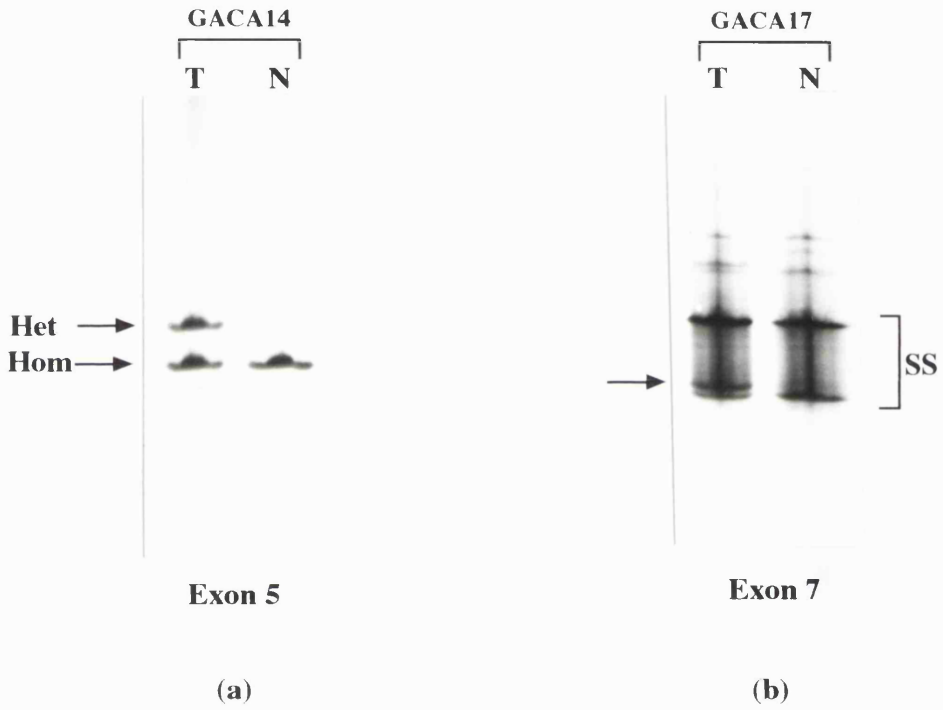


Figure 3.42. Detection of somatic variants in *p53* exon 5 and exon 7 in cases GACA14 and GACA17 respectively. (**a**) Heteroduplex analysis of *p53* exon 5 amplicon. T and N denote DNA samples isolated from tumour and matching normal tissues of case GACA14. A heteroduplex band (Het) of retarded mobility compared with homoduplex DNA (Hom) was observed in tumour DNA but not normal DNA. (**b**) SSCP analysis of *p53* exon 7 amplicon. T and N indicate DNA derived from tumour and normal tissues of case GACA17. A novel single-stranded (SS) DNA band was detected in the tumour DNA only as indicated by the arrow. The single-stranded DNA band of faster mobility than this variant band was weaker than the corresponding band in the normal DNA.

Figure 3.43.

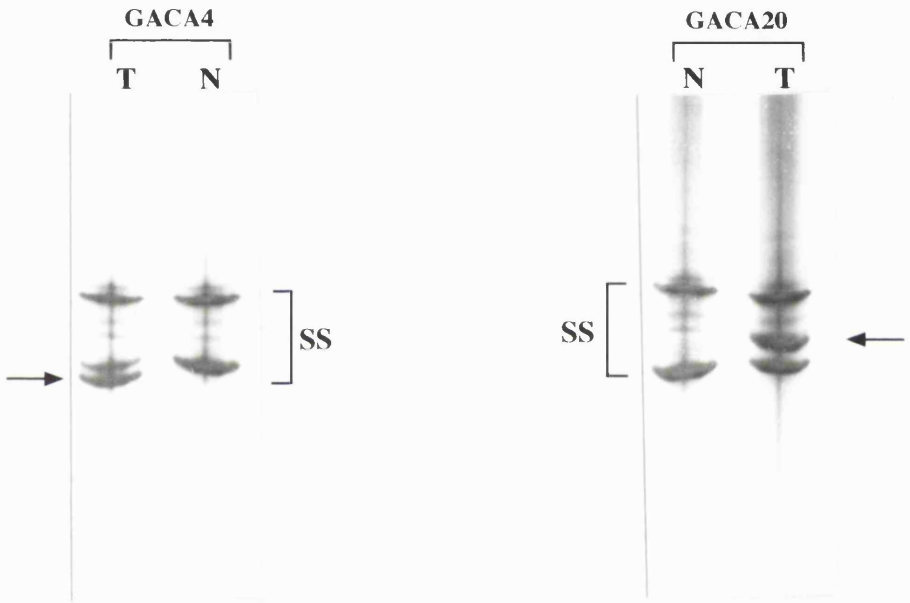
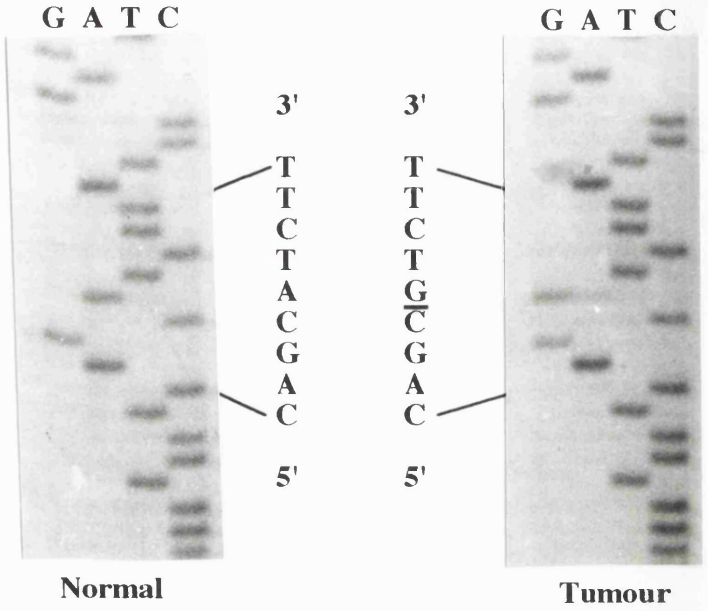
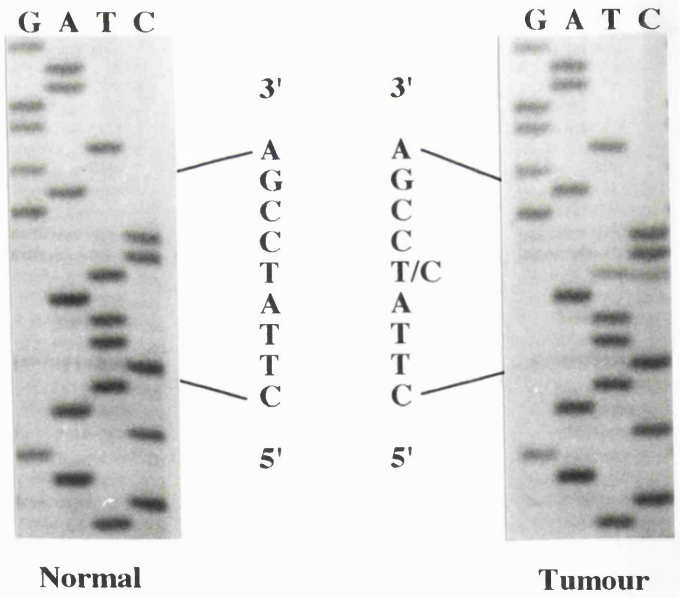


Figure 3.43. SSCP analysis of the exon 6 amplicon of the *p53* gene in tumour and normal DNA from cases GACA4 and GACA20. T, tumour tissue; N, corresponding normal tissue. Variant single-stranded (SS) DNA conformers of differing mobilities were seen in the tumours from both cases, as indicated by the arrows. In the tumour from case GACA4, the single-stranded DNA band directly above the variant band was weaker than the corresponding band in the normal DNA.

Figure 3.44.



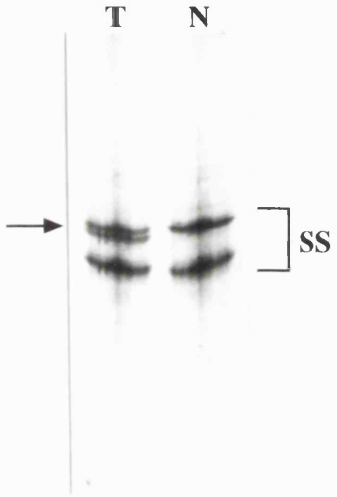
(a)



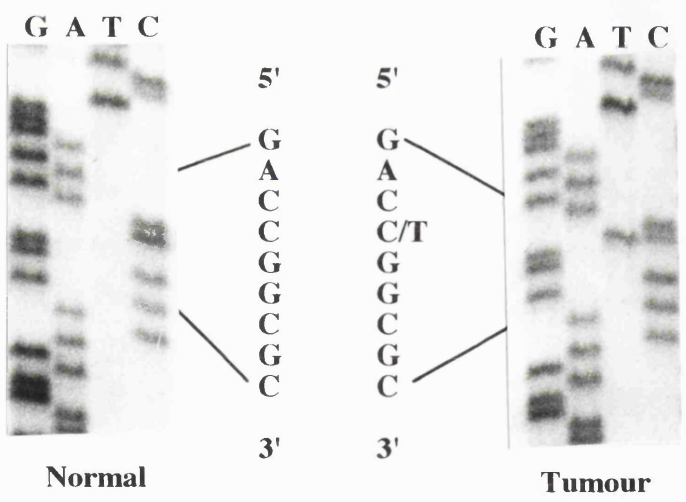
(b)

Figure 3.44. Sequence analysis of the exon 6 amplicon of *p53* in tumour and normal DNA from cases GACA4 and GACA20. (**a**) Case GACA4. An A to G transition was identified in the tumour DNA sequence resulting in an amino acid substitution of arginine (CGT) for histidine (CAT) at codon 193. The wild-type sequence (A) is very faint in the tumour DNA. (**b**) Case GACA20. Again a single base change was observed in the tumour DNA sequence, of T to C, causing an amino acid change from isoleucine (ATC) to threonine (ACC) at codon 195. This alteration was not present in the corresponding normal DNA sequence. Sequencing of the sense strand is shown.

Figure 3.45.



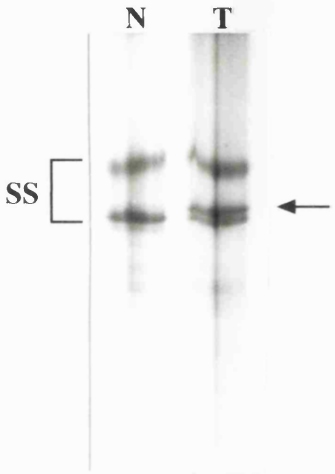
(a)



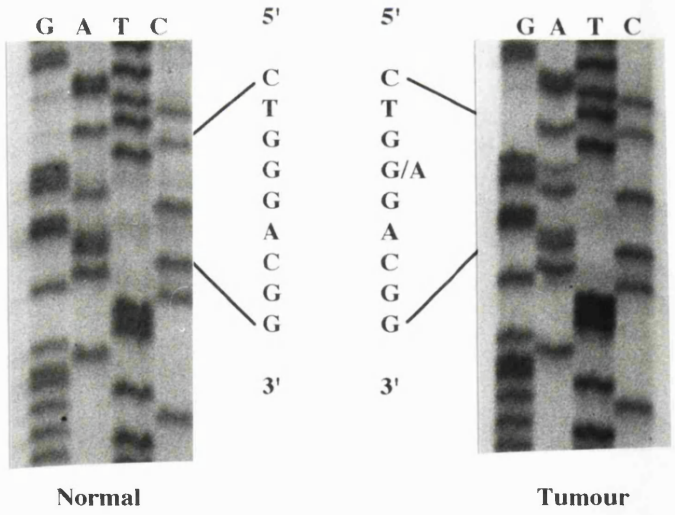
(b)

Figure 3.45. Somatic mutation identified in exon 8 of the *p53* gene in case GACA2. (**a**) SSCP analysis of exons 8-9 amplicon. Exons 8 and 9 of *p53* were amplified together using one set of primers. T and N denote DNA samples from matching tumour and normal tissues. A single-stranded (SS) DNA variant was observed in tumour DNA only from case GACA2. (**b**) Sequence analysis of exons 8-9 amplicon (antisense strand). A single base change of C to T was identified in the tumour DNA sequence at codon 282, resulting in an amino acid change from arginine (CGG) to tryptophan (TGG). Codon 282 is located in exon 8 of *p53*.

Figure 3.46.



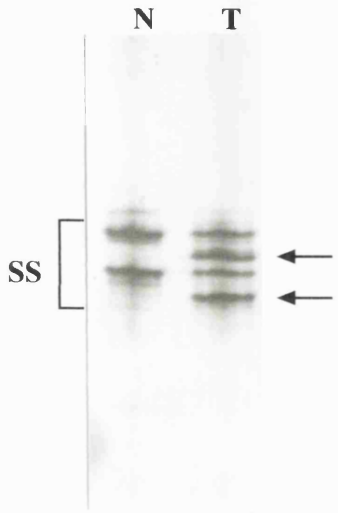
(a)



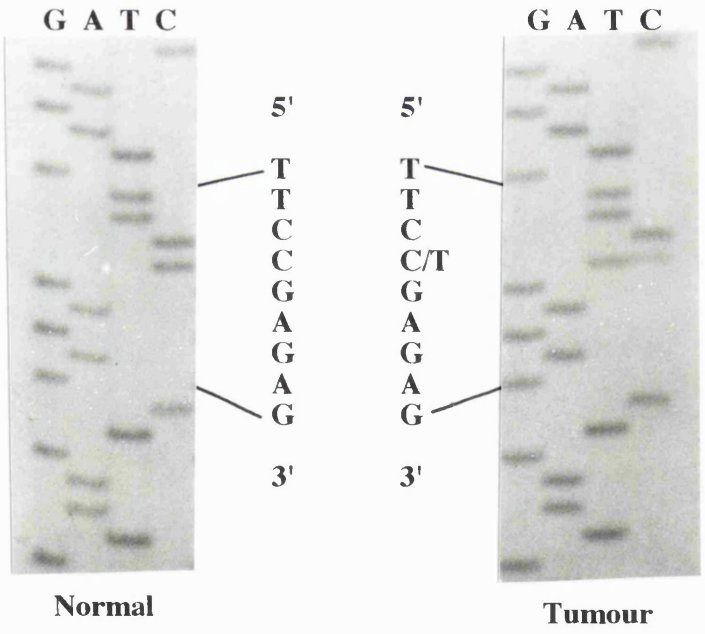
(b)

Figure 3.46. Detection of somatic mutation in exon 8 of *p53* in case GACA26. **(a)** SSCP analysis of exons 8-9 amplicon. T=tumour DNA from case GACA26; N=matching normal DNA. In addition to the wild-type single strand (SS) band pattern, a unique single-stranded DNA conformer was detected in the tumour (indicated by arrow). **(b)** Sequence analysis of exons 8-9 amplicon in tumour and normal DNA from case GACA26 (antisense strand). The variant detected above is the result of a single base subtitution of A for G which causes a missense mutation from Gly (GGA) to Arg (AGA) at codon 266. This codon is in *p53* exon 8. The mutation was not detected in the matching normal DNA sequence.

Figure 3.47.



(a)



(b)

Figure 3.47. Somatic mutation of the *p53* gene observed in case GACA23 in exon 10. (**a**) SSCP analysis of exon 10 amplicon. T, tumour DNA; N, corresponding normal DNA. SS denotes the single strands of DNA. As indicated by the arrows, novel single-stranded DNA conformers were detected in the tumour DNA. (**b**) Sequence analysis of exon 10 amplicon (antisense strand). A single base substitution of T for C was observed in the tumour DNA sequence which results in a change from arginine (CGA) to stop (TGA) at codon 342. The wild-type sequence ('C') is faint in the tumour DNA.

3.2.5. Loss of heterozygosity at the *p53* locus

LOH in the *p53* gene was investigated at polymorphic loci in intron 2 and exon 4 and at a dinucleotide repeat polymorphism at the *p53* locus (*p53CA*).

A polymorphic *Acc* II recognition site (CGCG) occurs at codon 72 in exon 4 of *p53* (de la Calle-Martin *et al.*, 1991). The polymorphism is due to a single base substitution of C for G at the second nucleotide of codon 72, resulting in an amino acid substitution of proline (CCC) for arginine (CGC) (Matlashewski *et al.*, 1987). In the present study the codon 72 polymorphism was detected by SSCP analysis of the exon 4 amplicon as shown in figure 3.48a. Different single-stranded DNA band patterns were seen in each case shown. Sequence analysis of the exon 4 amplicon in normal DNAs from the three cases indicated that the bands denoted 1 and 2 in the SSCP gel represented the polymorphic alleles. Case GACA3 was constitutionally heterozygous, case GACA7 was constitutionally homozygous for the allele with proline at codon 72, and case GACA24 was constitutionally homozygous for arginine. Thus the two homozygotes and heterozygote could be clearly distinguished by SSCP analysis.

Exon 2, intron 2, and exon 3 of *p53* were amplified together using one set of primers. A polymorphism at position 11827 in intron 2 of the gene, the result of a substitution of a C residue for a G residue, was detected by SSCP analysis as illustrated in figure 3.49a. Sequence analysis of the exons 2-3 amplicon in normal DNAs from the three cases shown indicated that the single-stranded DNA band denoted 1 represented the allele with G at position 11827 while the band denoted 2 represented the allele with C at this position. Case GACA6 was constitutionally heterozygous, case GACA10 was constitutionally homozygous for the 'G' allele, and case GACA7 was constitutionally homozygous for the 'C' allele. A more weaker staining single-stranded DNA band was always seen above the two main allele bands. This polymorphism in intron 2 was recently described by Oliva *et al.* (1995).

Analysis of normal DNA from the 26 gastric carcinoma cases demonstrated the polymorphisms in intron 2 and at codon 72 to be in linkage disequilibrium. As table 3.14 shows, the frequency of heterozygosity (informativity) at both loci was 27% (7 of 26 cases). LOH was detected in 3 of 7 (43%) informative tumours. At the *p53CA* locus, 22 of 26 (84.6%) gastric carcinoma cases were heterozygous and therefore informative. LOH was observed in 7 of 22 (31.8%) informative cases. A total of 24 gastric carcinoma cases (92.3%) were informative for at least one of the

three polymorphic loci (table 3.14). LOH of the *p53* gene was observed in 9 of 24 (37.5%) informative tumours.

Detection of LOH at the codon 72 polymorphic locus in exon 4 is shown in figure 3.48b. LOH was observed as a most significant reduction in intensity of an allele in the tumour from GACA4. Less extensive allele loss was detected in the tumour from case GACA14 and in the tumour from case GACA20. The residual signal observed could be due to contamination of the tumour sample with normal stromal cells, the presence of more than one neoplastic clone, amplification of the mutant allele without concomitant loss of the wild-type allele, or preferential duplication of one chromosome. Detection of LOH at the polymorphic locus in intron 2, in the tumour DNA from case GACA4, is depicted in figure 3.49b. LOH analysis at the *p53CA* repeat locus is illustrated in figure 3.50. LOH was observed as complete loss or reduction in intensity of bands in tumour DNA relative to corresponding normal DNA. The change in the allele:allele ratio in the tumour DNA from case GACA2 was not as extensive as that observed in tumour DNA from the other cases.

Sixteen of the 17 intestinal type gastric carcinomas were heterozygous for at least one polymorphic locus and therefore informative (table 3.14). LOH was detected in 8 tumours (50%). Eight of the 9 diffuse type gastric tumours were informative, and LOH was detected in one tumour (12.5%). The difference between the two histological types with regard to the frequency of LOH was not statistically significant ($p = 0.1$; Fisher's exact test).

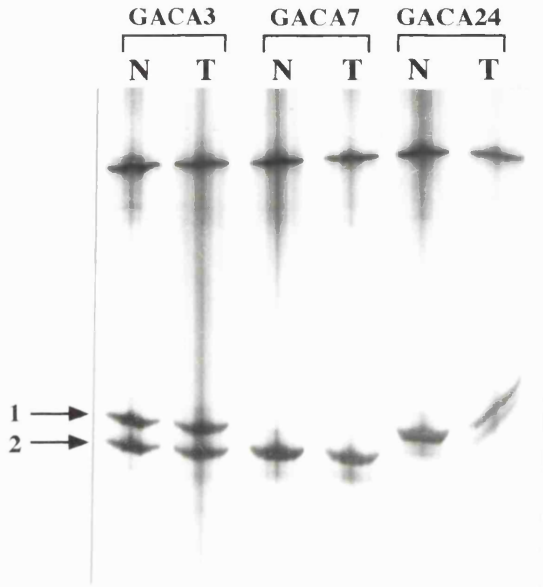
Somatic mutations of the *p53* gene were detected in 8 gastric carcinomas by SSCP and heteroduplex analysis as previously described in table 3.13. The LOH analysis demonstrated loss of the wild-type *p53* allele in 7 of these tumours (GACA2, GACA4, GACA14, GACA15, GACA17, GACA20, and GACA23). SSCP and/or sequencing analysis of tumour DNAs from cases GACA4, GACA15, GACA17, and GACA23 had also indicated that loss of the wild-type allele had occurred (section 3.2.4). The LOH analysis had demonstrated that loss in tumour DNA from cases GACA2, GACA14, and GACA20 was not extensive. Thus allele loss was not detected in these tumours during SSCP and sequencing analysis. The other gastric tumour that harboured a *p53* gene mutation, from case GACA26, showed retention of constitutional heterozygosity at the polymorphic loci. Two further gastric carcinomas, from cases GACA12 and GACA16, did not demonstrate *p53* gene mutations but did exhibit LOH (table 3.14).

Table 3.14. Results of LOH analysis at polymorphic loci of the *p53* gene in twenty-six gastric carcinoma cases.

