Colorectal Cancer Genetics: 
A Study of Chromosome 8p Tumour Suppressor Loci 
and Microsatellite Instability 

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Thesis submitted for the Degree of 
Doctor of Medicine 

University of Edinburgh 
1996
Dedication

I dedicate this thesis with all my love to my parents.
Declaration

I declare that this thesis was composed entirely by myself and that the work presented is my own unless otherwise stated. I have included some data generated by co-workers where it strengthens or complements the original research in this thesis. These include reference to allele loss on chromosomes 5q and 17p, p53 immunohistochemistry and some assessment of microsatellite instability.

Christopher Cunningham
October 1996

Presentation of this work was awarded the Patey Prize at the Surgical Research Society of Great Britain and Ireland, 1993.

The following publications are derived from this work.


Abstracts


Abstract

This thesis examines two types of genetic defect commonly acquired in colorectal cancer: loss of heterozygosity at putative tumour suppressor loci on chromosome arm 8p and microsatellite instability. Both lesions are placed in the context of clinicopathological features of colorectal cancer.

Colorectal cancer is a major cause of mortality and morbidity world-wide. Epidemiological and aetiological factors are reviewed and areas of importance which may be amenable to manipulation are highlighted. The possible application of presymptomatic diagnosis and adjuvant therapies are discussed as these are routes by which the disease process may be influenced. Evidence supporting the genetic basis of colorectal carcinogenesis and the adenoma-carcinoma sequence is presented. Research in familial adenomatous polyposis and hereditary non-polyposis colorectal cancer has dramatically improved our understanding of the genetic basis of colorectal carcinogenesis and these important syndromes are discussed in detail. Preliminary evidence suggesting the existence of putative chromosome 8p tumour suppressor loci is described, leading to an outline of the methodology employed in deriving a localisation of the tumour suppressor loci.

Fourteen chromosome 8 polymorphic loci were analysed for loss of heterozygosity in 119 colorectal cancers selected at random. Loss of heterozygosity was detected in 59.6% (59/99) of informative cases. Markers were of sufficient density to allow the construction of a deletion map which delineated two discrete regions likely to contain tumour suppressor loci. A 4cM region at 8p22-p21.3 between markers LPL and D8S133 and a further locus at 8p21-p11.2, between markers D8S137 and D8S136, estimated to span some 17cM.

Loss of heterozygosity on chromosome 8p was found to be independent of tumour site, Dukes' stage, patient age, sex and survival. Analysis of 50 sporadic colorectal adenomas revealed a low frequency of chromosome 8p loss of heterozygosity, suggesting that the tumour suppressor loci are preferentially involved in the later stages of colorectal carcinogenesis.

The role of defective DNA mismatch repair in colorectal cancer predisposition has been reported recently. These defects are manifested by microsatellite instability. Such instability was noted in tumour DNA during chromosome 8p loss of heterozygosity studies and the project was extended to investigate this phenomenon. The prevalence of microsatellite instability was determined in 245 colorectal cancers. 16.7% (41/245) displayed replication errors at one or more microsatellite loci, suggesting underlying errors in mismatch repair. Cancers displaying microsatellite instability tended to arise in the proximal colon, maintained nuclear diploidy and were associated with a significantly improved survival. The presence of replication errors was independent of patient age and sex, loss of heterozygosity at chromosomes 5q and 17p and immunohistochcmistry for p53 protein.
Loss of heterozygosity affecting chromosome 8p and defective DNA mismatch repair are frequent genetic abnormalities in colorectal cancers. This project provides important data localising the putative tumour suppressor genes to two discrete regions on chromosome 8p. This will aid future efforts towards cloning and identifying the genes involved. In addition, the prevalence and clinicopathological features of microsatellite instability have been established, in a large population of unselected colorectal cancers, allowing insight into the involvement of this mechanism in sporadic colorectal carcinogenesis.
Acknowledgements

This project was only possible through the help of many colleagues. Credit for the impetus to investigate chromosome 8p in colorectal cancer must go to Malcolm Dunlop, with Professor Andrew Wyllie and Professor Colin Bird who greeted the project enthusiastically and made subsequent research possible. Malcolm Dunlop and Andrew Wyllie have been on hand throughout the duration of this project with support, guidance and friendship. I also wish to thank Malcolm for his less subtle encouragement that I occasionally required. I received financial support from University of Edinburgh Postgraduate Fellowship (Leckie Mactier Scholarship); The Melville Trust for the Care and Cure of Cancer and Sir Stanley and Lady Davidson Research Fund. I wish to thank these funding bodies and hope that I have justified their support.