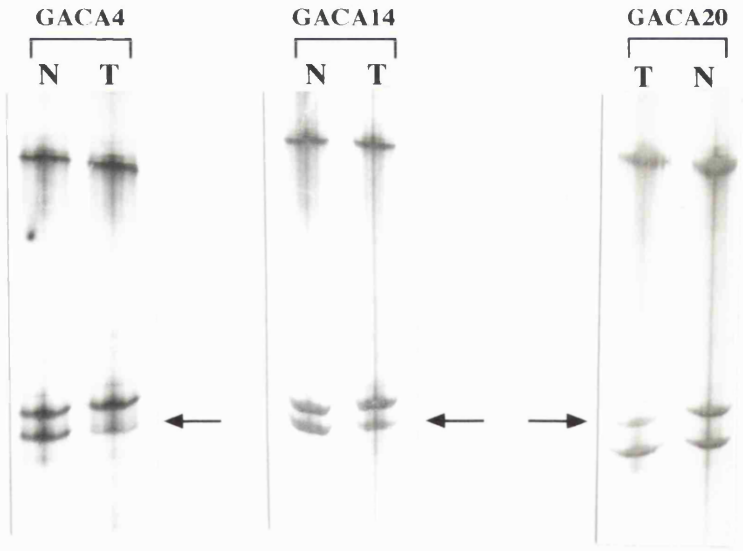
Case	Histological type[a]	Polymorphic site		
		Intron 2	Codon 72	p53CA
GACA1	D	hom	hom	HET
GACA2	D	hom	hom	**LOH**
GACA3	I	HET	HET	HET
GACA4	I	**LOH**	**LOH**	**LOH**
GACA5	D	hom	hom	HET
GACA6	D	HET	HET	HET
GACA7	D	hom	hom	HET
GACA8	I	hom	hom	hom
GACA9	D	HET	HET	HET
GACA10	I	hom	hom	HET
GACA11	I	hom	hom	HET
GACA12	I	hom	hom	**LOH**
GACA13	I	hom	hom	HET
GACA14	I	**LOH**	**LOH**	hom
GACA15	I	hom	hom	**LOH**
GACA16	I	hom	hom	**LOH**
GACA17	I	hom	hom	**LOH**
GACA18	D	hom	hom	HET
GACA19	D	hom	hom	hom
GACA20	I	**LOH**	**LOH**	hom
GACA21	I	hom	hom	HET
GACA22	I	HET	HET	HET
GACA23	I	hom	hom	**LOH**
GACA24	D	hom	hom	HET
GACA25	I	hom	hom	HET
GACA26	I	hom	hom	HET

[a] I, intestinal type; D, diffuse type. HET, constitutional heterozygosity retained in tumour; **LOH**, loss of heterozygosity observed in tumour; hom, constitutionally homozygous therefore uninformative.

Figure 3.48.



(a)



(b)

Figure 3.48. Analysis of polymorphism at codon 72 in exon 4 of the *p53* gene by PCR-SSCP. (**a**) SSCP analysis of the exon 4 amplicon in tumour (T) and normal (N) DNA from 3 gastric carcinoma cases. The band denoted 1 represents arginine at codon 72 and the band denoted 2 represents proline. Case GACA3 is constitutionally heterozygous and cases GACA7 and GACA24 are constitutionally homozygous for alternative alleles. (**b**) Three further gastric carcinoma cases are constitutionally heterozygous for the codon 72 polymorphism. In each case, LOH is observed as a reduction in intensity of one band in tumour DNA relative to corresponding normal DNA as indicated by the arrows.

Figure 3.49.

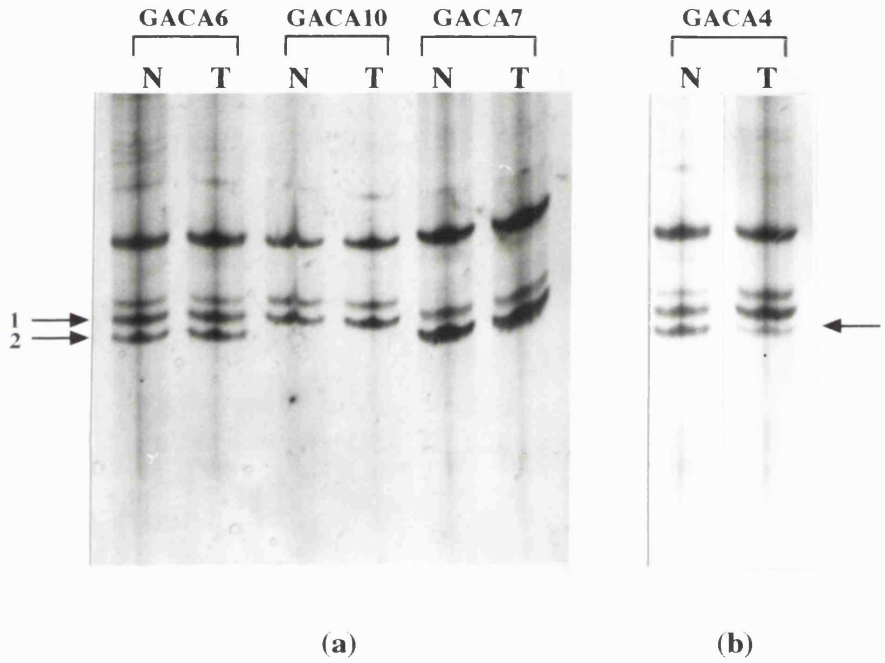


Figure 3.49. Detection of polymorphism at position 11827 in intron 2 of the *p53* gene by SSCP analysis. **(a)** SSCP analysis of the exons 2-3 amplicon in tumour (T) and normal (N) DNA from 3 gastric carcinoma cases. The band denoted 1 represents the allele with a G residue at position 11827 and the band denoted 2 represents the allele with a C residue at this position. Case GACA6 is constitutionally heterozygous and cases GACA10 and GACA7 are constitutionally homozygous for alternative alleles. Weaker single-stranded DNA bands are seen above the main allele bands. **(b)** Analysis of the exons 2-3 amplicon in normal DNA from case GACA4 indicates constitutional heterozygosity for the intron 2 polymorphism. In the corresponding tumour DNA, a significant reduction in intensity of the band representing the allele with C at position 11827 indicated LOH, as highlighted by the arrow.

Figure 3.50.

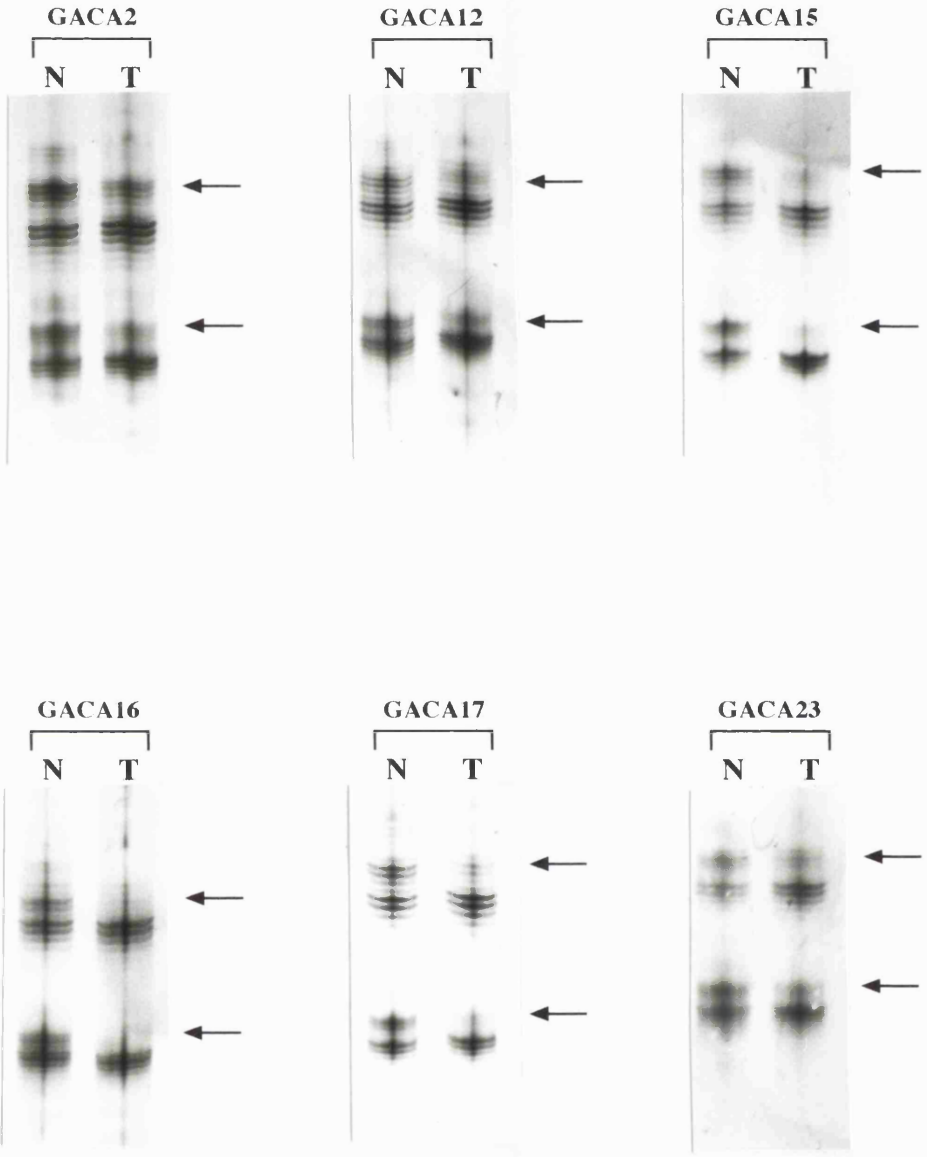


Figure 3.50. Analysis of LOH at the *p53CA* locus in gastric carcinomas. T and N denote DNA derived from corresponding tumour and normal tissues. The *p53CA* marker was analysed by PCR-SSCP. Each case shown is constitutionally heterozygous, each allele is represented by two bands in the normal DNA. Complete loss or a reduction in intensity of bands representing one allele in the corresponding tumour DNAs indicated LOH, as highlighted by the arrows.

3.2.6. Alterations at microsatellite repeat loci

The 26 gastric carcinoma and normal DNA pairs were investigated for replication error and loss of heterozygosity at twelve microsatellite repeat loci by PCR-SSCP analysis, as described for the colorectal carcinoma cases.

3.2.6 (a) Replication error

Replication error (RER) at one or more microsatellite repeat loci was detected in 3 of 26 (11.5%) gastric carcinomas. The results are summarized in table 3.15. RER was observed at 6 of 12 (50%) loci analysed in the tumour DNA from case GACA22, as illustrated in figure 3.51. RER is detected as new fragments in the tumour DNA not present in normal DNA from the same case. The tumour from case GACA22 was of the intestinal histological type. Figure 3.52 shows the detection of RER in tumour DNAs from cases GACA11 and GACA18. In both tumours, RER was observed at 1 of 12 (8.3%) loci analysed. Tumour GACA11 is an intestinal type gastric carcinoma and tumour GACA18 is a diffuse type carcinoma.

Table 3.15. Detection of RER in gastric carcinomas.

	Tumour		
Locus	**GACA11**	**GACA18**	**GACA22**
D2S119	-	-	-
D2S391	-	-	RER
D2S123	-	-	RER
D4S175	-	-	RER
D5S346	-	-	-
D9S156	-	-	-
D14S50	-	RER	-
D16S266	-	-	RER
p53CA	-	-	-
D18S61	-	-	RER
D19S49	RER	-	RER
D22S351	-	-	-

Information on family history of cancer was available for all three RER-positive (RER+) gastric carcinomas (table 2.1). However none of these cases showed a family history. Five of the RER-negative (RER-) gastric carcinoma cases had a family history but this was not strong in each case and none showed a history indicative of HNPCC.

Figure 3.51.

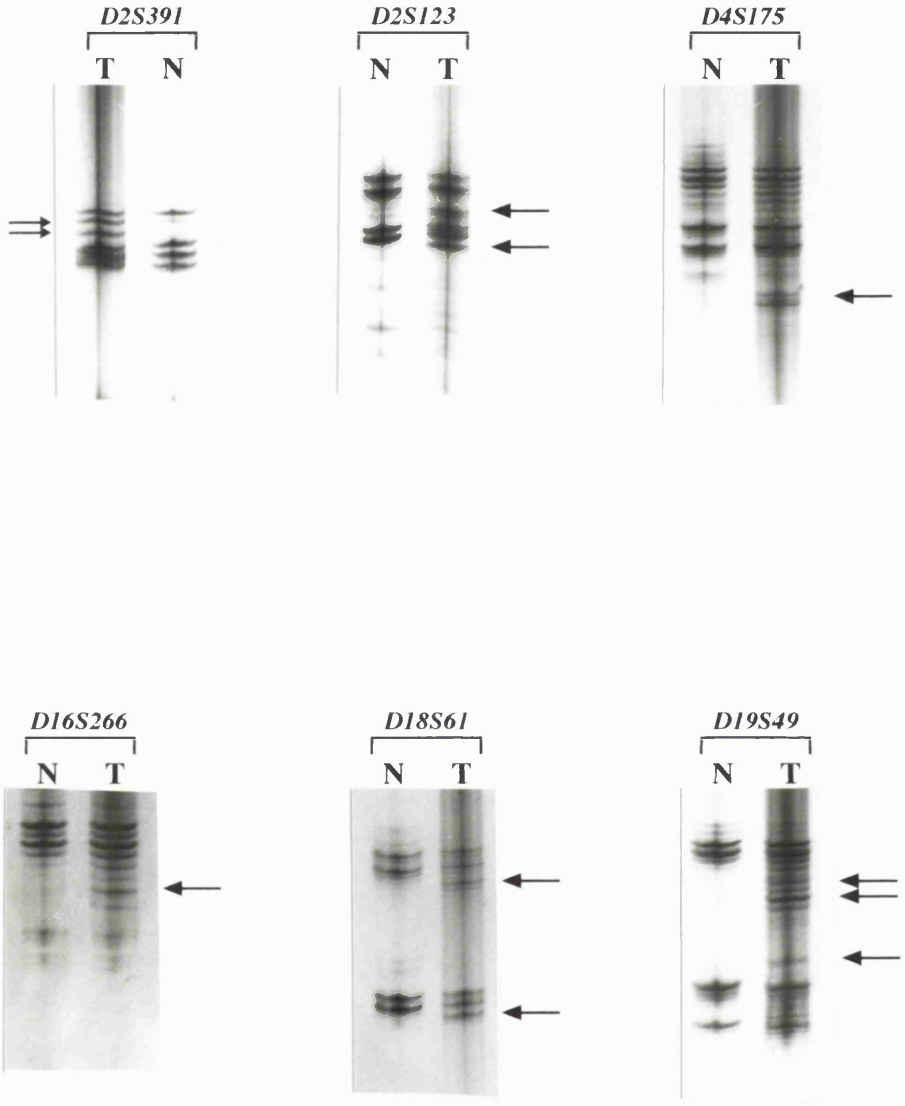


Figure 3.51. Analysis of RER at microsatellite repeat loci in case GACA22. T and N denote DNA samples derived from matching tumour and normal tissues of case GACA22. RER was observed in the tumour DNA at each locus shown as new bands not present in the matching normal DNA, as indicated by the arrows.

Figure 3.52.

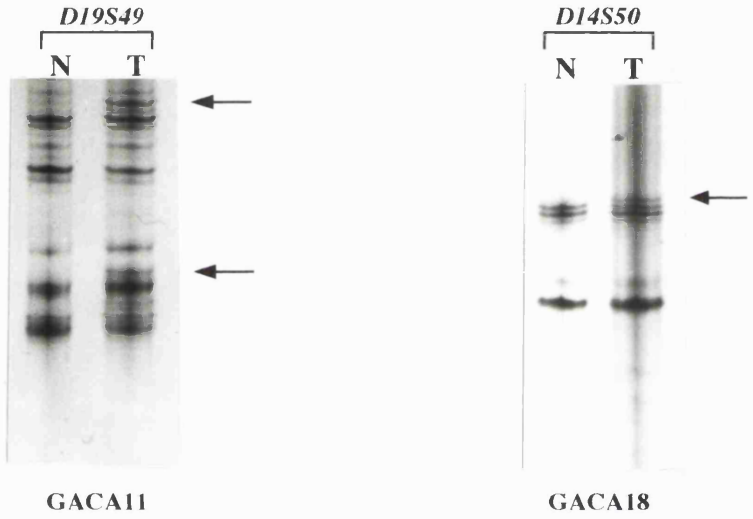


Figure 3.52. Detection of RER in gastric carcinoma cases GACA11 and GACA18. T, tumour DNA; N, corresponding normal DNA. RER was detected in the tumour DNA from case GACA11 at the *D19S49* microsatellite repeat locus and in the tumour DNA from case GACA18 at the *D14S50* locus. RER was observed as additional bands in tumour DNA compared with corresponding normal DNA, as indicated by the arrows.

3.2.6 (b) Loss of heterozygosity

The results of LOH analysis at 12 microsatellite repeat loci in 26 gastric carcinoma cases is summarized in table 3.16. Table 3.17 shows the percentage informativity and percentage LOH observed at each locus. Cases exhibiting RER at a given locus were excluded from LOH analysis at that locus and deemed uninformative. The percentage informativity ranged from 54 to 84%. The percentage LOH ranged from 0 to 31.8%.

Table 3.17. Frequency of informativity and LOH observed at microsatellite repeat loci in 26 gastric carcinoma cases.

Locus	No. of informative cases / Total No. of cases (%)	Cases with LOH (%)
D2S119	19 / 26 (73.1)	1 (5.3)
D2S391	18 / 26 (69.2)	2 (11.1)
D2S123	20 / 26 (76.9)	2 (10)
D4S175	21 / 26 (80.8)	4 (19.1)
D5S346	20 / 26 (76.9)	2 (10)
D9S156	18 / 26 (69.2)	0
D14S50	19 / 26 (73.1)	3 (15.8)
D16S266	14 / 26 (54)	0
p53CA	22 / 26 (84.6)	7 (31.8)
D18S61	19 / 26 (73.1)	1 (5.3)
D19S49	17 / 26 (65.4)	0
D22S351	20 / 26 (76.9)	6 (30)

The three microsatellite repeat markers from chromosome 2p16 are closely linked to the *hMSH2* gene. The order of the loci is telomere-*D2S119-D2S391-hMSH2-D2S123*-centromere. The genetic distance between *D2S119* and *D2S391* is 6.4 cM. *D2S391* is located approximately 2 cM distal to *hMSH2* and *D2S123* is approximately 6 cM proximal to *hMSH2* (Leach *et al.*, 1993). All 26 gastric carcinoma cases were constitutionally heterozygous and therefore informative for at least one of the chromosome 2p microsatellite repeat markers. LOH was observed in 2 of 26 (7.7%) informative cases. These were cases GACA4 and GACA15. LOH was observed at all 3 loci in the tumour from case GACA4, while case GACA15 was constitutionally homozygous for *D2S119* but exhibited LOH at *D2S391* and *D2S123*. Detection of LOH in each tumour is illustrated in figure 3.53.

Table 3.16. Results of analysis of twelve microsatellite repeat loci in twenty-six gastric carcinoma cases.

Case	Histological type[a]	*D2S119*	*D2S391*	*D2S123*	*D4S175*	*D5S346*	*D9S156*	*D14S50*	*D16S266*	*p53CA*	*D18S61*	*D19S49*	*D22S351*
GACA1	D	HET	hom	HET	HET	HET	HET	HET	hom	HET	hom	HET	HET
GACA2	D	HET	HET	HET	HET	hom	HET	HET	hom	**LOH**	hom	HET	**LOH**
GACA3	I	HET	HET	HET	HET	HET	HET	HET	HET	HET	HET	hom	hom
GACA4	I	**LOH**	**LOH**	**LOH**	**LOH**	HET	hom	HET	hom	**LOH**	HET	HET	**LOH**
GACA5	D	HET	HET	hom	HET	HET	hom	HET	HET	HET	HET	HET	hom
GACA6	D	hom	HET	HET	HET	HET	HET	HET	HET	HET	HET	hom	HET
GACA7	D	HET	HET	hom	HET	HET	HET	HET	HET	HET	HET	hom	hom
GACA8	I	HET	HET	HET	hom	HET	hom	HET	HET	hom	HET	HET	HET
GACA9	D	HET	hom	HET	HET	HET	hom	hom	hom	HET	HET	HET	HET
GACA10	I	hom	HET	HET	HET	hom	HET	HET	HET	HET	HET	HET	HET
GACA11	I	hom	hom	HET	**LOH**	HET	HET	hom	hom	HET	HET	**RER**	HET
GACA12	I	hom	HET	HET	**LOH**	hom	HET	**LOH**	HET	**LOH**	HET	HET	hom
GACA13	I	HET	hom	hom	HET	HET	hom	HET	hom	HET	HET	hom	HET
GACA14	I	HET	hom	HET	HET	hom	HET	**LOH**	hom	hom	HET	HET	**LOH**
GACA15	I	hom	**LOH**	**LOH**	HET	**LOH**	HET	**LOH**	HET	**LOH**	hom	HET	**LOH**
GACA16	I	HET	HET	HET	HET	hom	HET	hom	HET	**LOH**	**LOH**	hom	hom
GACA17	I	hom	HET	HET	**LOH**	HET	HET	HET	hom	**LOH**	hom	HET	**LOH**
GACA18	D	HET	hom	HET	hom	HET	hom	**RER**	HET	HET	HET	HET	HET
GACA19	D	HET	HET	hom	HET	HET	HET	HET	HET	hom	HET	HET	HET
GACA20	I	HET	HET	HET	HET	HET	HET	hom	hom	hom	hom	HET	hom
GACA21	I	HET	HET	hom	HET	hom	HET	hom	hom	HET	HET	HET	HET
GACA22	I	HET	**RER**	**RER**	**RER**	HET	hom	HET	**RER**	HET	**RER**	**RER**	HET
GACA23	I	HET	hom	HET	HET	**LOH**	HET	HET	HET	**LOH**	hom	HET	**LOH**
GACA24	D	HET	HET	HET	hom	HET	HET	HET	HET	HET	HET	hom	HET
GACA25	I	hom	HET	HET	HET	HET	hom	HET	hom	HET	HET	HET	HET
GACA26	I	HET	HET	HET	hom	HET	HET	hom	HET	HET	HET	hom	HET

[a] I, intestinal type; D, diffuse type. HET, constitutional heterozygosity retained in tumour; **LOH**, loss of heterozygosity observed in tumour; hom, constitutionally homozygous therefore uninformative; **RER**, replication error.

LOH was more frequently observed at the *D4S175* locus on chromosome 4q, in 4 of 21 (19.1%) informative tumours, as shown in figure 3.54. At *D14S50* on chromosome 14q, 19 of 25 gastric carcinomas were heterozygous and therefore informative. As illustrated in figure 3.55 LOH was seen in 3 (15.8%) informative cases. Figure 3.55 also illustrates detection of LOH at the *D18S61* locus on chromosome 18q in the tumour from case GACA16. LOH was infrequently observed at this locus, in 1 of 19 (5.3%) informative cases. *D18S61* is located more than 30 cM distal to the *DCC* gene on chromosome 18q (Jen *et al.*, 1994c). The incidence of LOH at *D22S351* on chromosome 22q was higher, being observed in 6 of 20 (30%) informative tumours (figure 3.56). No LOH was detected at the *D9S156* locus on chromosome 9p, the *D16S266* locus on chromosome 16q, nor at *D19S49* on chromosome 19q.

LOH was most frequently detected at the *p53CA* and *D22S351* loci. Seven gastric carcinomas exhibited LOH at *p53CA*, and as described in section 3.2.5, a further two tumours (GACA14 and GACA20) showed LOH at polymorphic loci in intron 2 and exon 4 of the *p53* gene. Six of the nine tumours also exhibited LOH at *D22S351*. These were tumours from cases GACA2, GACA4, GACA14, GACA15, GACA17, and GACA23 (table 3.16). The other three tumours, from cases GACA12, GACA16, and GACA20, were constitutionally homozygous at *D22S351*. The association between LOH of the *p53* gene and LOH at *D22S351* was highly significant statistically ($p < 0.0001$, Fisher's exact test; table 3.18).

Table 3.18. Correlation between LOH of the *p53* gene and LOH at *D22S351* in gastric carcinomas.

	p53 LOH analysis	
	LOH	No LOH
D22S351 LOH	6	0
No *D22S351* LOH	0	12

$p < 0.0001$; Fisher's exact test.

Table 3.19 shows the results of the LOH analysis at microsatellite repeat loci stratified according to histological type. Only one of the nine diffuse type gastric carcinomas, from case GACA2, exhibited LOH, at the *p53CA* and *D22S351* loci. In the intestinal type carcinomas, LOH was most frequently observed at *p53CA* and *D22S351*, but was also frequent at *D4S175* and *D14S50*.

Figure 3.53.

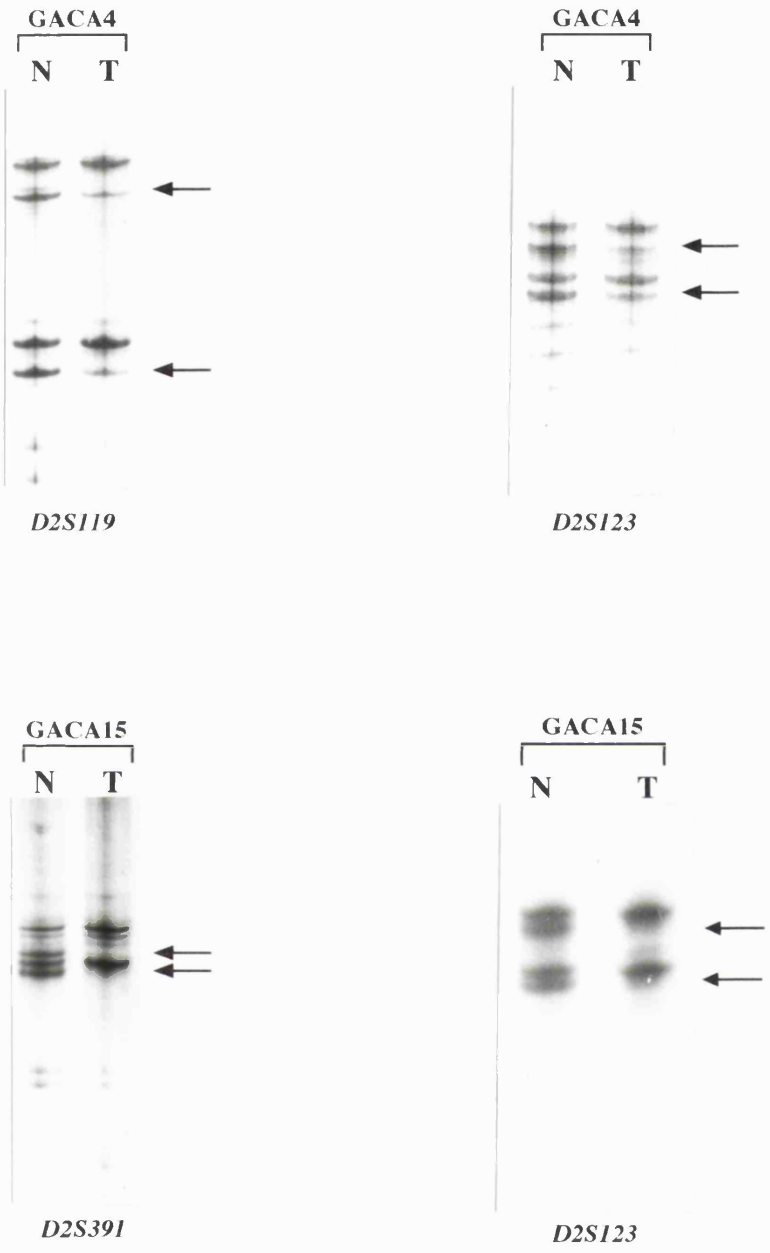


Figure 3.53. Detection of LOH at microsatellite repeat loci on chromosome 2p in gastric carcinoma cases GACA4 and GACA15. T and N denote DNA samples isolated from corresponding tumour and normal tissues. Both cases are constitutionally heterozygous at each locus shown, each allele being represented by two bands in the normal DNA. In the tumour DNA from case GACA4, a significant reduction in intensity of bands representing one allele at the *D2S119* and *D2S123* loci indicated LOH, as highlighted by the arrows. In the tumour DNA of case GACA15, complete loss of two bands representing one allele was observed at the *D2S391* and *D2S123* loci (see arrows). Both tumours were of the intestinal histological type.

Figure 3.54.

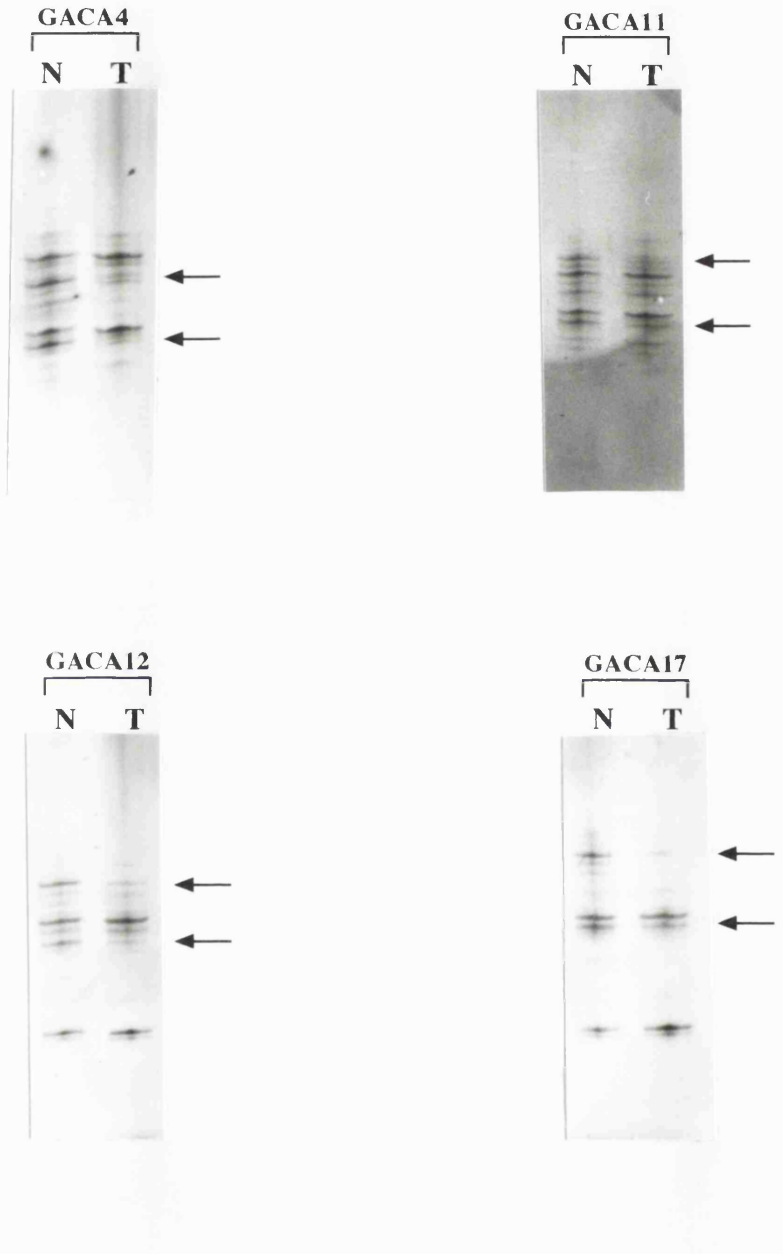


Figure 3.54. Analysis of LOH at the *D4S175* microsatellite repeat locus in gastric carcinomas. T, tumour DNA; N, corresponding normal DNA. All cases shown are constitutionally heterozygous, each allele being represented by two bands in the normal DNA. A significant reduction in intensity of two bands in tumour DNA relative to corresponding normal DNA indicates LOH, as highlighted by the arrows. All four tumours are intestinal type carcinomas.

Figure 3.55.

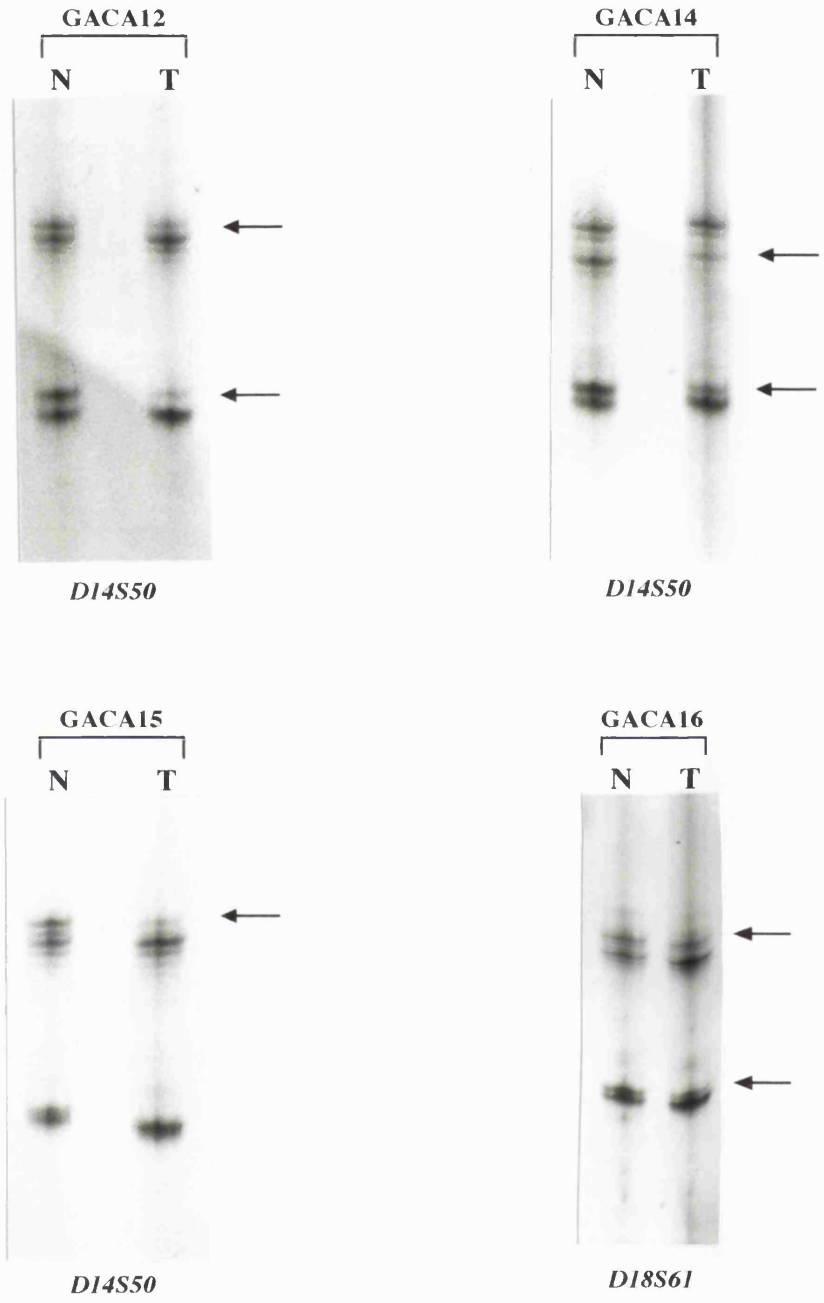


Figure 3.55. Detection of LOH at the *D14S50* and *D18S61* microsatellite repeat loci in gastric carcinomas. T=tumour DNA; N=matching normal DNA. Analysis of normal DNA from cases GACA12, GACA14, and GACA15 demonstrated constitutional heterozygosity at the *D14S50* locus, each allele being represented by two bands. In each case a reduction in intensity of bands representing one allele was observed in tumour DNA relative to matching normal DNA (highlighted by arrows), indicating LOH. All three tumours were of the intestinal histological type. Case GACA6 was constitutionally heterozygous at *D18S61*, each allele was represented by two bands in the normal DNA. A change in the allele:allele ratio was observed in tumour relative to normal DNA but extensive loss was not detected, as highlighted by the arrows. This tumour was also an intestinal type gastric carcinoma.

Figure 3.56.

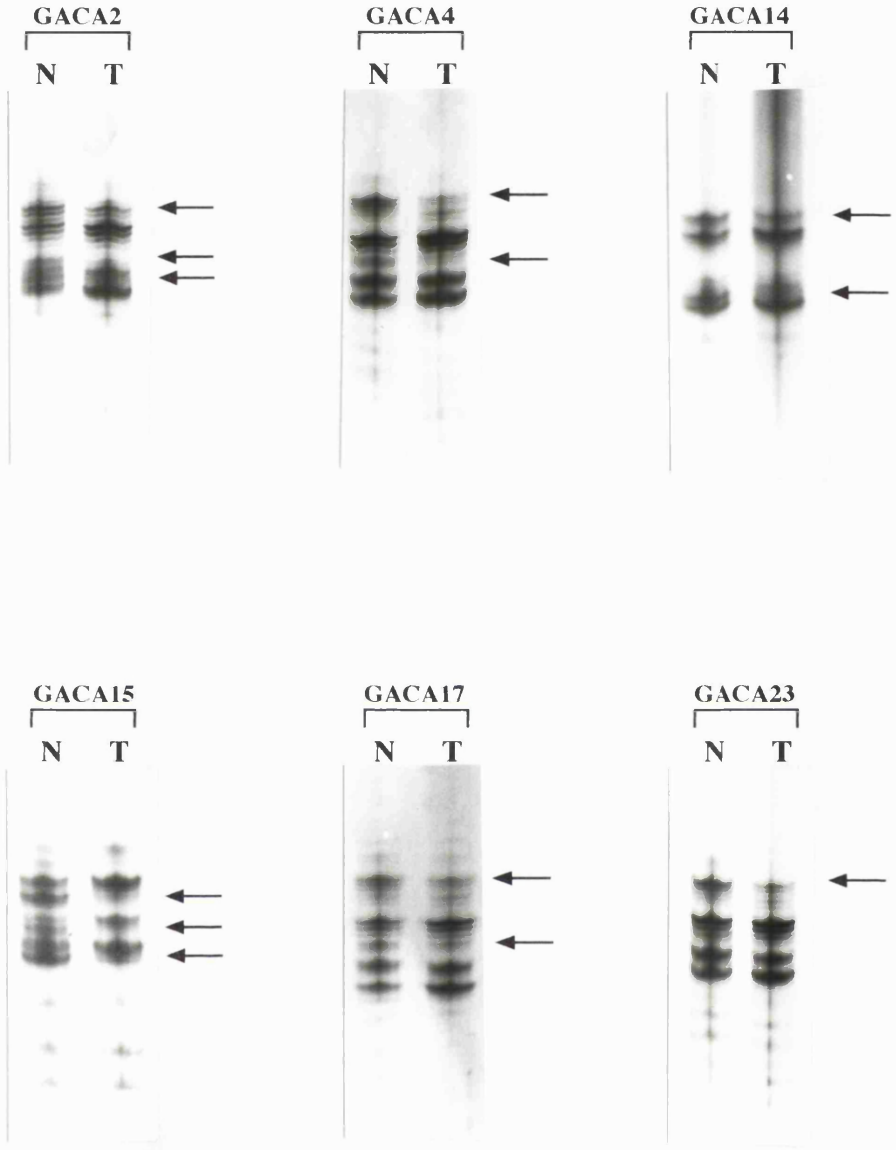


Figure 3.56. LOH analysis at the *D22S351* microsatellite repeat locus in gastric carcinomas. T, tumour DNA; N, corresponding normal DNA. Three bands are usually seen for each allele with the *D22S351* marker. Each case shown is constitutionally heterozygous. In the normal DNA from cases GACA2 and GACA15 each allele is represented by three bands. In the normal DNA from cases GACA4, GACA17, and GACA23 two bands have migrated to the same position, thus three bands are not seen for each allele. In the normal DNA from case GACA14 two bands are seen for each allele. In each case, complete loss or a reduction in intensity of bands representing one allele in tumour DNA relative to normal DNA indicated LOH, as highlighted by the arrows. The tumour from case GACA2 is of the diffuse histological type, the other tumours are intestinal type carcinomas.

Table 3.19. Detection of LOH at 12 microsatellite repeat loci in 17 intestinal type gastric carcinomas and 9 diffuse type gastric carcinomas.

	Cases with LOH / informative cases (%)	
Locus	**Intestinal type**	**Diffuse type**
D2S119	1 / 11 (9.1)	0 / 8
D2S391	2 / 12 (16.7)	0 / 6
D2S123	2 / 14 (14.3)	0 / 6
D4S175	4 / 14 (28.6)	0 / 7
D5S346	2 / 12 (16.7)	0 / 8
D9S156	0 / 12	0 / 6
D14S50	3 / 12 (25)	0 / 7
D16S266	0 / 8	0 / 6
p53CA	6 / 14 (43)	1 / 8 (12.5)
D18S61	1 / 12 (8.3)	0 / 7
D19S49	0 / 11	0 / 6
D22S351	5 / 13 (38.5)	1 / 7 (14.3)

3.2.7. Mutational analysis of the *hMSH2* gene

Exons 12-14 of the *hMSH2* gene were investigated for mutations in DNA derived from the 26 gastric carcinoma and matching normal tissues by SSCP and heteroduplex analysis. As previously described in section 3.1.4, a polymorphism was detected in the exon 13 amplicon of *hMSH2*, the result of a T to C transition in the intronic splice acceptor site, at a position six base pairs upstream of the beginning of exon 13. The polymorphism was detected as variation in the double-stranded DNA pattern. Six of the twenty-six gastric carcinoma cases were found to be constitutionally heterozygous for the T to C transition. Loss of heterozygosity was detected in the tumour DNA from one case, GACA15, as illustrated in figure 3.57a. Loss of the allele with T at the -6 position of the splice acceptor site was observed. LOH was also detected in this tumour at the *D2S391* and *D2S123* microsatellite repeat loci (figure 3.53) which are located close to the *hMSH2* gene on chromosome 2p. *D2S391* is distal to *hMSH2* and *D2S123* is proximal.

A different double-stranded DNA variant was detected in the exon 13 amplicon in both tumour and normal tissue DNA from case GACA6 (figure 3.57b). Two well separated heteroduplex DNA bands and a variant homoduplex DNA band were observed. Sequence analysis revealed the variation observed in case GACA6 to

Figure 3.57.

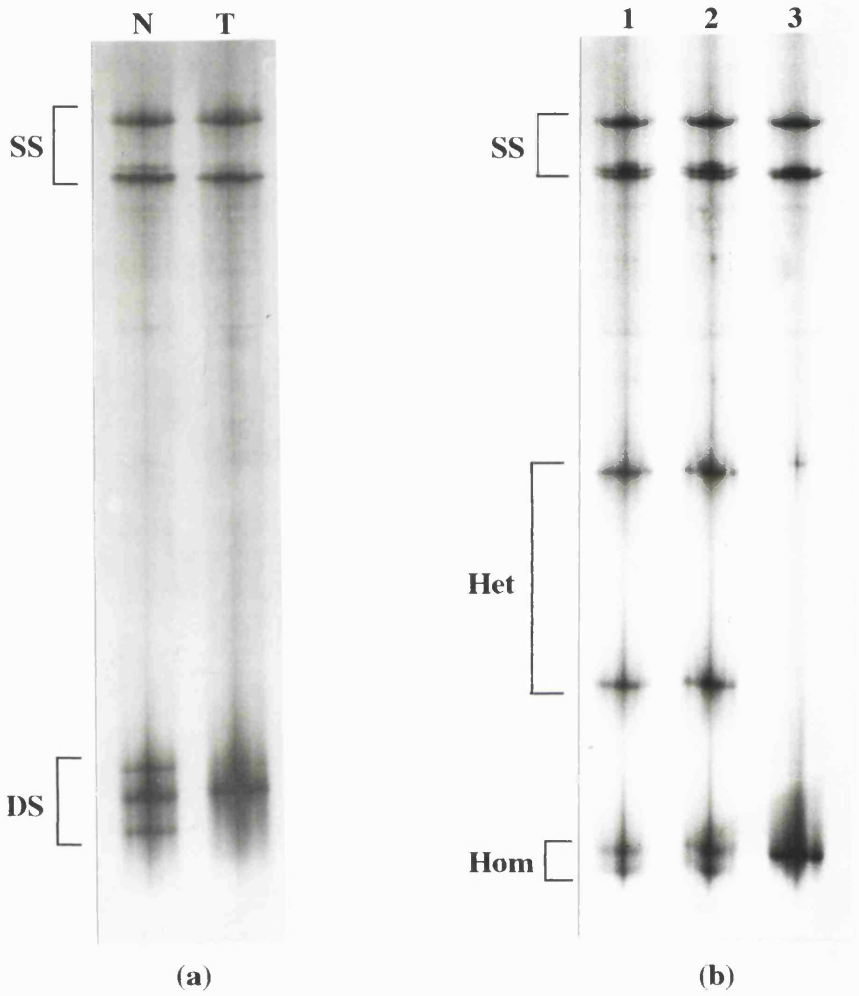


Figure 3.57. SSCP and heteroduplex analysis of the *hMSH2* exon 13 amplicon in tumour and normal DNA from cases GACA6 and GACA15. **(a)** Case GACA15. T and N denote DNA samples derived from tumour and normal tissues respectively. SS=single-stranded DNA; DS=double-stranded DNA. Case GACA15 is constitutionally heterozygous for the T to C transition at the -6 position of the intronic splice acceptor site of exon 13, as three double-stranded DNA bands are observed in the normal DNA. The band of fastest mobility represents the allele with T at the -6 position, the band of intermediate mobility represents the allele with C, and the band of slowest mobility probably represents heteroduplex DNA. In the corresponding tumour DNA, loss of the band representing the 'T' allele and of the heteroduplex DNA band is observed. **(b)** Lanes 1 and 2 correspond to normal and tumour tissue DNA respectively from case GACA6 and lane 3 corresponds to normal DNA from case GACA7. Two heteroduplex DNA bands (Het) and a variant homoduplex DNA band (Hom) were detected in both the tumour and normal DNA of case GACA6. In addition, the single-stranded DNA band pattern (SS) was different from that of case GACA7. On sequencing the variant observed in case GACA6 was characterized as a 4 bp duplication (TTAA) in intronic sequence, at 31 bp upstream of the beginning of exon 13. The sequence analysis additionally demonstrated that case GACA6 was constitutionally homozygous for T at the -6 position of the splice acceptor site of exon 13. Case GACA7 was also homozygous for T.

be the result of a 4 bp duplication in the intronic sequence, at 31 base pairs upstream of the beginning of exon 13: aagtttaaaatcttgctttctgatataatttgttttgtagGCC to aagtttaattaaaatcttgctttctgatataatttgttttgtagGCC, lower case letters represent intronic sequence, upper case letters represent exonic sequence, the four inserted bases are underlined. The sequence analysis also showed that case GACA6 was constitutionally homozygous for the allele with T at the -6 position of the intronic splice acceptor site of exon 13. Case GACA6 was the youngest patient in the series of gastric carcinoma cases studied, she had the gastrectomy at 35 years of age. The tumour was a diffuse type gastric carcinoma. No RER was detected in the tumour, and the patient had no family history of HNPCC (table 2.1).