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At the MRC HGU I was impressed with the concentration of expertise, and many people helped me with enquiries into new techniques and use of resources. Susan Farrington always provided lively debate and she was responsible for carrying the project through the final phases with creation of a YAC map. Stewart Morris introduced me to the use of automated laser fluorescent DNA sequencer and kindly gave up his own time and frequently his own scientific supplies to assist me. Andrew Carothers provided statistical advice and service at several points in the project, particularly with reference to survival analysis. This analysis was dependant on all involved in the Cancer Research Campaign Lothian Region Colorectal Cancer Project. I am indebted to the patients, pathologists, technical staff, auditors and surgeons involved in this project. At many points during this research I called up on the services of the Department of Photography at the MRC HGU. I cannot thank Norman Davidson, Sandy Bruce and Douglas Stuart enough for their tremendous help in producing slides and figures for presentations, publications and this manuscript.

The most protracted phase of this thesis was writing the final product. I would like to thank Andrew Wyllie for his most helpful suggestions regarding the manuscript. Finally, I wish to thank Lucy Curtis for her help, patience and support during the prolonged period of writing this thesis.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ALF</td>
<td>Automated laser fluorescence (DNA sequencer)</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>CHRPE</td>
<td>Congenital hypertrophy of the retinal pigment epithelium</td>
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<tr>
<td>DCC</td>
<td>Deleted in colon cancer</td>
</tr>
<tr>
<td>DDW</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>RSV</td>
<td>Rous sarcoma virus</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukaemia</td>
</tr>
<tr>
<td>ALV</td>
<td>Avian leukemia virus</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence <em>in situ</em> hybridisation</td>
</tr>
<tr>
<td>FOBT</td>
<td>Faecal occult blood test</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary nonpolyposis colorectal cancer</td>
</tr>
<tr>
<td>DM</td>
<td>Double minute (chromosomes)</td>
</tr>
<tr>
<td>HSR</td>
<td>Homogenously staining regions</td>
</tr>
<tr>
<td>IVSP</td>
<td><em>in vitro</em> synthesised protein (assay)</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base pairs</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
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<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>NAT</td>
<td>N-acetyl transferase</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PC</td>
<td>Phenol Chloroform</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PRLTSP</td>
<td>Platelet-derived growth factor receptor β tumour suppressor</td>
</tr>
<tr>
<td>RER</td>
<td>Replication errors</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RH</td>
<td>Retained heterozygosity</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium docecyl sulphate</td>
</tr>
<tr>
<td>SSC</td>
<td>Salt and sodium citrate buffer</td>
</tr>
<tr>
<td>STR</td>
<td>Short tandem repeats</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris Acetate EDTA buffer</td>
</tr>
<tr>
<td>TBE</td>
<td>Tri Borate EDTA buffer</td>
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</tbody>
</table>
TBE  Tri Borate EDTA buffer
TE   Tris EDTA
TEMED N,N,N',N' tetramethylethylenediamine
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Chapter 1

Introduction

This chapter deals with colorectal cancer as a major health issue in the Developed World. Epidemiology of the disease is described and possible aetiological factors discussed highlighting those which may be amenable to intervention, thereby offering a means through which the pattern of disease incidence may be altered. Colorectal cancer morbidity and mortality have changed little in recent years and it continues to be a significant drain on healthcare resources. In the long term there is hope that understanding the molecular mechanisms responsible for the development and progression of the disease may result in radical approaches to prevention, diagnosis and treatment. In the shorter term it is imperative that existing approaches to clinical management of colorectal cancer are influenced by new insights into the mechanisms of pathogenesis of the disease.

Although a genetic basis to colorectal cancer is now well established it is certain that other non-genetic factors have a significant influence. These may function independently, or their effects may be mediated through alteration in the function of cancer-related genes. Environmental, dietary and lifestyle factors have been associated with colorectal cancer risk, although, supporting evidence is frequently confused and contradictory. An effort will be made to summarise current thinking on the contribution of these factors in colorectal carcinogenesis.

Expansion in understanding the molecular genetic basis of colorectal cancer has provided an exciting insight into the mechanisms of carcinogenesis and predisposition to the disease. These areas are reviewed with particular reference to the adenoma-carcinoma sequence in sporadic colorectal cancer and the well characterised predisposition syndromes. The original work to be presented later in this thesis deals with two different genetic events in colorectal carcinogenesis: localisation of putative tumour suppressor loci on the short arm of chromosome 8 and microsatellite instability. The basis of interest, methods of study and clinical and pathological associations of these genetic lesions will be discussed.

1.1 Epidemiology of colorectal cancer and aetiological factors

1.1.1 Patterns in colorectal cancer incidence

In incidence of colorectal cancer

In the Developed World colorectal cancer is the most common malignancy in non-smokers, accounting for around 58,000 annual deaths in the USA and 19,000 in the UK. There
is great heterogeneity in the epidemiology of cancer of the colon and rectum. Internationally, a ten fold variation exists in the incidence of colon cancer and a five fold variation in rectal cancer. In general, they are diseases of affluence, common in North America and Northern Europe and rare in Asia, Africa and South America. The notable exception to this is Finland where the incidence is low but increasing (Weisburger, 1991). Incidence varies considerably within the United Kingdom. Scotland has one of the highest standardised incidence rates (34.2/100 000) and in certain areas within Scotland, such as the North East, colorectal cancer in males is more than twice as common as in the country as a whole (Kemp et al., 1985). These differences support the case for the existence of environmental carcinogenic forces, probably related to diet. However, in the relatively static populations in much of Scotland, they may also indicate clustering of predisposition genes.

Distribution of colorectal cancer

Colorectal cancer incidence increases with age, 50% of cases occur in the over sixties and only 5% in those less than 30 years of age. (Levin and Dozois, 1991). Gender differences are slight, but well substantiated (McMichael and Giles, 1994; Levin and Dozois, 1991; Jensen, 1984). The overall sex ratio for colon cancer is similar in most societies. There is a tendency for early onset disease (before age 50) to be more common in women. Cancer of the rectum is more prevalent in males and this is accentuated in the elderly. These differences have invited speculation on interaction between female reproductive physiology and colorectal carcinogenesis. In a prospective trial of over 100,000 American female nurses, no association was found between female reproductive history, use of oral contraception and colorectal carcinoma. However, in the same series, a protective effect of post-menopausal administration of oestrogens was identified (Chute et al., 1991a).

Trends in colorectal cancer incidence

Apparent trends in incidences and comparisons must be treated with caution as the true values may be hidden by inaccuracies in disease coding, the introduction of screening programmes and varying proficiencies in disease reporting. Chu et al. (1994) described patterns of incidence in a white American population with high overall incidence, using the Connecticut Cancer Registry. From 1950 to 1984 there was a steady increase in male colorectal cancer, of approximately 0.6% per year. This contrasted with a slight decline in female incidence of the disease over the same period, of approximately 0.3% per year. Subsequent to 1984, both sexes demonstrated a reduction in overall colorectal cancer incidence and specifically a decline in incidence of advanced stage disease. This period was also associated with a reduction in overall mortality which the authors attributed to the use of advanced diagnostic techniques, screening and lifestyle changes, augmented by high publicity following diagnosis of the disease in former
President Reagan. They proposed that the Connecticut population is typical of white high-risk groups, although as yet there is no evidence of similar decline in the UK.