3.2.8. Summary of genetic alterations in gastric carcinomas

Table 3.20 summarizes the somatic genetic alterations that were detected in the twenty-six gastric carcinomas. Between one and six separate alterations were identified in 13 (50%) tumours.

Somatic genetic changes were found in 11 of the 17 intestinal type gastric carcinomas. Greater than three alterations were detected in 6 of these tumours , two alterations were detected in 4 tumours, and a single alteration was detected in one tumour. The highest number of somatic changes was observed in tumours from cases GACA15 and GACA17, each demonstrating six alterations. LOH observed in the *APC/MCC* region on chromosome 5q in tumour GACA15 and in tumour GACA23 was regarded as a single genetic alteration. Tumour GACA15 was one of two carcinomas that exhibited LOH at microsatellite repeat loci on chromosome 2p. The region of loss observed in this tumour and in the tumour from case GACA4 included the *hMSH2* gene. The tumour from case GACA17 was the only carcinoma in which mutation of the *APC* gene and LOH of the *DCC* gene was observed. Another intestinal type carcinoma, from case GACA22, was the only tumour in which RER at multiple microsatellite repeat loci was detected. No other genetic alterations were observed in this tumour, however. The tumour from case GACA11 exhibited RER at one microsatellite repeat locus and LOH at one other. LOH and mutation of the *p53* gene were the most frequent alterations observed in intestinal type carcinomas.

Somatic genetic changes were detected in 2 of the 9 diffuse type gastric carcinomas. Three alterations were identified in the tumour from case GACA2, while a single alteration was observed in the tumour from case GACA18. Like five of the intestinal type carcinomas, tumour GACA2 demonstrated both alteration of the *p53*

gene and LOH at chromosome 22q. Tumour GACA18 exhibited RER at one microsatellite repeat locus.

Table 3.20. Summary of somatic alterations identified in twenty-six gastric carcinomas.

Case	Histological type[a]	*APC* mutation	LOH on chromosome 5q[b]			*DCC* LOH	*p53* mutation	*p53* LOH	RER at repeat loci	LOH at repeat loci	Total No. of alterations[c]
			APC	*D5S346*	*MCC*						
GACA1	D	-	-	-	-	-	-	-	-	-	0
GACA2	D	-	-	-	-	-	+	+	-	22q	3
GACA3	I	-	-	-	-	-	-	-	-	-	0
GACA4	I	-	-	-	-	-	+	+	-	2p, 4q, 22q	5
GACA5	D	-	-	-	-	-	-	-	-	-	0
GACA6	D	-	-	-	-	-	-	-	-	-	0
GACA7	D	-	-	-	-	-	-	-	-	-	0
GACA8	I	-	-	-	-	-	-	-	-	-	0
GACA9	D	-	-	-	-	-	-	-	-	-	0
GACA10	I	-	-	-	-	-	-	-	-	-	0
GACA11	I	-	-	-	-	-	-	-	+	4q	2
GACA12	I	-	-	-	-	-	-	+	-	4q, 14q	3
GACA13	I	-	-	-	-	-	-	-	-	-	0
GACA14	I	-	-	-	-	-	+	+	-	14q, 22q	4
GACA15	I	-	-	+	+	-	+	+	-	2p, 14q, 22q	6
GACA16	I	-	-	-	-	-	-	+	-	18q	2
GACA17	I	+	-	-	-	+	+	+	-	4q, 22q	6
GACA18	D	-	-	-	-	-	-	-	+	-	1
GACA19	D	-	-	-	-	-	-	-	-	-	0
GACA20	I	-	-	-	-	-	+	+	-	-	2
GACA21	I	-	-	-	-	-	-	-	-	-	0
GACA22	I	-	-	-	-	-	-	-	+	-	1
GACA23	I	-	+	+	-	-	+	+	-	22q	4
GACA24	D	-	-	-	-	-	-	-	-	-	0
GACA25	I	-	-	-	-	-	-	-	-	-	0
GACA26	I	-	-	-	+	-	+	-	-	-	2

[a] I, Intestinal type; D, diffuse type. [b] - indicates negative for LOH or not informative; + indicates positive for LOH. [c] LOH on chromosome 5q was detected at more than one locus in tumours GACA15 and GACA23 but was regarded as a single genetic alteration as the loci are in close proximity to each other.

Chapter 4

Discussion

4. DISCUSSION

4.1. Alterations of the *APC* gene in colorectal cancer

4.1.1. Somatic mutations of the *APC* gene

Since the *APC* gene was cloned in 1991 more than 300 germline mutations have been identified in familial adenomatous polyposis (FAP) patients with a similar frequency of somatic mutations in colorectal tumours (reviewed by Nagase and Nakamura, 1993; Polakis, 1995). In this study the prevalence and nature of mutations of the *APC* gene in the sporadic form of colorectal cancer was investigated. Using single-strand conformation polymorphism (SSCP) and heteroduplex analysis, over 40% of the coding region of *APC* was analysed in 43 colorectal carcinoma and normal DNA pairs. Due to the large size of the coding region of the *APC* gene (approximately 8.5kb), few investigators have screened the entire sequence (Miyoshi *et al.* 1992a and b; Nagase *et al.*, 1992a; Powell *et al.*, 1992; Groden *et al.*, 1993; Varesco *et al.*, 1993). These early published studies describing mutational analyses of *APC* in FAP patients and colorectal tumours had demonstrated that the majority of mutations detected were located in the 5' half of exon 15. This is the largest exon of *APC* (approximately 6.5kb).

In this study the 5' half of exon 15 (codons 653-1700) was screened for mutations as well as some earlier exons: exons 6, 8, 11, and 14. Somatic mutations of the *APC* gene were detected in 37.2% colorectal carcinomas. Miyoshi *et al.* (1992b) detected *APC* mutations in 58% sporadic colorectal carcinomas and 62.5% sporadic colorectal adenomas using RNase protection analysis, while Powell *et al.* (1992) detected *APC* mutations in 60% sporadic colorectal carcinomas and 63% sporadic colorectal adenomas using DNA sequencing analysis. These authors examined the entire coding region of the *APC* gene however. In the 5' half of exon 15 of *APC* Miyoshi *et al.* (1992b) detected mutations in 46.5% colorectal carcinomas and Powell *et al.* (1992) detected mutations in 48% colorectal carcinomas. In the present study mutations in the 5' half of exon 15 were observed at a lower frequency, in 35% colorectal carcinomas. Other studies have also reported investigations of sporadic colorecal carcinomas for mutations in the 5' half of exon 15 of *APC*. Ogasawara *et al.* (1994) detected mutations in a similar percentage of tumours as in the present study (38% carcinomas) as did Miyaki *et al.* (1994) (37.6% carcinomas). Both groups used SSCP analysis of radiolabelled PCR products. Tarmin *et al.* (1995) however,

observed mutations in the 5' half of exon 15 in 74% sporadic colorectal tumours, using the protein truncation test.

The majority of somatic mutations of the *APC* gene detected in this study (95%) were predicted to lead to truncation of the gene product, which is consistent with other studies (Miyoshi *et al.*, 1992b; Powell *et al.*, 1992; Miyaki *et al.*, 1994). Tarmin *et al.* (1995) used the protein truncation test (PTT) to screen the *APC* gene for mutations. This technique is specific for the detection of translation-terminating mutations. The higher percentage of tumours in which mutations were detected in the 5' half of exon 15 in the study of Tarmin *et al.* compared to other studies suggests that the PTT is a highly sensitive technique, particularly for the detection of mutations in a gene such as *APC*, in which most of the mutations thus far characterised are expected to result in protein truncation.

In this study 47% of the mutations detected were point mutations and 53% were frameshift mutations. These frequencies are similar to those observed in other studies using other mutation detection techniques. Miyoshi *et al.* (1992b) used RNase protection analysis and Powell *et al.* (1992) used DNA sequencing and both groups found that 49% of all mutations detected were point mutations while 51% were frameshift mutations. Two of the frameshift mutations detected in this study were insertions and eight were deletions. Most of these mutations occurred at sequences consisting of repeated bases. The sequences included mononucleotide, dinucleotide and pentanucleotide repeats. It is thought that such repeated sequences are prone to slippage by DNA polymerase during replication, resulting in misalignment. Hence repeated sequences are particularly prone to insertion and deletion mutagenesis. One mutation was a point mutation that created a cryptic splice acceptor sequence 8bp downstream of exon 8 (COCA35; table 3.1). This mutation would be expected to result in an *APC* transcript containing a frameshift.

Only 1 of the 9 point mutations that were identified was a missense mutation. The mutation is predicted to result in an amino acid change from valine, a non-polar residue, to isoleucine, another nonpolar residue. It is not known if such an alteration would significantly affect the structure and function of the APC protein. Nagase and Nakamura (1993) summarized somatic point mutations detected in colorectal tumours and reported that most (65%) of these mutations were a change from a cytosine residue to another residue. Approximately 37% of somatic point mutations were C to T transitions at CpG sites. Hydrolytic deamination of 5-methylcytosine in the CpG dinucleotide is thought to be the mechanism underlying this type of transition (Coulondre *et al.*, 1978). G:C to A:T transitions at CpG dinucleotide pairs occur

more frequently in the *p53* gene than in the *APC* gene however. In colorectal cancer such alterations of *p53* have been reported in 67% tumours (Hollstein *et al.*, 1991). In the present study 4 of the 9 *APC* point mutations were G:C to A:T transitions, of which 2 occurred at CpG dinucleotide pairs. Nagase and Nakamura (1993) reported that the frequency of somatic *APC* point mutations at CpG sites was significantly higher in American colorectal cancer patients than in Japanese patients, and suggested that this may reflect differences in dietary habits as the incidence of colorectal carcinoma is much higher in the United States than in Japan.

The majority of reported somatic mutations of *APC* appear to cluster in an area of exon 15 termed the mutation cluster region (MCR) (codons 1286-1513) which represents less than 10% of the coding region (Miyoshi *et al.*, 1992b; Powell *et al.*, 1992; Miyaki *et al.*, 1994). In accordance, three-quarters of somatic mutations detected in the present study were located in this region. Within the MCR, a 5bp deletion at codon 1307-1311 (more commonly known as the codon 1309 mutation) and a 2bp or 4bp deletion at codon 1462-1465 are the most frequently detected somatic mutations (Miyoshi *et al.*, 1992b; Powell *et al.*, 1992; Miyaki *et al.*, 1994). The codon 1309 mutation occurs within a tandem repeat of 5 nucleotides: AAAAGAAAAGAT. Deletion at codon 1462-1465 also occurs within a tandemly repeated sequence: AAAAGAGAGAGAGT. In this study the codon 1309 mutation was observed in 3 colorectal tumours and the codon 1462-1465 mutation was observed in one tumour.

It is possible that the colorectal carcinomas in which an *APC* gene mutation was not detected harboured a mutation in a part of the coding region that was not analysed. Mutations that affect the expression of the *APC* gene may also have occurred in intronic sequence or in the promoter region or in the 3' untranslated region which were not examined in this study. *APC* undergoes alternative splicing of coding as well as noncoding regions. An *APC* transcript that contains a previously unreported exon (exon 10A), located between exons 10 and 11, has been identified and is expressed in a variety of tissues (Sulekova and Ballhausen, 1995; Xia *et al.*, 1995). Inactivating mutations in this exon may occur in colorectal carcinomas. It is possible that the SSCP and heteroduplex assay used in this study was not sensitive enough to detect all mutations in the regions of the gene that were analysed. A lower frequency of mutation was detected in the 5' half of exon 15 by SSCP and heteroduplex analysis in colorectal tumours in this study than by RNase protection analysis, direct sequencing, and PTT in other studies (Miyoshi *et al.*, 1992b; Powell *et al.*, 1992; Tarmin *et al.*, 1995). SSCP and heteroduplex analysis was carried out using the PhastSystem™ (Pharmacia), an automated gel electrophoresis and staining

system. The advantages of this method are that the analysis does not involve the use of radioisotopes as the gels are silver stained, precast 20% non-denaturing polyacrylamide gels (PhastGel®) are available for use, the running conditions (temperature, voltage, and amperage) are accurately controlled, and the analysis can be performed much more rapidly than when using conventional, large gels. Up to twenty-four samples can be analysed in less than 3 hours. This system has been used to detect variants in the phenylalanine hydroxylase gene (Dockhorn-Dworniczak *et al.*, 1991), phosphoglucomutase-1 gene (March *et al.*, 1993), and the neurofibromatosis (NF1) gene (Ainsworth *et al.*, 1993).

SSCP has been reported to be less sensitive than other mutation detection methods but has the advantage of being more rapid and simple (Condie *et al.*, 1993). The sensitivity of SSCP was increased in the present study as double-stranded heteroduplex molecules could also be detected on the gels. All insertions and deletions were detected as heteroduplexes with little or no variation in the single strand DNA pattern. In contrast all of the single base substitutions were detected as single-stranded DNA variants only. One mutation resulted in both variation in the single-stranded DNA band pattern and heteroduplex DNA formation. This was a patricularly large deletion (29 base pairs). Insertions and deletions are thought to create stable heteroduplexes whereas heteroduplexes involving single base substitutions are thought to be more sensitive to environmental changes such as temperature and ionic strength of the buffer (Glavac and Dean, 1995). It is thought that SSCP analysis is less efficient at detecting single base pair substitutions than other techniques such as denaturing gel electrophoresis (DGGE) and chemical mismatch cleavage (CMC) analysis for which near 100% efficiencies for detecting point mutations have been reported (Grompe, 1993). As previously described a similar frequency of somatic point mutations in *APC* was detected in this study as in the studies of Miyoshi *et al.* (1992b) and Powell *et al.* (1992) in which RNase protection analysis and DNA sequencing analysis were used. Additionally, four germline variants that were detected in this study, two of which were common polymorphisms, were all due to single base substitutions.

In one carcinoma, COCA39, heteroduplex shifts were detected in the exon 15E and exon 15F amplicons (figure 3.10). As the 15E and 15F segments are overlapping it is possible that the same mutation was responsible for the variants that were observed in both amplicons. However sequencing analyses that were performed using forward and reverse primers for segments 15E and 15F did not identify a sequence variation in either amplicon. It is possible that the sequencing kits used were not efficient enough to detect all sequence alterations. Alternatively the variants

may have represented PCR artefacts. However for all other samples in which variants were detected by SSCP and heteroduplex analysis, the exact nature of the variation was determined by sequencing.

The sensitivity of SSCP analysis is affected by the length of the DNA fragment analysed (Sheffield *et al.*, 1993). To test the sensitivity of SSCP for detecting single base substitutions in different size PCR fragments, Sheffield *et al.* (1993) analysed point mutations in three different genes. The efficiency of base substitution detection was greatest in fragments ranging in size from 135bp to 200bp with an optimal size of approximately 150bp. The sensitivity of SSCP analysis was dramatically reduced in fragments greater or less than this range (Sheffield *et al.*, 1993). Sarkar *et al.* (1992a) also tested the sensitivity of SSCP for small (183bp) and large (307bp) DNA fragments at different running conditions. They found that the length of the PCR product greatly affected the efficiency of single base substitution detection (75% - 92% for the 183bp fragment and 50% - 64% for the 307bp fragment). In the present study the *APC* exon 6, 8, and 11 amplicons were between 215bp and 235bp in length while the exon 14 amplicon was 303bp. Segments 15A - J of *APC*, generated using primer sets described by Groden et al. (1991) were larger in size than 200bp (range 317bp -507bp). It cannot be excluded that the large size of these PCR fragments led to a decreased sensitivity of mutation detection. In the PCR fragment of greatest length, 15I (507bp), a polymorphism which was the result of a single base substitution was detected by SSCP analysis. However it is not likely that all sequence alterations in larger PCR fragments will be detected using SSCP analysis.

The temperature at which electrophoresis is carried out also affects the sensitivity of SSCP analysis. In the majority of studies in which SSCP analysis has been used, electrophoresis is performed at either room temperature or at 4°C and in general show sensitivity is greatest at 4°C (Condie et al., 1993; Gluvac and Dean, 1993; Hayashi and Yandell, 1993). The sensitivity of analysis at room temperature can be improved by addition of glycerol (5-10%) to the gel. It is not exactly clear how this influences the separation of single strands although it has been proposed that glycerol may act as a weak denaturant, due to -OH group interaction with DNA (Gluvac and Dean, 1993). In this study SSCP and heteroduplex analysis was carried out at temperatures of 4°C, 10°C, and 15°C. The PhastSystem can precisely control temperature during electrophoresis. PCR amplicons were analysed at two different temperatures at least. It was found that some variants detected at one temperature were not detected at another temperature. For example, a common two-allele polymorphism in amplicon 15I (codon 1493) was detected at 10°C as two well

separated single-stranded DNA bands. However at 15°C the bands were poorly separated while at 4°C the single-stranded DNA bands were diffuse and so the two alleles could not be distinguished. This study thus shows that temperature is an important factor in influencing the sensitivity of SSCP analysis.

It is now thought that mutation of the *APC* gene occurs in the great majority of common colorectal tumours. Powell *et al.* (1992) and Miyoshi *et al.* (1992b) found that sporadic colorectal adenomas were just as likely to acquire an *APC* mutation as sporadic colorectal carcinomas. Thus it is thought that mutation of the *APC* gene is an early, if not initiating, event in colorectal tumourigenesis. Powell *et al.* (1992) identified *APC* gene mutations in the smallest adenomas that were examined including those of 0.5cm in diameter. Furthermore mutations of *APC* have been detected in dysplastic aberrant crypt foci (ACF) which are thought to be precursors of colorectal adenomas and thus the earliest identifiable precursors of colorectal cancer (Jen *et al.*, 1994b; Smith *et al.*, 1994), providing further evidence that *APC* gene mutation is a very early event in colorectal tumourigenesis.

4.1.2. Germline alterations of the *APC* gene

Similar to somatic mutations of *APC*, the great majority of germline *APC* mutations that have been described in the literature are chain-terminating mutations. Two mutational hotspots have been identified: a 5bp deletion at codon 1061 and a 5bp deletion at codon 1309. Both mutations occur within sequences consisting of repeated bases. Together these two mutations now account for about 35% of FAP cases (Polakis, 1995). As previously described codon 1309 is frequently mutated somatically in colorectal tumours. Mutational analyses of the *APC* gene in FAP patients have determined a number of genotype - phenotype correlations. Patients with germline mutations between codons 1250 and 1464 were reported as having a severe phenotype in which thousands of colorectal adenomas developed whereas patients with mutations occurring outside this region developed fewer adenomas (Nagase *et al.*, 1992b; Gayther *et al.*, 1994). Germline mutation at codon 1309 was associated with the development of thousands of adenomas at a very young age and death from colorectal cancer about 10 years earlier than patients with other mutations (Caspari *et al.*, 1994; Gayther *et al.*, 1994). In contrast FAP patients with truncating mutations at the very 5' end of the *APC* gene (at or before codon 157) have been found to have an attenuated phenotype. These patients develop a relatively small number of adenomas (ranging from 1 or 2 to over 100) and have a later onset than most FAP patients (Spirio et al., 1993). Not many mutations in the 3' half of the *APC* gene have been published. However recent studies indicate that truncating mutations

beyond codon 1595 are also associated with an attenuated form of FAP (Friedl *et al.*, 1996; van der Luijt *et al.*, 1996). FAP patients develop a number of extracolonic lesions. Retinal lesions known as congenital hypertrophy of the retinal pigment epithelium (CHRPE) are associated with truncating mutations 3' to exon 9 and before codon 1444 (Wallis *et al.*, 1994; Caspari *et al.*, 1995). The development of desmoid tumours and dental anomalies has been asscoiated with mutations between codons 1444 and 1578 (Caspari *et al.*, 1995; Davies *et al.*, 1995).

The mechanisms underlying these genotype-phenotype correlations are not fully understood. It has been hypothesized that APC protein truncated at around codon 1300 (as in patients with a severe phenotype) can bind to wild type APC protein to form a complex with no or little suppressor activity (Nagase *et al.*, 1992b), thus such mutations may represent dominant negative mutations. Su *et al.* (1993) demonstrated that truncated APC proteins could associate with wild type APC, which is supportive of a dominant negative hypothesis. Oligomerization of the APC protein was found to be mediated by coiled coil structures at the amino-terminus of the protein. It was shown that the first 171 residues of APC are sufficient for APC oligomerization and that the first 55 amino acids are necessary for this interaction (Su *et al.*, 1993; Joslyn *et al.*, 1993). It was hypothesized that in patients with mutations at the 5' end of the gene, the small APC peptides may be unstable and thus may not be able to interfere with the wild type protein which would explain the less severe phenotype of these patients (Spirio *et al.*, 1993). However, paradoxically carriers of a complete deletion of the *APC* gene exhibit a classical FAP phenotype (Joslyn *et al.* 1991). Friedl *et al.* (1996) suggested that mutations at the 3' end of *APC* result in a stable truncated protein that still partially retains its normal function and therefore leads to an attenuated form of FAP. However van der Luijt *et al.* (1996) could not detect stable truncated proteins in patients with mutations located at the 3' end of the *APC*. They suggested that these mutations may lead to null alleles and that absence of a truncated APC protein with a possible dominant negative function may account for the less severe FAP phenotype rather than the presence of a partly functional protein.

It has also been suggested that the variations in FAP phenotype may be affected by an independent modifier locus (Spirio *et al.*, 1993). This has been demonstrated in the Min mouse. These mice carry a germline nonsense mutation in the murine homologue of *APC* (*mAPC*) at codon 580 and develop multiple intestinal adenomas (Su *et al.*, 1992). However depending on the inbred mouse strain carrying this mutation the numbers of adenomas developed was found to vary significantly. It was subsequently demonstrated as a result of linkage analysis that a single locus on

mouse chromosome 4 (*Mom-1*, for modifier of Min) accounted for 50% of this variation between strains (Dietrich *et al.*, 1993). Macphee *et al.* (1995) identified a candidate gene for Mom-1: mouse strains carrying the Min mutation and with large numbers of adenomas were found to also carry a truncating mutation in a gene encoding secretory phospholipase A2 (*Plag2g2a*). Phospholipase A2 is involved in lipid metabolism which is interesting as colorectal cancer has been associated with a high fat diet (Willett, 1989). The human homologue of the secretory phospholipase A2 gene (*PLAG2G2A*) was found to map to chromosome 1p35-36, a region that shows frequent allele loss in colorectal carcinomas (Praml *et al.*, 1995b). However no mutations of *PLAG2G2A* have been identified in individuals with FAP (Dobbie *et al.*, 1996; Spirio *et al.*, 1996) nor in sporadic colorectal tumours with loss of heterozygosity on 1p35 (Riggins *et al.*, 1995).

Interestingly the region of the *APC* gene in which germline mutations are associated with a severe phenotype corresponds to the mutation cluster region (MCR). Polakis (1995) suggested that tumour growth is, in part, driven by selection for a mutation in the MCR or alternatively that this region of *APC* is particularly susceptible to mutation. Miyaki *et al.* (1994) found that 94% of somatic mutations in tumours from FAP patients were located in the MCR compared to 77% for sporadic colorectal tumours. Polakis (1995) hypothesized that as a smaller percentage of germline mutations occur in the MCR, this suggests selective pressure for at least one allele to be mutated in this area of the gene.