Colorectal cancer incidence is increasing in those populations traditionally regarded as having a low risk. This was noted over the period 1950-1989, in the Mediterranean countries of Europe as well as those of Central and South America, Asia and Eastern Europe (Weisburger, 1991). As nations become more Westernised they appear to adopt a higher risk of colorectal cancer. This environmental influence is exemplified by migrant populations who acquire an increased incidence of colorectal cancer on passing from low to high risk regions. The acquired incidence approaches that of the indigenous population within one generation and has been documented in subsequent generations of Chinese immigrants to North America (Yeung et al., 1991); Japanese to Hawaii and California (McMichael and Giles, 1994) and Africans to North America (Burkitt, 1971). Indeed, Burkitt (1971) plots the dramatic rise in colorectal cancer incidence among black Americans which is now identical to whites (Levin and Dozois, 1991). Immigrants from countries of high incidence, moving to regions of lower incidence, similarly, adopt the rate of their new homeland. This was demonstrated in the movement of Scots to Australia (Weisburger, 1991).

1.1.2 Aetiology of colorectal carcinoma

Dietary factors

The importance of dietary influence was first proposed by Burkitt (1971) on the basis of studies comparing incidence of colorectal cancer in sub-Saharan Africa and the Developed World. The African diet, high in fibre and low in protein, fat and refined carbohydrate was believed to afford protection against the development of colorectal cancer. Burkitt also highlighted the possible role of bacterial degradation of bile salts, producing carcinogens within the bowel lumen. This source of carcinogen could be increased by high dietary fat increasing bile acid production or alteration in bacterial population by changes in dietary fibre and carbohydrate. Overall, these findings were supported by the apparent protection from rectal cancer described in the male Seventh Day Adventists and low incidence of colon cancer in Mormons, both of which adhere to a diet rich in cereal fibre and vegetable (Weisburger, 1991). One report, comparing immigrant Chinese in San Francisco with high colorectal cancer incidence with low risk indigenous Chinese in Sha Giao, identified the presence of increased dietary fat, cholesterol and protein and low dietary carbohydrate in the high risk population. However, there were no differences in stool bulk or fibre content (Yeung et al., 1991). Other series failed to substantiate the involvement of dietary animal fat or protein, but supported a protective role for dietary fibre (Little et al., 1993; Olsen et al., 1994). Overall, it would appear that high fibre, low fat diet is most likely to be protective against colorectal cancer. Other dietary factors have been implicated. Vitamins A, C and E are believed to protect against colorectal cancer by lowering intestinal pH,
thereby altering bacterial profile and high calcium intake is protective through changes in intestinal bile salt metabolism and reduction in cellular proliferation. However, evidence supporting these is frequently conflicting, suggesting that any real effects are fairly insignificant (Levin and Dozois, 1991; Little et al., 1993; Olsen et al., 1994).

Alcohol intake, particularly in the form of beer, is associated with a slight increase in rectal cancer but no effect on colon cancer (McMichael and Giles, 1994). Rectal mucosal hyperproliferation and tissue damage has been shown to result from alcohol consumption (Seitz and Simanowski, 1992).

Reports of dietary influences in colorectal cancer are often anecdotal and the underlying mechanisms likely to be complex and highly interactive. It is possible that some ingested chemicals may be directly carcinogenic, although more probable that procarcinogens are modified and activated in the gut. These physiological processes may be influenced by bacterial flora, micronutrients and other products of ingestion. It is also possible that the overall carcinogenic effects may be governed by the genetic profile of the individual, either in terms of functional enzyme polymorphisms or abnormalities in cancer genes.

**Physical activity and body habitus**

Physical activity, either during work or in leisure time, seems to offer protection against cancer of the colon but has no effects on rectal cancer (Markowitz et al., 1992; Lee et al., 1991). Weight appears to be positively associated with colon cancer, particularly in males. Adolescent obesity and height have been shown to predict an increased risk of colon cancer in a large series of females which was adjusted to account for the effects of dietary fats (Chute et al., 1992b).

### 1.1.3 Colorectal cancer mortality

Most countries have witnessed a modest improvement in survival from colorectal cancer, attributed to improvements in anaesthetic and surgical techniques. In Scotland, survival data show an improvement from 33% five year survival in 1968-1972, to 40% in 1983-1987 (Black et al., 1993). However, early detection by haemoccult and subsequent colonoscopy is likely to have contributed and this is supported by the diagnosis of cancers at an early stage in certain series (Chu et al., 1994).