During the course of screening the 5' half of exon 15 of *APC* for germline and somatic mutations in our laboratory two common polymorphisms were detected by SSCP analysis, in exon 15I (codon 1493) and exon 15J (codon 1678). From our data, linkage disequilibrium between these two polymorphisms and an *Msp* I polymorphism in exon 15N (codon 1960) appeared to be almost complete. Thus for haplotype analysis in FAP kindreds and for LOH analysis in tumours it is only necessary to type one of the three. In this study two further germline variants of the *APC* gene were detected in colorectal carcinoma cases. In case COCA33, a single base substitution of A for G at codon 653 did not result in an amino acid change. In case COCA35, a substitution of C for A resulted in an amino acid substitution of glutamic acid for glutamine at codon 1317. Groden *et al.* (1993) observed this sequence change in the normal population as well as in a FAP patient who also had a germline codon 1309 mutation and characterized the alteration as a rare variant. It has been suggested that a dominantly inherited susceptibility to common colorectal adenomas and cancers affets 19% of the population (Cannon-Albright *et al.*, 1988). Perhaps some germline amino acid substitutions in *APC* result in this inherited

colorectal tumour risk, or alternatively they might just represent variants of no consequence. Truncating germline mutations of the *APC* gene can result in an attenuated FAP phenotype in which only 1 or 2 adenomas develop, as previously described. It can be speculated from this data that certain germline mutations of *APC* or of a modifier of *APC* account for some apparently sporadic colorectal tumour cases.

4.1.3. The two - hit hypothesis

In FAP some but not all investigators believe that the rate-limiting step in tumour initiation is a somatic mutation of the wild type *APC* allele inherited from the unaffected parent which would be in accordance with Knudson's two-hit hypothesis (Knudson, 1971; 1985). One group detected somatic alterations of *APC* (intragenic mutation or allele loss) in 43% of adenomas from FAP patients (Ichii *et al.*, 1992; 1993). These authors concluded that both *APC* alleles must be inactivated for tumour formation. The identification of 'two hits' of the *APC* gene in tumours did not support the hypothesis of a dominant negative effect for truncated APC protein. Additionally, in mice with germline mutation of the murine homologue of *APC* (Min mice), loss of the wild type allele was observed in all tumours examined, including lesions that could only be observed at the microscopic level (Luongo *et al.*, 1994; Levy *et al.*, 1994).

There is evidence that inactivation of both alleles of *APC* occurs in the development of sporadic colorectal cancer. In the current study, three colorectal carcinomas were each found to contain two separate truncating mutations of the *APC* gene. In each tumour, it is not known whether the mutations occurred on different alleles as they were too distant to be analysed in one PCR product. In a fourth tumour, a truncating mutation and LOH were observed indicating complete inactivation of *APC*. In eight further carcinomas LOH at an intragenic polymorphic locus of *APC* or at the closely linked *D5S346* locus was observed. These tumours may have harboured a mutation in the remaining *APC* allele that was not detected in the present study. Powell *et al.* (1992) detected two *APC* mutations in 9 of 41 sporadic colorectal tumours. In 3 carcinomas the mutations were close enough to be included in one PCR product. Sequence analysis of individual PCR clones indicated that the mutations were on separate alleles in all 3 cases. Powell *et al.* also investigated allelic loss in the colorectal tumours. In total, 14 of 41 tumours (31% of adenomas, 36% of carcinomas) were predicted to have no normal functioning APC protein.

In the study of Miyoshi *et al.* (1992b) either two *APC* mutations or a single mutation and allelic loss were observed in 23 of 51 sporadic colorectal tumours. In 19 of 51 tumours (37% of adenomas, 37% of carcinomas) it could be determined that both *APC* alleles were inactivated. In a further study Smith *et al.* (1993) reported that 26 of 32 (81%) cell lines derived from colorectal tumours, mostly sporadic, did not contain detectable levels of normal, full-length APC protein using Western Blot analysis. Twenty-four cell lines contained truncated APC protein. Overall the evidence suggests that in sporadic colorectal neoplasia a growth advantage is afforded by mutating both copies of the *APC* gene but it is less clear if two mutations are required for tumour formation. The observation by Jen *et al.* (1994b) of two separate truncating *APC* mutations in a dysplastic ACF lesion, the earliest identifiable precursor of colorectal cancer, suggests that this is the case although further studies are needed.

4.1.4. Relation of *APC* mutations to protein structure and function

Kinzler and Vogelstein (1996) have hypothesized that APC acts as a 'gatekeeper' of colonic epithelial cell proliferation. Such a gatekeeper gene would normally be responsible for maintaining a constant cell number in renewing cell populations, ensuring that cells respond appropriately to situations requiring net cell growth, for example tissue damage. Inactivation of this gatekeeper would lead to a permanent imbalance of cell division over cell death (Kinzler and Vogelstein, 1996). A role for APC in programmed cell death (apoptosis) has been demonstrated. Morin *et al.* (1996) showed that introduction of wild-type *APC* into colorectal cancer cell lines containing only mutant *APC* alleles resulted in inhibition of cell growth due to the induction of apoptosis. The APC protein is cytoplasmic and is concentrated in the basolateral membrane in colorectal epithelial cells. Immunohistochemical studies with antibodies specific for the APC protein showed that staining of epithelial cells displayed a marked increase from the base of the crypt to the luminal surface, suggesting an association between APC expression and maturation of colorectal epithelial celss (Smith *et al.*, 1993). Morin *et al.* (1996) however suggested that this expression of APC is likely to result in apoptosis of cells migrating toward the top of the crypt, a process critical for achieving cellular equilibrium in an actively regenerating tissue.

The wild-type APC protein is thought to exist as an oligomer. A series of heptad repeats at the amino-terminus of APC mediate homo-oligomerization by a coiled-coil structure (Joslyn *et al.*, 1993; Su *et al.*, 1993). The majority of *APC* gene mutations that have been characterized occur downstream of the region coding for

this oligomerization domain thus it is expected that most mutant APC proteins will retain the domain. Furthermore Su *et al.* (1993) showed that truncated APC protein could associate with wild-type APC protein.

Two studies have demonstrated that wild-type APC when transiently overexpressed associates with the microtubule cytoskeleton but mutant APC protein does not (Smith *et al.*, 1994; Munemitsu *et al.*, 1994). This association was found to be mediated by the carboxy-terminus of APC, with residues 2130-2843 being sufficient. Munemitsu *et al.* (1994) also found that the APC protein was capable of inducing the polymerization of tubulin into microtubule arrays in vitro. Most mutant APC proteins detected in colorectal tumour cells, for example those truncated at the MCR, lack the carboxyl terminus. However it is not exactly clear how loss of binding of APC to microtubules could contribute to neoplasia. Two proteins that bind to the carboxy-terminus of APC have been identified. The first is EB1, a highly conserved 30kDa protein of unknown function (Su *et al.*, 1995). The most carboxy-terminal 284 residues of APC (residues 2560-2843) were sufficient for EB1 binding. The carboxy-terminal 72 residues of APC were demonstrated to bind the human homologue of the *Drosophila* discs large tumour suppressor protein (DLG) (Matsumine *et al.*, 1996b). The fact that the majority of *APC* mutations result in loss of the carboxy-terminus suggests that the EB1 and DLG proteins may be important for the tumour suppressor function of APC.

It is the interaction of APC with another protein, β-catenin, that has provided the greatest insight as to the function of APC. APC binds to b-catenin through two motifs in its central region. The first comprises three successive 15 amino acid repeats located between residues 1020 and 1169. The second putative binding region comprises seven related but distinct 20 amino acid repeats located between residues 1324 and 2075 (Rubinfeld *et al.*, 1993; Munemitsu *et al.*, 1995). Catenins were originally identified as cytoplasmic proteins that bind to cadherins, a family of cell adhesion molecules. Calcium-dependent cell-cell adhesion is maintained by interactions between transmembrane cadherin molecules which require the association of catenins with their cytoplasmic domains. Hulsken *et al.* (1994) reported that APC and E-cadherin (Epithelial-cadherin) compete for binding to the same region in β-catenin. Thus it is possible that APC may modulate E-cadherin-mediated cell adhesion.

Besides its role in cell adhesion, β-catenin has also been implicated in signal transduction. The *Drosophila* homologue of β-catenin, Armadillo, is a component of the Wingless pathway which controls anterior-posterior patterning within each

segment of the fly embryo. The homologous pathway in vertebrates is known as the Wnt signalling pathway and studies in *Xenopus* indicate that β-catenin is a part of this pathway (Heasman *et al.*, 1994). Expression of Wnt-1 (the human homologue of the Drosophila *Wingless* gene) results in stabilization and accumulation of b-catenin in the cytoplasm (Papkoff *et al.*, 1996). Studies in *Xenopus* and mice have indicated that free b-catenin associates with members of a family of high mobility group (HMG) box transcription factors, Tcf-1 and Lef-1 (Behrens *et al.*, 1996; Molenaar *et al.*, 1996). It was suggested that the complex of β-catenin with transcription factor translocates to the nucleus where it activates the transcription of target genes.

In contrast to Wnt, APC down-regulates β-catenin. Transfection of wild-type APC cDNA into a colorectal cancer cell line that contained only truncated, mutant form of APC caused a reduction in the level of β-catenin in these cells. This reduction was due to an enhanced rate of β-catenin protein degradation (Munemitsu *et al.*, 1995). The central region of APC that contains the 20 amino acid repeats was found to be responsible for down-regulation of β-catenin. An increase in the level of cytoplasmic β-catenin is thought to lead to an induction of the enzyme glycogen synthase kinase 3β (GSK3β), a human homologue of Drosophila zeste white 3, another member of the wingless signalling pathway (Rubinfeld *et al.*, 1996). This enzyme was shown to phosphorylate APC in its central region, which appeared to enhance the ability of this region of APC to interact with β-catenin in vitro (Rubinfeld *et al.*, 1996). This would be expected to enhance the β-catenin degrading activity of APC, thus the level of free cytoplasmic β-catenin is controlled.

As a result of the above studies it was hypothesized that APC in concert with GSK3β may regulate the signalling function of β-catenin. It was recently demonstrated that the nuclei from colon carcinoma cells lacking functional APC protein contained a stable complex of β-catenin with the transcription factor hTcf-4 that was constitutively active, as measured by transcription of a Tcf reporter gene. However reintroduction of wild-type APC removed β-catenin from hTcf-4 and abrogated the transcriptional transactivation (Korinek *et al.*, 1997; Morin *et al.*, 1997). Mutant APC proteins, including those truncated in the MCR, were defective in this inhibitory activity (Morin *et al.*, 1997). APC proteins that are truncated at the MCR retain the 15 amino acid β-catenin-binding repeats but lack most of the 20 amino acid repeats implicated in β-catenin binding and degradation. The above results indicated that part of the gatekeeper function of normal APC protein is to down-regulate the transcriptional activity of the β-catenin-Tcf complex. In the absence of

wild-type APC, uncontrolled transcription of target genes could contribute to colorectal tumourigenesis.

Morin *et al.* (1997) additionally examined two colorectal cancer cell lines which expressed wild-type APC for mutations in the gene encoding b-catenin (*CTNNB1*). Both lines were found to contain mutations affecting residues previously implicated in the down-regulation of b-catenin in *Xenopus* (Yost *et al.*, 1996). Moreover in further experiments Morin *et al.* (1997) demonstrated that these mutations were dominant, resulting in an increase in β-catenin-hTcf-4-mediated transcriptional activity. The results suggested that β-catenin acquires oncogenic activity when it is mutated or when it is upregulated by inactivation of APC. Identification of the genes that are activated by hTcf-4-β-catenin complexes and inhibited by wild-type APC should provide an even greater insight into the tumour suppresive function of APC.

4.2. LOH at microsatellite repeat loci in colorectal cancer

Twelve microsatellite repeat loci were analysed for replication error (see next section) and for LOH in 43 colorectal carcinoma and normal DNA pairs. The microsatellite markers were analysed by SSCP analysis using the PhastSystem in this study. LOH was most frequently observed at the *p53CA* locus on chromosome 17p13 and at the *D18S61* locus on chromosome 18q22. A high incidence of allele loss on chromosomes 17p and 18q has been observed in previous studies of colorectal tumours and has been associated with tumour progression. Allelic deletion of chromosome 18q has been associated with progression from intermediate stage adenoma (an adenoma without associated foci of carcinoma) to late stage adenoma (an adenoma with associated foci of carcinoma) while allelic deletion of chromosome 17p has been associated with progression from late adenoma to carcinoma (Fearon and Vogelstein, 1990). The *p53* gene has been demonstrated to be the target of allelic deletion on chromosome 17p in colorectal tumours (Baker *et al.*, 1990a and b). On chromosome 18q, three candidate tumour suppressor genes that are contained within a minimally lost region, namely *DCC*, *DPC4* and *JV18-1*, have been proposed as targets (Fearon *et al.*, 1990; Thiagalingam *et al.*, 1996; Riggins *et al.*, 1996). A low incidence of mutations in each of these genes has been described in colorectal tumours however, suggesting that other gene(s) on chromosome 18q21 may be targets for allelic deletion in colorectal tumourigenesis too.

In the current study LOH at *p53CA* was detected in 58% of informative tumours, and at *D18S61* in 33% of informative tumours. The *D18S61* locus is located more than 30cM distal to the *DCC* gene on chromosome 18q. The putative tumour suppressor genes *JV18-1* and *DPC4* are both located proximal to *DCC*. In other studies allele loss on chromosome 18q has been described in 52% to 73% colorectal carcinomas (Law *et al.*, 1988; Vogelstein *et al.*, 1988; Thiagalingam *et al.*, 1996). LOH on chromosome 17p has been described in 56% to 75% colorectal carcinomas (Law *et al.*, 1988; Vogelstein *et al.*, 1988; Fey *et al.*, 1989). Cawkwell *et al.* (1994) and Leggett *et al.* (1995) also analysed the *p53CA* marker in colorectal carcinoma cases and detected LOH in 66% and 68% tumours respectively. A possible reason for the lower rate of detection of LOH at the *p53* locus and at chromosome 18q in this study compared to some others is that the tumour specimens were contaminated with normal stromal cells which mask detection of allele loss. Indeed a residual signal was often seen in tumours that exhibited LOH which could have reflected normal cell contamination.

In order to compare the SSCP method of analysing microsatellite repeat markers with another method the *D22S351* marker was also analysed by denaturing capillary electrophoresis using an automated laser DNA analyser. This marker was chosen as with SSCP analysis each allele was represented by 3 bands but often two bands migrated to the same position thus a complex pattern was seen. No difference in results was seen between the SSCP method and the capillary electrophoresis method. It is possible with the other markers that were analysed by SSCP analysis that bands representing alternative alleles comigrated resulting in misclassification of a heterozygote as a homozygote. Thus potentially a case demonstrating LOH in the tumour could have been missed. A high incidence of informativity (heterozygosity) was observed at each locus, however. For *D22S351* heterozygotes and homozygotes corresponded using the two methods of analysis. Although LOH and RER were detected at microsatellite repeat loci with SSCP analysis, the capillary electrophoresis method is preferable as it gives a more accurate interpretation of the alleles. Each allele peak can be quantified in terms of size, area, and height.

LOH at *D22S351* was detected in only 13% informative colorectal carcinomas in this study. In some other studies LOH on chromosome 22q has been observed at a higher frequency in colorectal tumours (approximately 30%) (Vogelstein *et al.*, 1989; Miyaki *et al.*, 1990). These studies suggested that a tumour suppressor gene for colorectal cancer may exist on this chromosome. Yana *et al.* (1995) examined 16 polymorphic DNA markers from chromosome 22q in sporadic colorectal carcinomas and detected LOH in 41% of tumours. The commonly deleted region was located

between two telomeric loci. The *D22S351* marker analysed in the present study is located more proximally, at 22q11.2, which may partially explain why the incidence of 22q LOH was so low.

Three microsatellite repeat markers (*D2S119*, *D2S391*, *D2S123*) that are closely linked to the *hMSH2* DNA mismatch repair gene on chromosome 2p16 were also analysed in the 43 colorectal carcinoma cases. LOH was detected in only 1 tumour, at the *D2S391* locus, consistent with other studies (Aaltonen *et al.*, 1993; Tomlinson *et al.*, 1996) and suggests that allele loss at *hMSH2* does not significantly contribute to colorectal tumourigenesis. A low rate of LOH was detected at loci on chromosomes 4q (9.4%), 9p (7.4%), 14q (15.6%), and 16q (4.4%). Vogelstein *et al.* (1989) observed a low frequency of LOH on these chromosomes in colorectal carcinomas (10-20%). In other studies LOH on chromosome 14q has been detected at a higher rate, in 30%-53% colorectal carcinomas (Sasaki *et al.*, 1989; Young *et al.*, 1993a). Young *et al.* (1993a) identified a minimally lost region at the distal portion of chromosome 14q, at 14q32, suggesting that a tumour suppressor gene involved in colorectal neoplasia resided there. The *D14S50* locus analysed in the current investigation however is located proximally, at 14q11.

4.3. RER at microsatellite repeat loci in colorectal cancer

The observation of RER at microsatellite repeat sequences in tumours is thought to reflect a defect in DNA mismatch repair, resulting in errors of DNA replication not being repaired at these sites. Mismatch repair deficiency due to mutation in a DNA mismatch repair gene is thought to result in widespread genomic instability. Six mismatch repair (MMR) genes have been shown to be involved in HNPCC and sporadic colorectal cancer: *hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*, *hMSH3*, and *hMSH6* (*GTBP*) (see section 1.4.4).

RER at one or more microsatellite repeat loci was detected in 6 of 43 (14%) colorectal carcinomas. In the literature RER at 1 or more loci has been described in 12% to 28% apparently sporadic colorectal carcinomas, thus the frequency detected in the present study is within this range (Aaltonen *et al.*, 1993; Thibodeau *et al.*, 1993; Ionov *et al.*, 1993; Lothe *et al.*, 1993; Kim *et al.*, 1994; Liu *et al.*, 1995a; Cawkwell *et al.*, 1995; Bubb *et al.*, 1996; Bocker *et al.*, 1996). Two of the RER+ colorectal tumours identified in this study were however found not to be sporadic cases but had family histories that were indicative of HNPCC. These two tumours exhibited RER at multiple microsatellite repeat loci. In other studies RER has been

observed in about 86% of colorectal cancers from HNPCC patients (Aaltonen *et al.*, 1993; 1994). These HNPCC tumours exhibit RER at the majority of loci analysed.

The variation in the frequency of RER in apparently sporadic colorectal carcinomas that is observed in the literature may reflect differences in criteria used in classification of a tumour as RER+, different types of loci analysed, variation in the number of loci analysed, or different methods of detection. Some authors have analysed only 4-6 loci. No consensus exists as to how many loci should be analysed and how many of these should show alteration for the tumour to be classified as RER+. In the current investigation RER was observed at 1 of 12 (8%) loci in one tumour, at 2 of 12 (17%) loci in each of two tumours, at 5 of 12 (42%) loci in one tumour, at 8 of 12 (67%) loci in one tumour, and at 10 of 12 (83%) loci in one tumour. The latter two carcinomas were from the patients with the HNPCC-type family histories. Many authors define a tumour as being RER+ if at least two of the markers analysed exhibit electrophoretic mobility shifts (Kim *et al.*, 1995; Liu *et al.*, 1995a; Bocker *et al.*, 1996). Using this criterion the frequency of RER+ sporadic colorectal carcinomas in most of the published studies would be 10% to 15%.

The detection of RER at only 1 locus may represent a PCR artefact. Also it has been suggested that a background instability of microsatellite repeats exists (Weber and Wong, 1993). A total of 12 loci were analysed in this study. It is not known if the mechanism responsible for the RER at 2 of 12 loci which was observed in two colorectal tumours would be different from that causing RER at 1 of 12 loci which was observed in one colorectal tumour, thus this latter tumour was classified as RER+ along with the other tumours. It has also been suggested that tumours should be considered as RER+ if at least 30% of markers showed bands of altered mobility in the tumour compared to normal (Moslein *et al.*, 1996). Using this criterion, in the present study the tumours exhibiting RER at 2 of 12 loci analysed as well as at 1 locus would not be regarded as RER+. It is possible that these tumours were RER+ at further loci but the SSCP method was not efficient at detecting all cases of RER. One marker, *D22S351*, was analysed by a different method too as discussed in the previous section. Tumours that were RER- at *D22S351* using the SSCP method were also found to be RER- at this locus using the denaturing capillary electrophoresis method. In addition SenGupta *et al.* (1997) used the same detection method as in the present study, SSCP analysis using the PhastSystem, and observed RER in 17 of 24 (71%) colorectal cancers from individuals with metachronous cancer.

It can be speculated that in carcinomas with RER at only a few loci the mismatch repair defect responsible occurred at a later stage of tumourigenesis than in

carcinomas with RER at multiple loci, thus there may not have been enough time for widespread RER to occur. An alternative explanation is that tumours which are RER+ at a few loci could have mutations in different mismatch repair (MMR) genes from tumours which are RER+ at multiple loci, or they could have alterations in the same gene but in different regions which may account for the difference in RER phenotype. It is possible that other genes involved in DNA replication or repair may be mutated in the tumours and be responsible for the RER. It has been reported that tumours with mutations in both alleles of one known MMR gene, *hMSH6*, demonstrate a low degree of RER at dinucleotide repeat loci and reduced mononucleotide repeat instability compared to tumours with mutations in other MMR genes (Papadopoulos *et al.*, 1995; Akiyama *et al.*, 1997a). Bhattacharyya *et al.* (1995) analysed two colorectal tumour cell lines, one of which had homozygous mutation of the *hMSH6* gene and the other had homozygous mutation of the *hMLH1* gene, but both demonstrated an elevated rate of mutation of the gene encoding the purine salvage enzyme hypoxanthine guanine phosphoribosyl transferase (hprt) compared with MMR proficient cell lines. Single base substitutions were frequent in both cell lines but frameshift mutations were more frequent in the *hMLH1* mutant line (39% total mutations) than in the *hMSH6* mutant line (6% total mutations) (Bhattacharyya *et al.*, 1995). The tumours that are RER+ at 1 or 2 dinucleotide repeat loci in this study may have mutations of the *hMSH6* gene.

That tumours which are RER+ at multiple loci arise through a different mechanism to those that are RER+ at a few loci is supported by the report that with respect to certain clinicopathological variables, tumours that are RER+ at 1 locus show features more in common with RER- tumours than with tumours that are RER+ at 2 or more loci (Lothe *et al.*, 1993). The latter tumours demonstrated characteristics commonly shown by HNPCC tumours. These include location in the proximal (right side) colon (Lothe *et al.*, 1993; Ionov *et al.*, 1993; Thibodeau *et al.*, 1993; Aaltonen *et al.*, 1993; Kim *et al.*, 1994), poor differentiation (Lothe *et al.*, 1993; Ionov *et al.*, 1993; Kim *et al.*, 1994), diploidy (Aaltonen *et al.*, 1993; Lothe *et al.*, 1993), and increased survival rates (Lothe *et al.*, 1993; Thibodeau *et al.*, 1993).