There has been a demonstrable "rightward shift" in tumour location, accompanied by a slight decrease in the detection of rectal lesions, especially over the past 15 years (Sariego et al., 1992; Kee et al., 1992; Waalen, 1994). This trend is believed to be due in part to the increasingly widespread use of surveillance colonoscopy (Sariego et al., 1992). If correct, there will be a lag period before the incidence of proximal lesions falls. Proximal cancers are associated with a more favourable prognosis than distal cancers (Cotran et al., 1994) which may be contributing to improved survival rates.
1.2 Methods towards reducing the burden of colorectal cancer

Long term hopes of reducing colorectal cancer incidence, morbidity and mortality rest with the practical application of strategies derived from advances in molecular genetics. This is already apparent in the inherited predisposition syndromes familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). However, benefits can also be achieved through optimising conventional means of early diagnosis and treatment. This section outlines several areas where this may be achieved.

Screening

Colorectal cancer is more common than breast or cervical cancer, yet there is no formal screening protocol in the UK (Atkin et al., 1993). Moreover, colorectal cancer would appear to be an ideal candidate for clinical screening. It is a common cancer, with a well defined premalignant state, which can be readily diagnosed, and progression may be prevented by endoscopic removal. In addition, cancers identified at an early stage are associated with an improved survival following colectomy (Hardcastle et al., 1989). Formal randomised trials designed to measure the efficacy of colorectal cancer screening are in progress, in Nottingham (UK), Goteborg (Sweden) and Funen (Denmark). Results are likely to be available in the mid to late 1990's. However, it is likely to require a prolonged, randomised, nation-wide screening programme before robust conclusions will be delivered which will be acceptable to most practitioners.

Clinical screening strategies may take several forms. Overall benefits must be balanced against clinical efforts and costs, prevalence of false negatives and morbidity and mortality of the procedure. Faecal occult blood tests (FOBT) have been employed in several centres. In the Nottingham, UK study (Hardcastle et al., 1989), a non-hydrated FOBT has been offered to over 50,000 individuals between the ages of 50 to 75 years with an uptake of 53%. Of these, 2.3% had positive FOBT and proceeded to colonoscopy. As an approximate guide, if such a system were applied to the population in Scotland between ages 50-75 (1 597 074 individuals, Registrar General for Scotland Figures, June 1995) there would be 846 449 FOBT's (53% uptake) leading to 19 468 colonoscopies (assuming 2.3% positivity). The Nottingham study has yet to show a significant reduction in mortality among screened patients. The sensitivity of FOBT may be improved using rehydrated annual FOBT as described by Mandel (Mandel et al., 1993) which has resulted in a 30% reduction in mortality. More recently, testing with immunological FOBT has demonstrated even higher sensitivity (Robinson et al., 1995). These approaches generate more colonoscopic examinations as there is no improvement in specificity. This is a considerable expense (over £100.00 per colonoscopy) and it is likely that current health care resources could not cope with this burden. In addition, it is prudent to remember that colonoscopy has inherent
morbidity and indeed mortality. In the Funen adenoma follow-up study, a total of 3959 colonoscopies were performed with 1801 of these including polypectomy. Serious complications occurred in 12 cases (mainly colonic perforation following polypectomy) leading to death in two patients from septicaemia and myocardial infarction (Jorgensen et al., 1993).

Although colonoscopy offers many diagnostic and therapeutic benefits, it is likely that the availability of colonoscopists will be a limiting feature as screening programmes move into less specialised centres. As a possible alternative, the Goteborg trial places emphasis on the combined use of barium enema examination and flexible sigmoidoscopy, both techniques being more readily available outside of major centres. The overall cost of these investigations is less, being around £70, and, even in specialist centres, barium enema examination is more successful in visualising the caecum (McCarthy, 1992). Furthermore, Maule (1994) demonstrates that non-medical staff may be adequately trained in the use of flexible sigmoidoscopy with no loss in sensitivity or specificity. As most colonic neoplasia is within the 60 cm reach of the flexible sigmoidoscope, this technique offers a significant cost saving whilst still offering a large proportion of the diagnostic and therapeutic performance of colonoscopy. Therefore, the combination approach of the Goteborg trial may be a reasonable compromise. However, the trend towards more proximal lesions (Sariego et al., 1992; Kee et al., 1992; Waalen, 1994) and the high prevalence of proximal cancers in hereditary states support the use of colonoscopy.

In the United States, a more aggressive policy has been advocated with routine five yearly flexible sigmoidoscopy in addition to annual Haemoccult testing (Byers and Gorsky, 1992). It is estimated that this could result in a 40% reduction in mortality in the test group (50-70 years of age) who took up the option for screening. However, this approach is expensive, estimated to cost $48 for each person per year for screening and follow up testing. Recent recommendations were made for the introduction of a national screening campaign in the UK (Atkin et al., 1993). It is proposed that a single flexible sigmoidoscopic examination between age 55-60 with further colonoscopic examination of the 3-5% found to have high-risk polyps (villous or >1cm diameter) could reduce colon cancer deaths in the UK by 3500 per year. This order of mortality reduction is similar to that expected in the more rigorous use of annual faecal occult testing and 3-5 yearly flexible sigmoidoscopy in the United States. However, it is suggested that this would incur little cost to the National Health Service as it would result in a reduction in advanced disease requiring treatment. In addition, recent reports suggest that individuals showing a tendency to develop colonic polyps may only require 5 yearly examination of the colon without loss in sensitivity (Rex et al., 1994).

A national randomised trial may be the only means through which screening methods may be assessed. As the trial period is likely to be at least 10-15 years, it is imperative that this be undertaken as soon as possible. In the meantime, screening protocols are left to the discretion of individual practitioners. With recent developments in understanding the genetic basis of
colorectal cancer, particularly predisposition, it is likely that a second generation of screening strategies will emerge, designed to target those individuals at high risk of disease. This area is of particular interest to the major themes of this thesis and will be discussed later.

Optimum surgical treatment and adjuvant therapies

Surgery remains the principal treatment for colorectal cancer offering cure in a significant proportion of cases. Disease may progress through local recurrence or secondary liver deposits, which are most likely present as occult micrometastases at the time of resection. Reduction of local recurrence is addressed through radical surgical resection or the use of radiotherapy. Efforts towards controlling occult liver metastases are based on the use of adjuvant chemotherapy.

Patient survival and local recurrence of disease are surgeon dependent, being determined by operative skill and specific surgical techniques employed (Quirke et al., 1986). It is reasonable that radical resection, including draining lymph nodes is likely to offer superior control of local disease and this is borne out by clinical studies (Heald and Ryall, 1986). In the colon, such resection is relatively straight-forward and local recurrence is less common. Rectal cancer is associated with higher rates of local recurrence and complete excision of the mesorectum, although technically demanding should be the universal aim.

Adjuvant treatment

In colorectal cancer, radiotherapy is used mainly to reduce local recurrence of rectal cancer. It may also be employed to down-stage advanced rectal tumours, often with the hope of permitting resection. Unfortunately, this control is often transient, associated with a high local failure rate. In colon cancer, radiotherapy has little to offer except a role in palliation of metastases, particularly in bone.

In recent years there has been considerable interest in the assessment of adjuvant chemotherapy in colorectal cancer, aimed at reducing the burden of occult liver metastases. Cumulative data demonstrate an overall improved survival rate in Duke's B and C cancers of 10-15% (International multicentre pooled analysis of colon cancer trials investigators, 1995). All regimens employ 5-FU either alone or in combination with folinic acid and/or levamisole. Agents may be administered as intermittent boluses (usually in 2-3 week cycles) or protracted infusion either into central vein or hepatic portal venous system through indwelling venous catheters. Initial criticisms suggested that these trials were simply delaying the inevitable growth of hepatic metastases, such that a 'catch-up' would occur in mortality over the following months or years. The most recent multicentre North American trial with a minimum follow up of 5 years (median 6.5 years) suggests this is not the case (Moertel et al., 1995). The authors advocate their regimen of 5FU and levamisole as standard adjuvant therapy for stage III patients.
Methods of manipulating colorectal carcinogenesis

In the broadest sense, the carcinogenic process in the human colon and rectum may be modified by dietary changes, referred to in 1.1.2, with increase in dietary fibre and reduction of fat and inclusion of supplementary vitamins A, C and E. However, several reports have suggested benefits from more specific pharmacological interventions.