In HNPCC patients, the RER+ phenotype is believed to occur at an early stage of tumourigenesis as RER has been detected in 57% of adenomas (Aaltonen *et al.*, 1994). In contrast RER has been detected at a low frequency in sporadic colorectal adenomas (0-3%) (Young *et al.*, 1993b; Aaltonen *et al.*, 1994). These studies are however at variance with others that report the detection of RER in 10-15% aberrant crypt foci (ACF), the earliest identifiable precursor of colorectal cancer (Augenlicht *et al.*, 1996; Heinen *et al.*, 1996).

At the beginning of this study it was thought that all the colorectal carcinomas being investigated were sporadic cases. Two of the RER+ carcinomas (COCA9 and COCA26) were subsequently found to occur in patients from HNPCC-type families. It is possible that these patients had inherited germline mutations in one of the MMR genes that have been found to be responsible for HNPCC. Mutations of the *hMLH1* and *hMSH2* genes account for the majority of germline mutations identified in HNPCC patients thus far (55% and 42% mutations respectively) (reviewed by Papadopoulos and Lindblom, 1997). Germline mutations of the *hPMS1*, *hPMS2*, and *hMSH6* genes have been identified in only a few HNPCC kindreds while no germline mutations of *hMSH3* have been reported. Most mutations of MMR genes are truncating mutations. The mutations are spread throughout the length of the *hMSH2* and *hMLH1* genes and most are unique. However a splice donor site mutation of *hMSH2* exon 5 has been found to occur in 12% English and North American HNPCC kindreds (Froggatt *et al.*, 1995) and two *hMLH1* mutations account for more than half of Finnish kindreds with HNPCC (Nystrom-Lahti *et al.*, 1996). In this study skin lesions were noted to occur in the family of case COCA9. Muir-Torre syndrome, considered to be a variant of the HNPCC syndrome, is characterized by the occurence of skin lesions and a tumour spectrum similar to that in HNPCC. Analysis of the mutational spectrum of the MMR genes has not demonstrated a correlation with phenotype, for example an identical *hMSH2* mutation has been detected in both Muir-Torre and HNPCC families (Papadopoulos and Lindblom, 1997).

The RER+ colorectal carcinomas identified in this study that did not have family histories of cancer may have resulted from somatic mutations in the known MMR genes. Exons 12-14 of the *hMSH2* gene were screened for mutations in RER+ and RER- colorectal tumours (see next section). Other studies investigating mutations of the MMR genes in sporadic RER+ colorectal carcinomas report somatic mutations of the *hMLH1* and *hMSH2* genes in 26-42% tumours (Liu *et al.*, 1995b; Borresen *et al.*, 1995; Wu *et al.*, 1997; Herfarth *et al.*, 1997). Similar to HNPCC tumours, somatic alteration of the second allele of the MMR gene (either mutation or allele loss) has been detected in many of these tumours, supporting the hypothesis that complete inactivation of MMR genes is necessary for the manifestation of defective mismatch repair and RER (Leach *et al.*, 1993). Somatic mutations of the *hMSH6* gene have been detected in 3 colorectal cancer cell lines (Papadopoulos *et al.*, 1995) The *hMSH6* and *hMSH3* genes respectively contain tracks of 8 deoxyadenosines and 8 deoxycytosines in their coding regions. Malkhosyan *et al.* (1996) identified frameshift mutations consisting of either a deletion of insertion of one nucleotide within the monotonic run in *hMSH3* in 39% RER+ colorectal tumours and in the monotonic run in *hMSH6* in 30% RER+ colorectal tumours. Because

these frameshift mutations occurred in short repetitive DNA sequences similar to microsatellite repeats it was suggested that they may be the result of a previous defect in mismatch repair, for example mutation of the *hMSH2* or *hMLH1* gene. Indeed Malkhosyan *et al.* (1996) did identify one tumour with mutation in both the *hMSH2* and *hMSH3* genes, as well as 8 *hMSH3/hMSH6* double mutants.

Liu *et al.* (1995b) did not detect mutations of the *hPMS1* and *hPMS2* MMR genes in sporadic RER+ colorectal carcinomas. In addition the frequency of mutation of *hMSH2* and *hMLH1* in RER+ sporadic colorectal cancer is much less than in HNPCC, suggesting the involvement of other genes, possibly as yet unidentified MMR genes. In a second study the aforementioned group reported the identification of missense mutations in the proofreading exonuclease domain of polymerase δ in two RER+ colorectal cancer cell lines (da Costa *et al.*, 1995). In one case the mutation was also present in the normal colon. These findings thus support the hypothesis that other genes involved in replication or repair, in addition to the known MMR genes, may contribute to the RER+ phenotype.

Aaltonen *et al.* (1993) reported that the frequency of mutation of the *APC* gene detected in colorectal tumours from HNPCC patients was similar to that seen in sporadic colorectal cancers, thus ruling out the possibility that tumours progress along either an 'APC' or 'HNPCC' pathway. In accordance, in the present study somatic mutations of the *APC* gene were identified in 3 of the 6 RER+ colorectal carcinomas. Two of the 3 cases appeared to belong to HNPCC-type kindreds (COCA9 and COCA26). Huang *et al.* (1996) similarly reported that the prevalence of *APC* mutations was similar in RER+ colorectal tumours derived from sporadic and HNPCC patients and RER- colorectal tumours. However these authors found that frameshift mutations of the *APC* gene were significantly more frequent in RER+ than RER- tumours. Most of these insertions and deletions were within simple repeated sequences. It was suggested that these findings indicate that the RER phenotype precedes, and is responsible for *APC* mutation in RER+ colorectal tumours (Huang *et al.*, 1996). In contrast in the current study the *APC* mutations detected in the RER+ carcinomas from cases COCA9 and COCA26 were point mutations (missense and nonsense mutation respectively). The *APC* gene mutation detected in tumour COCA38, which exhibited RER at 2 microsatellite repeat loci, was a frameshift mutation, however.

Some other molecular differences between RER+ and RER- colorectal carcinomas have been reported. A negative correlation between the presence of RER and mutations in the *p53* and K-*ras* genes has been found (Ionov *et al.*, 1993).

Mutations of the transforming growth factor β (TGFβ) type II receptor gene have been observed in RER+ but not RER- colorectal tumours (Markowitz *et al.*, 1995; Parsons *et al.*, 1995b). TGFβ inhibits the growth of many epithelial cell types and loss of this regulation is believed to contribute to tumour development. The mutations detected in the TGFβ type II receptor gene were insertions or deletions of adenines within a polyadenine tract located in the coding region and were predicted to result in protein truncation (Parsons *et al.*, 1995b). Markowitz *et al.* (1995) showed that 8 of 11 RER+ colon cancers with such inactivating mutations of the TGFβ type II receptor gene were resistant to the growth inhibitory effects of TGFβ. These studies showed that the mechanism underlying the RER+ phenotype in cancer cells, a defect in the DNA mismatch repair system, induces not only an accumulation of mutations at microsatellite repeat sequences in non-coding areas of the genome but also inactivating mutations in genes likely to play critical roles in tuour suppression. Such mutations would be expected to accelerate tumour progression. Indeed similar frameshift mutations to those observed in the TGFβ type II receptor gene have been detected in mononucleotide repeat sequences located in the coding regions of other genes in RER+ colorectal tumours. These genes include *Bax*, a gene that promotes apoptosis (Rampino *et al.*, 1997) and the gene encoding insulin-like growth factor II receptor which appears to have a growth-suppressing function (Souza *et al.*, 1996).

The above results, together with the finding that RER+ colorectal cancer cells exhibit a mutation rate two to three orders of magnitude higher than in normal cells (Bhattacharyya *et al.*, 1994; Bhattacharyya *et al.*, 1995; Eshleman *et al.*, 1995) have led to the general belief that RER+ colorectal tumour cells have a true mutator phenotype. It is thought that HNPCC patients develop colorectal carcinomas much earlier than the general population because mismatch repair deficiency leads to a rapid accumulation of somatic mutations in tumour suppressor genes and oncogenes and this genetic instability causes a rapid progression to malignancy (Kinzler and Vogelstein, 1996). RER- colorectal carcinomas are generally aneuploid whereas RER+ carcinomas are often diploid (Aaltonen *et al.*, 1993; Bocker *et al.*, 1993). Aneuploidy in tumours is thought to reflect an underlying chromosomal instability (Lengauer *et al.*, 1997). It has been proposed that there are two ways for a colorectal tumour to develop the multiple genetic alterations required for malignancy: subtle alterations due to mismatch repair deficiency occur in a minority of cases (those with RER) while gross chromosomal alterations (chromosomal gains and losses) occur in the majority (those without RER) (Kinzler and Vogelstein, 1996; Lengauer *et al.*, 1997).

4.4. Alterations of the *hMSH2* gene in colorectal cancer

The *hMSH2* gene was screened for mutations using SSCP and heterduplex analysis. Exons 12, 13, and 14 were analysed in this study as they encode the most conserved region of the hMSH2 protein (Fishel *et al.*, 1993; Leach *et al.*, 1993). The only sequence variation that was detected in colorectal cancer cases was a T to C transition polymorphism at the -6 position of the splice acceptor site of exon 13. In this study, two-allele polymorphisms in the *APC* and *p53* genes were detected as single-stranded DNA variants. Thus unusually the *hMSH2* splice site polymorphism was not detected as variation in the single-stranded DNA pattern, but as double-stranded DNA variants.

The splice site T to C transition was first described by the two groups that cloned the *hMSH2* gene. Leach *et al.* (1993) identified the T to C transition in an affected individual from a HNPCC kindred and also in 2 of 20 unrelated normal individuals and concluded that the substitution was a polymorphism. Fishel *et al.* (1993) however detected the T to C transition in two sporadic colorectal tumours but not in corresponding normal tissues, and also detected this sequence change in constitutional DNA of affected individuals from two HNPCC kindreds. The authors suggested that the T to C transition represented a mutation rather than a polymorphism. Furthermore, Brentnall *et al.* (1995) reported that the presence of the T to C transition increased the risk of colorectal cancer in patients with ulcerative colitis. Fishel *et al.* (1993) and Brentnall *et al.* (1995) suggested that the substitution may cause aberrant splicing of *hMSH2*. However analysis of splice acceptor sites from other human genes showed that there is a nearly equal probability of observing C or T at the -6 position, which would argue against the above hypothesis (Rogan and Schneider, 1995).

The group of Fishel *et al.* subsequently analysed 111 sporadic colorectal cancer cases and 114 unaffected individuals for the splice site sequence variation and found that an equal proportion of the two groups (about 25%) were constitutionally heterozygous at this site, concluding that the T to C transition is a common polymorphism (Hall *et al.*, 1994). In the current study, 50 unaffected unrelated individuals as well as 43 colorectal carcinoma cases and 26 gastric carcinoma cases were analysed for the splice site T to C transition. A similar proportion of individuals in the 3 groups were found to be heterozygotes (28% of the group of unaffected individuals, 21% of colorectal carcinoma cases, 23% of gastric carcinoma cases). In another study Borresen *et al.* (1995) identified the T to C transition in a similar

proportion of unaffected individuals (24%). It is concluded that the overall evidence indicated that the T to C transition is a polymoprhism that is common in the general population and does not increase susceptibility to the development of cancer.

4.5. Alterations of the *APC* and *MCC* genes in gastric cancer

Twenty-six gastric carcinoma and normal DNA pairs were screened for mutations in exons 6, 8, 11, 14 and the 5' half of exon 15 of *APC* using SSCP and heteroduplex analysis. The protein truncation test (PTT) is a method that specifically detects translation-terminating mutations. PTT was used as a secondary screening technique to analyse a specific region of *APC* exon 15 for mutations, as the great majority of mutations that had been detected in colorectal tumours and FAP patients in published studies were predicted to lead to truncation of the protein product (Nagase and Nakamura, 1993). Using the combination of mutation detection techniques, a somatic mutation of *APC* was identified in only 1 of 26 (4%) gastric carcinomas. Additionally, restriction enzyme digest analysis of PCR products was carried out for the detection of specific chain-terminating mutations in exons 6, 8, and 14 of *APC*. However none of the 4 mutations that were tested for were detected in the gastric carcinomas. Of note, one of these mutations, a nonsense mutation at codon 302 (exon 8), was detected in one colorectal carcinoma in this study using SSCP and heteroduplex analysis.

Several studies have now been published describing investigations of gastric tumours for mutations of the *APC* gene. The 5' half of exon 15 has been screened for mutations in these studies as well as in the present study as it includes a region where most somatic mutations detected in colorectal tumours are clustered (MCR) (Nagase and Nakamura, 1993). Horii *et al.* (1992) detected somatic *APC* gene mutations in 3 of 44 (7%) gastric carcinomas using RNase protection analysis. In a later study these authors carried out microdissection to isolate tumour cells from formalin fixed and paraffin-embedded tissues from contaminating normal cells in the tumour tissue. Mutations of the *APC* gene were detected in 12 of 57 (21%) gastric cancers (Nakatsuru *et al.*, 1992). Microdissection was not carried out in the present study thus contamination of tumour tissue with normal cells may have contributed to the low incidence of *APC* gene mutations by masking mutations. However in two further studies tumour cell enrichment was carried out using either microdissection or flow cytometric cell sorting but no mutations were detected in the 5' half of exon 15 in gastric carcinomas (Ogasawara *et al.*, 1994; Powell *et al.*, 1996). Ogasawara *et al.* (1994) used SSCP analysis and Powell *et al.* (1996) used PTT analysis. In a second

study, the former group analysed 72 gastric carcinomas for *APC* gene mutations but detected such alterations in only 1 case (1.4%) (Maesawa *et al.*, 1995).

Taken together the findings indicate that *APC* gene mutations do not occur in the majority of gastric cancers. They may be frequent only in certain histological types. In this study mutation of *APC* was detected in an intestinal type gastric cancer. Nakatsuru *et al.* (1992) identified somatic *APC* gene mutations in differentiated carcinomas (intestinal type according to the classification of Lauren) and signet ring cell carcinomas but not in poorly differentiated carcinomas. These authors further divided differentiated type carcinomas into 'very well differentiated' and 'well or moderately differentiated' types. *APC* mutations were found to be significantly more frequent in very well differentiated carcinomas (41%) than in well or moderately differentiated carcinomas (11%). Mutations were detected in 30% signet ring cell carcinomas (Nakatsuru *et al.*, 1992). Maesawa *et al.* (1995) also detected an *APC* gene mutation in a signet ring cell carcinoma. Furthermore, gastric adenomas are thought to be precursors of some differentiated types of gastric cancer. *APC* mutations have been detected in 20-40% of gastric adenomas (Nakatsuru *et al.*, 1993; Tamura *et al.*, 1994).

The mutation detected in the intestinal type gastric carcinoma in this study was located at a particular hotspot in the MCR (codon 1462-1465). A similar mutation at this position was identified in two flat adenomas of the stomach by Nakatsuru *et al.* (1993). It does remain possible that mutations in gastric carcinomas are frequent in areas of the *APC* gene other than those that correspond to mutation cluster regions in colorectal carcinomas. Mutations were not identified in exons 6, 8, 11, and 14 in this study, however. In addition to the 5' half of exon 15, Horii *et al.* (1992) screened exons 8 to 14 of *APC* for mutations in gastric carcinomas but did not detect alterations in these regions.

As previously mentioned the vast majority of *APC* gene mutations in colorectal carcinomas (>95%) are truncating in nature. The *APC* mutation detected in a gastric carcinoma in this study was also a protein truncating mutation. In contrast Nakatsuru *et al.* (1992) found that 9 of the 17 (53%) mutations that they detected in gastric carcinomas using RNase protection analysis were missense mutations. Furthermore, three separate somatic *APC* mutations were detected in each of two gastric carcinomas, a phenomenon which has not been noted in colorectal tumours (Nakatsuru *et al.*, 1992). These differences in the type of *APC* mutation between gastric and colorectal tumours may reflect the effect of specific mutagens, possibly in food, on gastric mucosal cells. The sensitivity of SSCP for the detection of single

base substitutions has been reported to be greatest for DNA fragments of shorter than 200bp (Sheffield *et al.*, 1993). As discussed in section 4.1.1, in this study PCR fragments for *APC* exon 15A-J were larger in size (range 317bp to 507bp). It is possible that this resulted in a decreased sensitivity of base substitution and hence missense mutation detection in gastric carcinomas. In addition, the PTT assay which was also used for screening a section of *APC* exon 15 for mutations in gastric carcinomas does not detect missense mutations. Although SSCP and heteroduplex analysis identified somatic mutation of the *APC* gene in only 1 (4%) gastric carcinomas it was effective in detecting mutations in colorectal carcinomas in this study (37%).

In this study gastric tumours from predominantly British patients were studied. Japan has the highest rate of gastric cancer in the world. The higher frequency of *APC* gene mutations detected in some Japanese gastric carcinomas and adenomas (Nakatsuru *et al.*, 1992; Nakatsuru *et al.*, 1993; Tamura *et al.*, 1994) than in the present study may reflect the influence of environmental, possibly dietary, carcinogens. However Maesawa *et al.* (1995) detected *APC* gene mutations in only 1 of 72 Japanese gastric carcinomas. Powell *et al.* (1996) failed to detect *APC* gene mutations in gastric carcinomas from patients living in a high gastric cancer risk region in North-Central Italy.

LOH of *APC* was detected in only 8% of informative gastric carcinomas and of *MCC* in 10% of cases. One gastric tumour demonstrated LOH at *APC* and at *D5S346*, which is located between the *APC* and *MCC* genes on chromosome 5q, but was constitutionally homozygous at *MCC* intragenic polymorphic loci. Another gastric tumour exhibited LOH at *MCC* and at *D5S346* but was homozygous for the *APC* intragenic polymorphisms. Thus these two tumours may have allele loss of both the *APC* and *MCC* genes. Analysis of further markers flanking both genes would clarify the situation. Another gastric tumour, however, was constitutionally heterozygous at intragenic polymorphic loci of *APC* and *MCC* and at *D5S346* but only exhibited LOH at *MCC*. Similar discrepancy of allelic loss at *MCC* and *APC* loci has been reported in oesophageal cancer (Huang *et al.*, 1992) and small cell lung cancer (D'Amico *et al.*, 1992).

A low frequency of alteration of the *MCC* gene as well as the *APC* gene was detected in this study. Once again a reason for the low rate of LOH could be contamination of the tumour specimens with normal cells. DNA was extracted from frozen tumour tissue as a mass but histological analysis of adjacent cryostat sections indicated that all tumour samples used in this study consisted of at least 50%

neoplastic cells. In other gastric cancer studies the frequency of LOH on chromosome 5q varies probably reflecting differences in sample preparation and methods of analysis. Interestingly in another study of British gastric carcinomas Fey *et al.* (1989) also described infrequent LOH on chromosome 5q (10% of cases), although other studies on Western gastric cancers report higher rates (25% tumours by Schneider *et al.*, 1995; 58% tumours by Gleeson *et al.*, 1997). In studies of Eastern gastric cancers, Rhyu *et al.* (1994a) detected LOH at both *APC* and *MCC* loci in 30% tumours, while Tamura *et al.* (1993) observed LOH at *APC* in 86% tumours and at *MCC* in 100% tumours. The low incidence of *APC* gene mutations that has been observed in gastric carcinomas suggests that mutation in another gene(s) on chromosome 5q is responsible for the frequent LOH in these studies, possibly *MCC*. In colorectal cancer however very few mutations of the *MCC* gene have been reported (Kinzler *et al.*, 1991b; Nishisho *et al.*, 1991; Curtis *et al.*, 1994. A role for *MCC* in tumourigenesis remains unclear although in a recent study it was shown that overexpression of the MCC protein blocked cell cycle progression from G1 to S phase (Matsumine *et al.*, 1996a).

In several other tumour types, similar diverging observations of frequent chromosome 5q LOH but infrequent *APC* gene mutations have been reported, including oesophageal cancer (Powell *et al.*, 1994), ovarian cancer (Allan *et al.*, 1994), breast cancer (Kashiwaba *et al.*, 1994), and lung cancer (Cooper *et al.*, 1996). Thus it does appear that another tumour suppressor gene exists in this region. Tamura *et al.* (1996b) investigated LOH at 9 microsatellite repeat loci on chromosome 5q in gastric carcinomas. Although many tumours exhibited large interstitial deletions on 5q that included *APC* (5q21-22), two minimum regions of deletion distinct from the *APC* locus were identified, at 5q14 and at 5q31.1 (Tamura *et al.*, 1996b). It is possible that tumour suppressor genes at these sites may play a role in the development of gastric carcinomas.

In the present study, no alterations of the *APC* and *MCC* genes were observed in diffuse type gastric cancers. This histological type usually contains a higher proportion of non-neoplastic stromal cells which can inhibit the detection of genetic alterations. However alterations of *APC* and *MCC* were also infrequent in intestinal type gastric carcinomas, thus these genes do not appear to play an important role in the development of gastric cancer in patients from the British population.

4.6. Alterations of the *DCC* gene in gastric cancer

LOH at the *DCC* locus was observed in only 1 of 20 (5%) informative gastric tumours. This was an intestinal type carcinoma. The *D18S61* locus was also investigated for LOH in gastric carcinomas. *D18S61* is located approximately 30cM distal to *DCC* on chromosome 18q. LOH was again observed in only 1 tumour (5.3%), also an intestinal type carcinoma. Thus this study does not demonstrate that allele loss of chromosome 18q is a significant event in gastric tumourigenesis. A much higher frequency of LOH at polymorphic loci within the *DCC* gene has been reported in other studies of gastric carcinomas (27% to 58% informative tumours) (Maesawa *et al.*, 1995; Cho *et al.*, 1996; Wu *et al.*, 1997a). In these investigations neoplastic cells were isolated by either microdissection or flow cytometric cell sorting methods. Such analysis was not performed in the current study which may explain the low rate of *DCC* LOH. In other studies investigating allele loss on chromosome 18q in gastric carcinomas, a varying frequency of LOH has been described (22-68%) (Uchino *et al.*, 1992; Ranzani *et al.*, 1993; Schneider *et al.*, 1995; Gleeson *et al.*, 1997).

Recent studies have indicated a role for the DCC protein in the development of the nervous system but have questioned a role in tumour suppression. DCC is thought to be a component of a netrin receptor involved in the guidance of developing axons (Keino-Masu *et al.*, 1996; Kolodziej *et al.*, 1996). Transgenic $Dcc^{+/-}$ mice did not show an increase in tumour predisposition compared to $Dcc^{+/+}$ mice, with only 1 of 200 $Dcc^{+/-}$ mice developing a gastrointestinal tumour. However $Dcc^{-/-}$ mouse embryos showed developmental defects in the nervous system (Fazeli *et al.*, 1997).

Investigations of colorectal and pancreatic carcinomas have shown that two other genes on chromosome 18q, *DPC4* and *JV18-1*, are targets of frequent 18q deletion, in addition to *DCC* (Thiagalingam *et al.*, 1996; Riggins *et al.*, 1996; Hahn *et al.*, 1996). As other studies have shown that 18q LOH is frequent in gastric carcinomas, these two genes may be altered in some gastric tumours too.

4.7. Alterations of the *p53* gene in gastric cancer

Mutations of the *p53* gene are the most common genetic alterations known to occur in human cancer. In contrast to the *APC* gene, the majority of *p53* mutations are missense mutations, and these are clustered in the central region of the gene that

corresponds to the sequence-specific DNA binding domain (Hollstein *et al.*, 1991; 1994). In this study, the entire coding region of the *p53* gene was screened for mutations using SSCP and heteroduplex analysis. Somatic mutations of *p53* were identified in 8 of 26 (31%) gastric carcinomas. A similar frequency of *p53* mutation has been reported in other studies of gastric cancer (33%-38%) (Tamura *et al.*, 1991; Seruca *et al.*, 1992; Uchino *et al.*, 1993; Schneider *et al.*, 1994; Poremba *et al.*, 1995). However other studies have reported higher frequencies (50%-65%) (Kim *et al.*, 1991; Matozaki *et al.*, 1992; Imazeki *et al.*, 1992; Renault *et al.*, 1993; Hongyo *et al.*, 1995).

The varying frequency of *p53* gene mutations in gastric cancer could reflect differences in processing of tumour tissue samples for DNA extraction or in mutation detection technique used. Generally a higher incidence of *p53* mutation has been detected in gastric carcinomas using sequencing or DGGE analysis (Kim *et al.*, 1991; Matozaki *et al.*, 1992; Imazeki *et al.*, 1992; Renault *et al.*, 1993) than by using SSCP analysis (Tamura *et al.*, 1991; Uchino *et al.*, 1993; Schneider *et al.*, 1994; Poremba *et al.*, 1995). Hongyo *et al.* (1995) however detected somatic mutations of *p53* in 65% of gastric carcinomas, but these authors carried out microdissection to enrich for tumour cells. As previously discussed the sensitivity of SSCP analysis (section 4.1.1) is affected by the length of the PCR fragment being analysed (Sheffield *et al.*, 1993; Sarkar *et al.*, 1992a). Sheffield *et al.* reported that the efficiency of detection of single base substitutions is greatest in fragments of 135-200bp. In the current study, the p53 exon 6, 7, 10, and 11 amplicons were between 139bp and 236bp and were thus of optimal length for SSCP analysis. The exons 2-3, 4, 5, and 8-9 amplicons were larger in size (271bp-330bp) which may have led to a decreased sensitivity of base substitution detection.

As observed for other tumour types, the majority of somatic *p53* mutations (8 of 9) detected in gastric carcinomas in this study were located in the sequence-specific DNA binding domain which spans amino acid residues 102 to 292 (Pavletich *et al.*, 1993; Bargonetti *et al.*, 1993). Wild-type p53 acts as a transcription factor. A transcriptional activation domain is located at the amino-terminus of the p53 protein (Fields and Jang, 1990; Raycroft *et al.*, 1990). The major tumour suppressive function of p53 appears to be in mediating a response to DNA damage, by inducing either cell cycle arrest or apoptosis, thereby preventing the accumulation of potentially oncogenic mutations and genomic instability (see section 1.3.2d). The ability of p53 to activate the transcription of genes containing p53 binding sites appears to be required for mediating this response, thus mutations in the sequence-specific binding domain would be expected to severely disrupt the biological activity

of p53. p53 is thought to bind to DNA as a tetramer. An oligomerization domain has been localised to the carboxy-terminus of the protein (residues 312-365) (Pavletich *et al.*, 1993; Cho *et al.*, 1994). One of the 9 mutations detected in this study, a nonsense mutation at codon 342, is predicted to cause truncation of the p53 protein within this domain. Thus this mutation could alter the biological function of the p53 protein.

The majority of mutations detected in the sequence-specific DNA binding core domain (5 of 8) were missense mutations, as observed in previous studies of gastric and other tumour types. One missense mutation occurred at codon 282 which is a particular mutational hotspot accounting for approximately 4% of *p53* mutations (Hollstein *et al.*, 1994). Analysis of a crystal structure of a complex containing this p53 core domain and a DNA binding site showed that the wild-type arginine residue at codon 282 appears to play a critical role in stabilizing the structure of the DNA binding surface of p53 (Cho *et al.*, 1994). Studies of tumour-derived p53 mutants which contain missense mutations in the DNA binding domain have shown that they are defective in sequence-specific DNA binding, and consequently they cannot activate transcription of genes downstream of a p53 DNA binding site (Kern *et al.*, 1992; Unger *et al.*, 1992). One mutation that was detected in the sequence-specific DNA binding domain, at codon 161, was a 2bp deletion predicted to result in truncation of the p53 protein. Another tumour, from case GACA15, was found to contain two truncating (nonsense) mutations at codons 103 and 104. Both mutations are thought to occur on the same allele as sequencing analysis demonstrated loss of the wild-type sequence at both codons. These mutations in adjacent codons may have been induced by a specific mutagen, possibly present in food. It is predicted that the mutant gene product would lack the sequence-specific DNA binding core domain and the carboxy-terminus as the truncating mutations occur at the beginning of the core domain which is located between residues 102 and 292.

Analysis of an extensive database of somatic mutations of the *p53* gene in human tumours and cell lines (Hollstein *et al.*, 1994) revealed that the mutations detected at codons 193, 195, 237, 266, 282, and 342 in this study had previously been observed in a variety of malignancies. At codon 161 missense mutations have been observed in other studies. Only one study reported a mutation at codon 103 (a missense mutation in a bladder carcinoma) and one other study reported a mutation at codon 104 (a 1bp deletion in a skin tumour). Thus the point mutations detected at codons 103 and codon 104 in tumour GACA15 may indeed have been caused by a gastric-specific carcinogen. It is interesting that both mutations were protein truncating mutations.

Although mutations of the *p53* gene are commonly detected in human cancer the nature of *p53* point mutation varies among different malignancies and appears to be related to organ site and specific aetiological agent. G:C to A:T transitions at CpG dinucleotide pairs are frequent in colorectal, brain, and lymphoid malignancies (Hollstein *et al.*, 1991). G to T transversion at codon 249 predominantly occurs in hepatocellular carcinomas in individuals in the high-risk regions. Infection with hepatitis B virus and exposure to the potent liver carcinogen aflatoxin B1 are thought to be the responsible factors. G:C to T:A transversions also predominate in lung cancers and are thought to be induced by carcinogens in tobacco smoke (Hollstein *et al.*, 1991). Mutations at dipyrimidine sequences, particularly CC to TT transitions, are common in skin cancers and appear to be induced by ultraviolet light (Brash *et al.*, 1991).

Hongyo *et al.* (1995) summarized ten published studies on p53 gene mutations in gastric cancers and reported that the pattern of mutations differed significantly between European and Oriental cases. G:C to A:T transitions were significantly more frequent in European gastric tumours than in Oriental tumours whereas A:T to G:C transitions and transversions both represented a significantly greater proportion of Oriental tumours. This difference in mutation type may reflect the action of specific carcinogens possibly in the diet. The European studies that Hongyo *et al.* (1995) summarized were on gastric cancers from patients living in high risk areas in Italy and Portugal. In the present study on British gastric carcinomas most of the single base substitutions (5 of 8) were G:C to A:T transitions. G:C to A:T transitions are specifically induced by N-methyl-N'-nitro-N-nitrosoguanidine (Kohalmi and Kunz, 1988). It is believed that carcinogenic N-nitroso compounds are produced in the stomach from nitrite and other nitrogen-containing compounds derived from food (Mirvish, 1983). Two of the C to T transitions occurred at CpG dinucleotide pairs. Spontaneously occurring deamination of 5'-methylcytosine in the CpG dinucleotide has been proposed as a mechanism underlying C to T transition (Coulondre *et al.*, 1978).

In this study although *p53* gene mutations were detected more frequently in intestinal type than diffuse type gastric carcinomas, the difference was not statistically significant. Imazeki *et al.* (1992) and Tamura *et al.* (1991) also reported no correlation between *p53* mutation and histological type of gastric cancer but Uchino *et al.* (1993b) reported an association with the intestinal type.

During screening of the *p53* gene for mutations using SSCP and heteroduplex analysis, common polymorphisms were detected in intron 2 (position 11827) and

exon 4 (codon 72). Analysis of 26 gastric carcinoma cases found these two polymorphisms to be in linkage disequilibrium. The frequency of heterozygosity at both loci was 27%. The codon 72 polymorphism has been detected by *Acc* II restriction enzyme digestion in previous studies in which the reported frequency of constitutional heterozygosity varies from 27% to 52% (Meltzer *et al.*, 1991; Oka *et al.*, 1991; Dockhorn-Dworniczak *et al.*, 1994). Oliva *et al.* (1995) also detected the intron 2 polymorphism using SSCP analysis and reported that 41.5% of individuals tested were heterozygous at this site, a higher rate than observed in the present study although their sample size was larger (135 individuals). In addition to the codon 72 and intron 2 polymorphisms a microsatellite repeat polymorphism at the *p53* locus (*p53CA*) was analysed for LOH. LOH of *p53* was detected in 37.5% (9 of 24) gastric carcinomas. A similar frequency of LOH at polymorphic loci of *p53* has been reported in other studies on gastric carcinomas: 26%-37% cases in the studies of Dockhorn-Dworniczak *et al.* (1994) and Renault *et al.* (1993), and 45% cases in the study of Tamura *et al.* (1996a). Higher rates have been described though: 64% cases in the study of Rhyu *et al.* (1994a).

Loss of the wild-type *p53* allele was observed in the majority of gastric carcinomas (7 of 8) harbouring *p53* gene mutations in this study. Similar observations have been made in most tumour types (Nigro *et al.*, 1989; Baker *et al.*, 1990a). In two gastric carcinomas that exhibited *p53* LOH, mutation was not detected in the retained *p53* allele. It is possible that *p53* mutations in these tumours are located in regions of the gene that were not examined, for example the promoter region, intronic sequence, or the 3' untranslated region. The PCR primers used for amplifying the *p53* coding region are those described by Williamson *et al.* (1994) and most lie across intron-exon borders, thus it is possible that mutations at splice sites or at the 5' or 3' ends of exons could not be detected. Alternatively it is possible that mutations present in the parts of the gene analysed were not detected by the SSCP and heterodupex assay used. One gastric carcinoma was found to contain a *p53* gene mutation but did not exhibit LOH. It is possible that this tumour harboured an intragenic mutation in the other *p53* allele that was not detected in the present study.

There is evidence however that some *p53* missense mutations are dominant negative mutations of wild-type p53 function. Milner and Medcalf (1991) demonstrated that cotranslation of mutant and wild-type p53 resulted in mixed oligomers, and the mutant form apparently drove the wild-type form into the mutant conformation within the complex. Harvey *et al.* (1993) mated p53-deficient mice (either nullizygous or heterozygous) with mice containng multiple copies of a *p53* mutant transgene (codon 135 Val to Ala). Intercrosses between the F1 offspring of

these mice generated F2 mice of six possible genotypes: $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$, each either with or without the mutant transgene. Expression of the mutant protein accelerated tumour development in $p53^{+/+}$ and $p53^{+/-}$ animals but had no such effect in $p53^{-/-}$ mice. Thus the mutant protein demonstrated a dominant negative effect in the presence of wild-type p53 but showed no gain of function in the absence of wild-type p53. Furthermore although the mutant transgene accelerated the rate of tumour development in $p53^{+/-}$ mice, this rate remained slower than that of the $p53^{-/-}$ mice indicating the dominant negative effect was only partial (Harvey *et al.*, 1993). This observation may explain why there is selection for loss of the wild-type *p53* allele in human tumours with a mutation in the other *p53* allele.

4.8. RER at microsatellite repeat loci in gastric cancer

RER was detected in 3 of 26 (11.5%) gastric carcinomas. One carcinoma exhibited RER at multiple loci (6 of 12 loci analysed, 50%). However two other tumours each exhibited RER at only 1 of 12 loci (8.3%) analysed. In these tumours RER at 1 locus could represent a PCR artefact and not reflect a true RER phenotype. Alternatively as discussed in section 4.2 it could reflect mutation in a gene involved in DNA repair or replication that does not result in extensive instability at short repeated sequences. Inactivation of one known DNA mismatch repair gene, *hMSH6*, is associated with a low degree of RER (Papadopoulos *et al.*, 1995; Akiyama *et al.*, 1997a). It is also possible that the two tumours in which RER was detected at 1 locus did in fact exhibit RER at further loci but this was not detected by the SSCP method used.

In other studies investigating RER in gastric carcinomas, the majority of authors have defined RER+ tumours asthose exhibiting RER at one or more microsatellite repeat loci. Most studies report an RER frequency of approximately 30% (Han *et al.*, 1993; Chong *et al.*, 1994; Rhyu *et al.*, 1994b; Seruca *et al.*, 1995; Lin *et al.*, 1995; Dos Santos *et al.*, 1996; Semba *et al.*, 1996; Renault *et al.*, 1996; Wu *et al.*, 1997b; Ottini *et al.*, 1997) although some others report a lower frequency (14%-18%) tumours (Mironov *et al.*, 1994; Strickler *et al.*, 1994; Nakashima *et al.*, 1995; Tamura *et al.*, 1995; Buonsanti *et al.*, 1997). Using more stringent criteria for definition of RER+, some studies report that RER at 2 or more loci has been observed in 23%-30% gastric carcinomas (Rhyu *et al.*, 1994b; Seruca *et al.*, 1995; Dos Santos *et al.*, 1996; Ottini *et al.*, 1997; Wu *et al.*, 1997b). In the present study RER at 2 or more loci was observed in just one (5%) gastric tumour. Some other studies report

low frequency of RER at 2 or more loci too: 4-5% carcinomas in the studies of Mironov *et al.* (1994), Keller *et al.* (1995), Buonsanti *et al.* (1997), and 8-12% carcinomas in the studies of Tamura *et al.* (1995) and Semba *et al.* (1996).

The varying frequency of RER observed in gastric carcinomas may reflect differences in methodology. In the various studies different loci have been analysed, the number of loci analysed varies, and different methods of sample preparation were used, for example some authors performed microdissection of tumour specimens. Interestingly in another study of British gastric carcinomas, 12 microsatellites were analysed in 10 cases but no RER was detected (Hayden *et al.*, 1997) which together with the results from the present study may suggest that defective DNA mismatch repair is not important in the development of gastric cancer in the British population. Hayden *et al.* (1997) however only analysed tumours from patients under 40 years of age, therefore there may not have been enough time for RER to occur. On the other hand, RER has been detected in precancerous lesions including intestinal metaplasia, dysplasia, and gastric adenoma, suggesting that it is an early event in gastric tumourigenesis (Rhyu *et al.*, 1994b; Semba *et al.*, 1996; Ottini *et al.*, 1997). Dos Santos *et al.* (1996) analysed 6 microsatellite repeat loci in 61 gastric carcinoma cases and reported that carcinomas with RER at 1 or 2 loci shared clinicopathologic features with RER- carcinomas that differed from those of carcinomas with multiple RER+ loci. Similar observations have been made in colorectal cancer (Lothe *et al.*, 1993). The gastric carcinomas with RER at multiple loci were all of the intestinal type, had a lower DNA content, less prevalent lymph node metastasis, and better survival rates than RER+ at 1 or 2 loci and RER- carcinomas. The authors concluded that the finding of RER at a single or few loci does not qualify a gastric cancer case as a mutator phenotype from a clinical standpoint (Dos Santos *et al.*, 1996).

In this study, the tumour that displayed RER at multiple loci was of the intestinal type as was one of the RER+ at 1 locus tumours. The other tumour with RER was a diffuse type gastric carcinoma. In contrast to Dos Santos *et al.* (1996) many studies report no difference between intestinal and diffuse type carcinomas with respect to the prevalence of RER (Rhyu *et al.*, 1994b; Lin *et al.*, 1995; Nakashima *et al.*, 1995; Renault *et al.*, 1996; Wu *et al.*, 1997b).

Keller *et al.* (1995) and Ottini *et al.* (1997) both reported that in families of patients with RER gastric cancer was found significantly more frequently in first degree relatives than in patients without RER, but Chong *et al.* (1994) and Strickler *et al.* (1994) found no correlation between family history of gastric cancer and RER. A positive association between RER+ phenotype and family history may reflect

exposure to aetiological factors shared by close relatives. Alternatively given the fact that gastric cancer occurs commonly in the HNPCC syndrome, the RER+ phenotype may reflect a genetically determined gastric cancer susceptibility in some cases. In support of this suggestion Keller *et al.* (1996) identified a germline missense mutation of the *hMLH1* gene in a patient with an RER+ gastric carcinoma and a family history consisting of one second degree relative affected with gastric cancer. In the current study none of the 3 gastric carcinoma cases with RER was reported to have a family history of cancer.

Overall the evidence suggests that RER is an important event in a subset of gastric cancer. It remains to be seen which mismatch repair gene is most frequently altered in these tumours. In a few cases, the responsible gene may be mutated in the germline. Furthermore, it has been shown that similar to RER+ colorectal carcinomas, RER+ gastric carcinomas harbour frameshift mutations in mononucleotide repeat tracks within the coding regions of the TGFβ receptor type II gene, *Bax* gene, and *hMSH3* and *hMSH6* genes (Myeroff *et al.*, 1995; Yamamoto *et al.*, 1997) suggesting that a similar mutator phenotype is involved in the development of these two types of tumours.

4.9. LOH at microsatellite repeat loci in gastric cancer

In gastric carcinomas, the highest rates of LOH were observed at *p53CA* and at a microsatellite repeat locus on chromosome 22q (*D22S351*) (30% tumours). In addition statistical analysis demonstrated that LOH at *D22S351* was significantly associated with LOH at *p53*. This may suggest that alteration of a putative gastric cancer tumour suppressor gene on chromosome 22q and alteration of the *p53* gene may occur at a similar stage of gastric cancer development. Interestingly mutations of the *p53* gene have been detected in gastric cancer precursor lesions: Shiao *et al.* (1994) observed *p53* gene mutations in 37.5% of intestinal metaplasia cases and in 58.3% of gastric dysplasia cases and Tohdo *et al.* (1993) detected mutations of *p53* in 30% gastric adenomas.

Some other studies on gastric carcinomas report a similar frequency of chromosome 22q LOH to that observed in the current investigation: 26% cases in the study of Schneider *et al.* (1995) and 17% cases in the study of Tamura *et al.* (1996a). The neurofibromatosis 2 (NF2) tumour suppressor gene is located at chromosome 22q12. Patients with germline mutations of the *NF2* gene characteristically develop tumours of the brain and nervous system (Trofatter *et al.*, 1993; Rouleau *et al.*, 1993).

The *NF2* gene could be the target of the allelic loss observed in gastric carcinomas. The *D22S351* marker analysed in this study has been localised to chromosome 22q11.2. LOH studies in a number of tumour types, including ovarian cancer, colorectal cancer, breast cancer, and meningioma have suggested the existence of a tumour suppressor gene distal to the *NF2* locus on chromosome 22q (Akagi *et al.*, 1995; Yana *et al.*, 1995; Bryan *et al.*, 1996). It is possible that this gene may be altered in gastric carcinomas too. Alternatively an unmapped tumour suppressor gene on chromosome 22q may be the target of LOH in gastric tumours.

LOH at a microsatellite repeat locus on chromosome 14q11 was detected in 16% gastric carcinomas. Schneider *et al.* (1995) detected 14q LOH in gastric carcinomas at a similar frequency (17%) but Tamura et al. (1996a) and Gleeson *et al.* (1997) observed higher frequencies (38-47%). LOH studies in several tumour types including carcinomas of the colorectum, endometrium, bladder, and ovary indicate the presence of a tumour suppressor gene at the distal portion of chromosome 14q (14q32) (Young *et al.*, 1993a; Fujimo *et al.*, 1994; Chang *et al.*, 1995; Bandera *et al.*, 1997). Furthermore investigation of ovarian carcinomas and bladder carcinomas suggests that a second tumour suppressor gene resides more proximally, at 14q12 (Bandera *et al.*, 1997; Chang *et al.*, 1995). These two genes may play a role in gastric tumourigenesis too.

LOH at a microsatellite repeat locus on chromosome 4q21 was observed in 19% gastric carcinomas. A similar frequency of 4q LOH in gastric cancer has been reported by Tamura *et al.* (1996b) (17%) and Schneider *et al.* (1995) (21%). However Gleeson *et al.* (1997) analysed 6 microsatellite repeat markers from chromosome 4q in gastric carcinomas and detected LOH in 70% cases. Interestingly deletion mapping studies in other tumour types such as oesophageal cancer, head and neck carcinoma, cervical carcinoma, and bladder carcinoma suggest the presence of a tumour suppressor gene(s) on the long arm of chromosome 4 (Hammoud *et al.*, 1996; Perhouse *et al.*, 1997; Mitra *et al.*, 1994; Polascik *et al.*, 1995).

Three microsatellite repeat markers from chromosome 2p16 which are closely linked to the *hMSH2* gene were analysed in gastric carcinomas. LOH on 2p16 was detected in 2 (7%) gastric carcinomas (GACA4 and GACA15). In both cases the deletion at 2p16 encompassed the *hMSH2* gene. One case, GACA15, was constitutionally heterozygous for a T to C transition polymorphism within the *hMSH2* gene that was detected by SSCP and heteroduplex analysis. Allele loss was detected in tumour GACA15 at this locus. The low level of chromosome 2p LOH detected in gastric carcinomas may suggest that the loss observed in tumours GACA4 and

GACA15 are nonsignificant random events. Alternatively it is possible that the two gastric carcinomas had a mutation in the retained *hMSH2* allele. Mutation in both alleles of the *hMSH2* gene has been observed in tumours from HNPCC patients (Leach *et al.*, 1993). Information on family history of cancer was available for GACA4 and GACA15 but both cases showed a negative history. RER would be expected to be observed if the *hMSH2* mismatch repair gene was inactivated but RER was not detected in the two gastric carcinomas. It is possible that somatic alterations of the *hMSH2* gene occurred at a very late stage of tumour development in these two carcinomas so that a mismatch repair deficiency was not manifest as widespread RER. Alternatively another tumour suppressor gene on chromosome 2p may have been the target of allele loss. Another DNA mismatch repair gene, *hMSH6*, is located on chromosome 2p16 at a distance of approximately 0.5Mb from *hMSH2* and could be the target of LOH. Interestingly tumour cell lines with mutations in both alleles of *hMSH6* demonstrate a less severe phenotype compared to cells with other mismatch repair gene defects (Papadopoulos *et al.*, 1995). The tumours from GACA4 and GACA15 demonstrate LOH at several other microsatellite repeat loci on different chromosomes (table 3.16) rather than RER thus these tumours probably arose through genetic mechanisms involving mutation in tumour suppressor genes followed by LOH rather than a defect in the DNA mismatch repair system.

No LOH was observed at microsatellite loci on chromosomes 9p, 16q, and 19q. In other gastric cancer studies a varying frequency of LOH on each of these chromosomes has been described: 24-65% for chromosme 9p, 5-40% for chromosome 16q, 15-44% for chromosome 19q (Schneider *et al.*, 1995; Tamura *et al.*, 1996a; Gleeson *et al.*, 1997). LOH at microsatellite repeat loci was predominantly detected in intestinal type gastric carcinomas in this study. Only 1 of 9 diffuse type carcinomas (GACA2) demonstrated LOH. This was at *p53CA* and *D22S351*. Infiltration of diffuse type carcinomas with non-neoplastic stromal cells is usually much greater than in intestinal type carcinomas which most likely explains why a low frequency of LOH was detected in diffuse gastric carcinomas in this study. An alternative explanation is that the results reflect a differing molecular pathogenesis for intestinal and diffuse type carcinomas. The carcinoma from case GACA2 may have been histologically heterogenous and the part of the tumour from which DNA was extracted showed diffuse type characteristics.

4.10. Alterations of the *hMSH2* gene in gastric cancer

A mutation in the *hMSH2* gene was detected in both tumour and normal tissue DNA from 1 of 26 gastric carcinoma cases (GACA6) using SSCP and heteroduplex analysis. The mutation was characterized as a duplication of 4 bases in intron 12, at 31 base pairs upstream of the beginning of exon 13. This intronic alteration could represent either a pathogenic mutation or a sequence variation of no consequence. It is possible that the insertion could result in a defect in splicing. In addition to the intronic 5' splice donor site and 3' splice acceptor site, an intronic branch site is essential for splicing out of introns from mRNA precursors. The 2'-OH of an adenosine residue in the branch site attacks the 5' splice site to form a lariat intermediate. Splicing occurs within a spliceosome, a complex assembly of small ribonucleoprotein particles (snRNPs) composed of a variety of small nuclear RNAs and associated proteins. U2 snRNP binds to the branch site. Branch site sequences are typically located 20 to 50 nucleotides upstream of the 3' splice site (Sharp, 1987). As the 4bp insertion detected in case GACA6 is 31bp upstream of a 3' splice site it is possible that it led to the branch site not being recognised resulting in defective splicing.

Unfortunately a blood sample was not available to analyse from case GACA6 to verify the germline status of the *hMSH2* gene alteration. Several factors are supportive of the alteration being a causative mutation. Case GACA6 was only 35 years of age at the time of the gastrectomy, much younger than most of the other gastric cancer patients investigated in this study. Gastric cancer occurs commonly in the HNPCC syndrome. Patient GACA6 is not believed to have a family history of HNPCC, however the alteration could represent a de novo mutation. Case GACA6 developed a diffuse type gastric carcinoma. Interestingly in previous studies it is gastric cancer cases with the diffuse type that have been associated with a familial clustering of gastric cancer (Lehtola, 1978; Mecklin *et al.*, 1988; Zanghieri *et al.*, 1990). However if the *hMSH2* gene alteration is an inactivating mutation this would not be compatible with the fact that RER was not detected in the tumour from case GACA6.

4.11. Conclusions

The development of cancer is a multistep process involving the accumulation of multiple genetic alterations. This has been demonstrated in the present study on gastric and colorectal cancer. Multiple genetic changes were observed in many tumours. In the generally accepted model of colorectal tumourigenesis, inactivation of the *APC* gene is proposed to be a very early if not initiating event, and mutation of the K-*ras* gene, a gene on chromosome 18q, and the *p53* gene are proposed to occur at progressively later stages of tumour development. An accumulation of alterations in other oncogenes and tumour suppressor genes occurs too. In a subset of colorectal tumours mismatch repair deficiency occurs, manifest as replication error (RER) at short repeated sequences. The results of this study were supportive of alteration of the *APC* gene and RER being important events in colorectal tumourigenesis. LOH on chromosome 18q and the the *p53* locus were also frequently observed.

In gastric cancer however a low frequency of alteration of the *APC* and *MCC* genes on chromosome 5q and of the *DCC* gene on chromosome 18q was observed in this study, indicating that these genes do not play a significant role in gastric tumourigenesis. Alterations of the *p53* gene were frequently observed in gastric carcinomas suggesting that this gene is important in gastric tumourigenesis. However *p53* gene alterations are commonly detected in may different types of human cancer. LOH involving the *hMSH2* mismatch repair gene was observed in two gastric carcinomas. In a further gastric carcinoma case a sequence variation of *hMSH2* was detected in both tumour and normal tissue. Although the significance of this alteration is not known, these results indicate that *hMSH2* is involved in the development of gastric cancer as well as colorectal cancer. A lower prevalence of RER was detected in this series of gastric carcinomas than has been found in colorectal carcinomas. As the intestinal type of gastric cancer shares histological features with tumours of the large intestine it has been suggested that this type of gastric carcinoma in particular may share a common pathogenesis with colorectal cancer. However the results of the present study are not supportive of this hypothesis. In other studies of gastric cancer a lower frequency of *APC* gene mutation and of LOH in the region of the *DCC* gene on chromosome 18q has been observed than in studies of colorectal cancer. Although mutations of the K-*ras* gene were not investigated in this study others have reported that they are infrequent in gastric carcinomas, further support for a differing molecular pathogenesis for gastric and colorectal cancer.

Somatic genetic alterations were not detected in one half (13 of 26) of the gastric carcinomas that were analysed in this study. It is highly probable that contamination of tumour specimens with normal cells contributed to the low frequency of detection of genetic alterations. Because of the infiltrating nature of its growth diffuse type gastric carcinoma in particular contains a high proportion of non-neoplastic stromal cells. Somatic genetic alterations were detected in only 2 of 9 diffuse gastric carcinomas. In future microdissection of neoplastic cells from tumour tissue should be performed. LOH in tumour cells in particular may be masked by contaminating normal cells. Nevertheless LOH analysis demonstrated frequent LOH at a locus on chromosome 22q11 in gastric carcinomas. Analysis of further microsatellite repeat markers from chromosome 22q may identify a minimally deleted region and indicate a locus for a tumour suppressor gene that may play a significant role in the development of gastric cancer. It is concluded that tumour suppressor genes other than those investigated in the present study must play a more important role in gastric tumourigenesis. Identification of these genes represents a major challenge for the future.

The relatively new technique of comparative genomic hybridization (CGH) could help in the detection of such genes. This method involves screening the tumour genome for DNA sequence copy number changes, providing a map of chromosomal regions that are gained or lost in a DNA sample. A mixture of tumour and normal DNA, each labelled with a different fluorochrome, is hybridized to a normal metaphase chromosome spread. Differences in copy number between the tumour and normal genomes are indicated as differing ratios of the fluorescence intensity of the two probes along the normal metaphase chromosomes. The consistent observation of gain or loss of a particular chromosomal region in gastric cancers may indicate the presence of genes important in gastric tumourigenesis.

5.

Appendix

5. APPENDIX

A1. Standard buffers and solutions

TBE: 90mM Tris-HCl pH 8
90mM Boric acid
2mM EDTA

TE: 1mM Tris-HCl pH 8
0.1mM EDTA

TAE: 40mM Tris-HCl pH 8
20mM sodium acetate
6.25% (v/v) glacial acetic acid
2mM EDTA

Tris-glycine buffer: 0.02M Tris base
0.192M glycine
0.1% (w/v) SDS

STE: 150mM NaCl
10mM Tris-HCl pH 8
10mM EDTA

PBS: 10xPBS (Gibco) diluted to working strength with sterile distilled deionised water and neutralised with sterile 1M NaOH

Loading buffer for agarose gels (10x): 40% (w/v) sucrose
0.025% (w/v) bromophenol blue
0.025% (w/v) xylene cyanol

Loading buffer for SDS-polyacrylamide gels: 0.06M Tris-HCl pH 6.8
10% (v/v) glycerol
2% (w/v) SDS
5% (v/v) b-mercaptoethanol
0.025% (w/v) bromophenol blue

Formamide stop solution: 95% formamide
20mM EDTA
0.05% (w/v) bromophenol blue
0.05% (w/v) xylene cyanol

A2. Solutions used for DNA extraction

Phenol:

400ml of a TE buffer (1M Tris, 0.25M EDTA was added to 500g of solid phenol. This mixture was shaken vigorously and placed at 37°C to dissolve the phenol. 0.1% (w/v) hydroxyquinilone was added to the phenol and mixed.

Chloroform:

24 parts of chloroform to 1 part isoamyl alcohol were mixed, prior to use.

Reagent B:

400mM Tris-HCl pH 8
60mM EDTA
150mM NaCl
1% (w/v) SDS

A3. Polymerase chain reaction (PCR)

A3.1. Oligonucleotide primers

All primers are read in the 5' to 3' direction. The forward primer in each pair lies 5' of the DNA segment to be amplified, and the reverse primer lies 3' of the DNA segment to be amplified.

A3.1.1. Primers for amplification of segments of the *APC* gene

A3.1.1 (a) Primers used for SSCP and heteroduplex analysis

Exon	Codons contained in amplicon	Primer sequences[b]	Annealing temp. used (°C)	Length of amplicon (bp)	Reference
6	216-243	CAAGGATCCTGAGCTTTTAAGTGGTAG CTGAAGCTTTTCTCAGAATAACTACCTA	53	235	Ando *et al.* (1993)
8	279-311	CATGATGTTATCTGTATTTACC CTTAGCAAAGTAGTCATGGC	51	215	Ando *et al.* (1993)
11	470-516	GATGATTGTCTTTTTCCTCTTGC CTGAGCTATCTTAAGAAATACATG	53	215	Kraus and Ballhausen (1992)
14	582-653	CAACTCTAATTAGATGACCCA GAGAGTATGAATTCTGTACTT	53	303	Ando *et al.* (1993)
15A[a]	653-759	GTTACTGCATACACATTGTGAC GCTTTTTGTTTCCTAACATGAAG	52	371	Groden *et al.* (1991)
15B	736-851	AGTACAAGGATGCCAATATTATG ACTTCTATCTTTTTCAGAACGAG	52	346	Groden *et al.* (1991)
15C	825-957	ATTTGAATACTACAGTGTTACCC CTTGTATTCTAATTTGGCATAAGG	52	397	Groden *et al.* (1991)
15D	927-1054	CTGCCCATACACATTCAAACAC TGTTTGGGTCTTGCCCATCTT	53	381	Groden *et al.* (1991)
15E	1028-1171	AGTCTTAAATATTCAGATGAGCAG GTTTCTCTTCATTATATTTTATGCTA	51	429	Groden *et al.* (1991)
15F	1139-1283	AAGCCTACCAATTATAGTGAACG AGCTGATGACAAAGATGATAATG	53	434	Groden *et al.* (1991)
15G	1256-1383	AAGAAACAATACAGACTTATTGTG [c] ATGAGTGGGGTCTCCTGAAC	53	381	Groden *et al.* (1991)
15H	1359-1499	ATCTCCCTCCAAAAGTGGTGC TCCATCTGGAGTACTTTCTGTG	61	420	Groden *et al.* (1991)
15I	1471-1640	AGTAAATGCTGCAGTTCAGAGG CCGTGGCATATCATCCCCC	62	507	Groden *et al.* (1991)
15J	1595-1700	CCCAGACTGCTTCAAAATTACC GAGCCTCATCTGTACTTCTGC	61	317	Groden *et al.* (1991)

[a] Exon 15 is very large (approximately 6.5 kb) therefore Groden *et al.* (1991) designed oligonucleotide primers to amplify this exon in 23 overlapping segments (termed 15A - W). In this study primer pairs 15A to 15J were used.

[b] The first primer listed in each pair is the forward primer, the second is the reverse primer. Primers for amplifying exon 6, 8, 11, and 14, and the forward primer for exon 15A lie in intronic sequence flanking the exons. The other primers are located within exon 15. PCR conditions used for each primer set were as described in section 2.2.3 (b).

[c] Instead of this primer, the following forward primer was used to generate PCR product for sequencing: GGCTGCCACTTGCAAAGTTTC. This primer lies 32 bp upstream of the published primer.

A3.1.1 (b) Primers used for protein truncation test (PTT) analysis

The forward primer was described by van der Luijt *et al.* (1994) and was the 15E forward primer modified to include a T7 promoter sequence for transcription initiation and Kozak consensus sequence for translation initiation at the 5' end, in frame with *APC* unique sequence:GGATCCTAATACGACTCACTATAGGAACAG ACCACCATGCTTAAATATTCAGATGAGCAGTTGAA. The reverse primer used was the 15J reverse primer (A3.1.1a). The PCR conditions were an initial denaturation step at 94°C for 4.5 minutes followed by 35 cycles of:

94°C for 30 seconds (denaturation)
63°C for 45 seconds (primer annealing)
72°C for 1 minute (elongation).

On completion of the final cycle a further elongation at 72°C for 10 minutes was carried out. A 2 kb product encompassing codons 1028-1700 was generated.

A3.1.1 (c) Primers used for LOH analysis at polymorphic sites in exon 15N and the 3' untranslated region

Primer sequences for the *Msp* I polymorphic site in exon 15 region N (codon 1960) were described by Cottrell *et al.* (1992):
Forward primer: ATGATGTTGACCTTTCCAGGG
Reverse primer: CTTTTTTGGCATTGCGGAGCT.
PCR conditions were an initial denaturation step at 94°C for 4 minutes followed by 30 cycles of:

94°C for 1 minute (denaturation)
60°C for 1 minute (primer annealing)
72°C for 1 minute (elongation).

On completion of the final cycle a further elongation at 72°C for 10 minutes was carried out. A product of 541 bp was generated.

Primer sequences for the *Ssp* I polymorphic site in the 3' untranslated region of *APC* were described by Heighway *et al.* (1991):
Forward primer: GCATTAAGAGTAAAATTCCTCTTAC
Reverse primer: ATGACCACCAGGTAGGTGTATT.
PCR conditions were an initial denaturation step at 94°C for 4.5 minutes followed by 30 cycles of:

94°C for 45 seconds (denaturation)
55°C for 45 seconds (primer annealing)
74°C for 1.5 minutes (elongation).

A product of 850 bp was generated.

A3.1.2. Primers for amplification of polymorphic sites in the *MCC* gene

Polymorphic site	Primer sequences	Annealing temperature	Length of amplicon	Reference
3' untranslated region	CCAATGAAACTTCGCTTTAATCAG CTGGATACAGTCCACAATGACAC	63°C	210 bp	Curtis *et al.* (1994)
Exon 10	TACGAATCCAATGCCACA CTGAAGTAGCTCCAAACA	57°C	79 bp or 93 bp	Greenwald *et al.* (1992)

The first primer listed in each pair is the forward primer, the second is the reverse primer. PCR conditions used for each primer set were as described in section 2.2.3 (b).

A3.1.3. Primers for amplification of *Msp* I polymorphic sites in the *DCC* gene

Polymorphic site	Primer sequences	Annealing temperature	Length of amplicon	Reference
M2	TGCACCATGCTGAAGATTGT AGTACAACACAAGGTATGTG	55°C	396 bp	Huang *et al.* (1992)
M3	CGACTCGATCCTACAAAATC TCTACCCAGGTCTCAGAG	55°C	240 bp	Parry *et al.* (1991)

The first primer listed in each pair is the forward primer, the second is the reverse primer. PCR conditions used for each primer set were as described in section 2.2.3 (b).

A3.1.4. Primers for amplification of exons 12-14 of the *hMSH2* gene

Exon	Codons contained in amplicon	Primer sequences	Annealing temp. used (°C)	Length of amplicon (bp)	Reference
12	587-669	ACAGGCTATGTAGAACCAATGC AAAAACAAAACGTTACCCCC	58	297	This study
13	669-737	CGCGATTAATCATCAGTG GGACAGAGACATACATTTCTATC	58	352	Fishel *et al.* (1993)
14	737-820	TGTTACCACATTTTATGTGATG CATACCTTTCTTCACCTGATAA	58	303	This study

The first primer listed in each pair is the forward primer, the second is the reverse primer. The forward primer for amplifying exon 12 and the reverse primer for amplifying exon 14 lie across intron-exon borders, while all other primers lie in intronic sequences flanking the exons. PCR conditions used for each primer set were as described in section 2.2.3 (b).

A3.1.5. Primers for amplification of the *p53* gene

The primers used were those described by Williamson *et al.* (1994):

Exon	Codons contained in amplicon	Primer sequences	Annealing temperature used (°C)	Length of amplicon (bp)
2-3	1-32	TGGATCCTCTTGCAGCAGCC AACCCTTGTCCTTACCAGAA	62	271
4	33-125	ATCTACAGTCCCCCTTGCCG GCAACTGACCGTGCAAGTCA	63	293
5-6	126-201	TTCCTCTTCCTGCAGTACTC GCAAATTTCCTTCCACTCGG	61	325
5-6	179-224	ACCATGAGCGCTGCTCAGAT AGTTGCAAACCAGACCTCAG	61	236
7	225-261	GTGTTGTCTCCTAGGTTGGC CAAGTGGCTCCTGACCTGGA	63	139
8-9	262-331	CCTATCCTGAGTAGTGGTAA CCAAGACTTAGTACCTGAAG	58	330
10	332-367	TGTTGCTGCAGATCCGTGGG GAGGTCACTCACCTGGAGTG	64	139
11	368-393	TCTCCTACAGCCACCTGAAG CTGACGCACACCTATTGCAA	62	202

The first primer listed in each pair is the forward primer, the second is the reverse primer. The primers all lie across intron-exon borders with the exception of the reverse primer used to amplify exon 5 which lies in exon 6, the forward primer used to amplify exon 6 which lies in exon 5, and the reverse primer for amplifying exon 11 which lies in the 3' untranslated region sequence flanking the exon. Exon 2, intron 2, and exon 3 were amplified together using one primer pair, as were exon 8, intron 8, and exon 9. PCR conditions used for each primer set were as described in section 2.2.3 (b).

A3.1.6. Primers for amplification of microsatellite repeat loci

Locus	Chromosomal localization	Primer sequences	Annealing temp. (°C)	Size range of alleles (bp)	Heterozygosity	Reference
D2S119	2p16	CTTGGGGAACAGAGGTCATT GAGAATCCCTCAATTTCTTTGGA	58	214-232	0.80	Gyapay *et al.* (1994)
D2S391	2p16	ATGGAGCCAGTAGGTTACAGC GGTGAGAGGGTATGATGGAA	51	142-152	0.79	Gyapay *et al.* (1994)
D2S123	2p16	AAACAGGATGCCTGCCTTTA GGACTTTCCACCTATGGGAC	58	197-227	0.76	Gyapay *et al.* (1994)
D4S175	4q21	ATCTCTGTTCCCTCCCTGTT CTTATTGGCCTTGAAGGTAG	56	112-134	0.82	Mills *et al.* (1992)
D5S346	5q21-22	ACTCACTCTAGTGATAAATCGGG AGCAGATAAGACAAGTATTACTAGTT	53	96-122	0.83	Spirio *et al.* (1991)
D9S156	9p22-23	ATCACTTTTAACTGAGGCGG AGATGGTGGTGAATAGAGGG	56	133-155	0.79	Gyapay *et al.* (1994)
D14S50	14q11	AACACCCCTAATTCACCACT ATGATTCCACAAGATGGCAG	58	166-180	0.77	Wang and Weber (1992)
D16S266	16q23	AGCTTTACAGATGAGACCAG CAGCCAATTTCTTGAGTCCG	56	94-104	0.59	Weber *et al.* (1990a)
p53CA	17p13	AGGGATACTATTCAGCCCGAGGTG ACTGCCACTCCTTGCCCCATTC	64	103-135	0.90	Jones and Nakamura (1992)
D18S61	18q22	ATTTCTAAGAGGACTCCCAAACT ATATTTTGAAACTCAGGAGCAT	58	157-179	0.86	Gyapay *et al.* (1994)
D19S49	19q12	ACTCATGAAGGTGACAGTTC GTGTTGTTGACCTATTGCAT	58	106-122	0.74	Weber *et al.* (1990b)
D22S351	22q11	GTCAGGGCAGGTAAGGTTGA CTCCTGCCCTCGAAAGTCAT	60	145-163	0.76	Sainz *et al.* (1993)

All repeat sequences are of the $(dC\text{-}dA)_n$ type. The CA-strand primer is listed first for each marker except for *p53CA* and *D22S351*. PCR conditions used for each primer set were as described in section 2.2.3 (b) with the exception of *p53CA*. The conditions for amplifying this marker were an initial denaturation step at 94°C for 4 minutes followed by 35 cycles of 94°C for 1 minute (denaturation), 64°C for 1 minute (primer annealing), 72°C for 1 minute (elongation). On completion of the final cycle a further elongation at 72°C for 10 minutes was carried out.

A3.2. Deoxynucleoside triphosphates

Deoxynucleoside triphosphates (dATP, dCTP, dGTP, dTTP) were obtained as 100mM stock solutions from Pharmacia, St. Albans, Herts., and diluted to 2mM working strength.

A3.3. Super *Taq* reaction buffer

10x Super *Taq* DNA polymerase reaction buffer was purchased from HT Biotechnology Limited, Cambridge, and contained 50mM Tris-HCl pH 9.0, 50mM potassium chloride, 7mM magnesium chloride, and 16mM ammonium sulphate.

A4. Method for silver staining of PhastGel® homogenous 20

Step No.	Solution	Time (min.)	Temp. (°C)	Remarks
1	20% (w/v) TCA	5	20	Fixing solution
2	50% (v/v) ethanol, 10% (v/v) HAc	2	50	Wash solution
3	10% (v/v) ethanol, 5% (v/v) HAc	2	50	Wash solution
4	10% (v/v) ethanol, 5% (v/v) HAc	4	50	Wash solution
5	5% (v/v) gluteraldehyde	6	50	Substance sensitization
6	10% (v/v) ethanol, 5% (v/v) HAc	3	50	Wash solution
7	10% (v/v) ethanol, 5% (v/v) HAc	5	50	Wash solution
8	Distilled deionised water	2	50	Wash solution
9	Distilled deionised water	2	50	Wash solution
10	0.4% (w/v)silver nitrate	10	40	Staining solution
11	Distilled deionised water	0.5	30	Wash solution
12	Distilled deionised water	0.5	30	Wash solution
13	2.5% (w/v) sodium carbonate, 2% (v/v) formaldehyde	1	30	Developing solution
14	2.5% (w/v) sodium carbonate, 2% (v/v) formaldehyde	10	30	Developing solution
15	2.5% (w/v) sodium thiosulphate, 3.7% (w/v) Tris-HCl	2	30	Background reducing solution
16	10% (v/v) glycerol	5	50	Stop solution

Abbreviations used: TCA, trichloroacetic acid; HAc, glacial acetic acid. All solutions were prepared fresh on the day of use with distilled deionised water. 75ml of solution is used in each step.

A5. Formulation of SDS-polyacrylamide gels used in Protein Truncation Test (PTT) analysis

Component	10% acrylamide gel mix	20% acrylamide gel mix	Stacking gel mix (4% acrylamide)
Distilled deionised water	6.8ml	-	7.5ml
30% (w/v) acrylamide: bisacrylamide 37.5:1	5.6ml	11.2ml	1.3ml
1.87M Tris pH 8.8	3.3ml	3.3ml	-
1M Tris pH 6.8	-	-	1.3ml
Glycerol	0.9ml	2.7ml	-
10% SDS	200ml	200ml	100ml
TEMED	15ml	15ml	10ml
25% AMPS	15ml	5ml	40ml

SDS, sodium dodecyl sulphate; TEMED, NNN'N' tetramethylethylenediamine; AMPS, ammonium persulphate.

6.

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