Chemical carcinogenesis studies in animals suggests that aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) have a strong protective effect in colorectal cancer (Pollard et al., 1981). These effects are believed to be mediated by inhibition of prostaglandin H synthetase. Several studies suggest that aspirin prophylaxis reduces death from colorectal cancer in humans (Thun et al., 1991; Schreinemachers and Everson, 1994). Incidental observations from studies primarily examining aspirin prophylaxis in ischaemic heart disease, failed to reveal significant reduction in colorectal cancer incidence (Gann et al., 1993). However, follow-up was limited to only five years and the study only considered the use of low dose aspirin. Ultimately, the potential benefits from higher doses of aspirin or longer duration of use can only be addressed by more detailed epidemiological studies and prevention trials with randomised participants. In familial adenomatous polyposis, regression of polyps has been noted on treatment with NSAID's which may have important implications for disease control.

1.3 Predisposing conditions

Patients with chronic ulcerative colitis have a higher risk of developing colorectal cancer. Traditional teaching states a 5% cancer incidence in patients suffering ulcerative colitis for 10 years reaching 25% incidence at 25 years (Cotran et al., 1994). Incidence is higher in those with extensive disease involving the proximal colon (Levin and Dozois, 1991). However, Langhoz et al., (1992) suggests that adequate modern treatment may reduce the risk of colorectal cancer to that of the general population. This Danish group of ulcerative colitics were subjected to maximal medical therapy, under intensive review, had a colectomy rate of 32.4% after 25 years and a cumulative incidence of only 3.1% colorectal cancer after 25 years.

Crohn's disease, although not classically premalignant in the large bowel, confers a 4 to 20 times increased risk of colorectal cancer compared with a healthy population (Levin and Dozois, 1991). Although no definite mechanisms have been determined, it is possible that these effects may be mediated through alteration of the bacterial activity in abnormal segments or loops created as a consequence of surgery or as complication of the disease process itself.

The most striking predispositions to colorectal cancer are those with a genetic basis. Familial adenomatous polyposis and hereditary non-polyposis coli are the most comprehensively understood. Elucidation of the carcinogenic mechanisms involved in these conditions is likely to
alter dramatically the clinical approach to colorectal cancer management. These syndromes will be considered in detail below.

1.4 The adenoma-carcinoma sequence

For many years colorectal cancer has been suspected to arise within premalignant colorectal adenomas (Burkitt, 1971) and this is exploited in the many approaches to colorectal cancer screening. Evidence for the progression from adenoma to carcinoma comes from several sources. On a world-wide scale, the distribution of colorectal adenomas reflects that for colorectal cancer and, indeed, those populations acquiring increased risk on moving to regions with high cancer incidence also demonstrate increasing incidence of colorectal adenomas (Sato, 1976; Stemmerman and Yetani, 1973). In addition, adenomas are more prevalent in sigmoid colon and rectum, sites most commonly producing malignant disease and adenomas are found in resected cancer-bearing bowel at an unexpectedly high frequency (Goligher, 1984). Furthermore, histopathological assessment has established the coexistence of adenomatous tissue and malignant change in the same lesions (Lane, 1976). Finally, the average age of onset of adenomas is approximately five years earlier than carcinoma (Muto, 1975) a temporal relationship which would be consistent with sequential progression.

However, it is certain that not all adenomas have a strong tendency to undergo malignant change. Atkin et al. (1992) demonstrated that in those individuals with a single tubular adenoma the risk of colorectal cancer was low. In contrast, those with adenomas greater than 1cm in size, particularly with a villous or tubulovillous architecture displaying severe dysplasia were at a high risk of developing a subsequent carcinoma. Indeed, the corollary is also likely to be true ie, not all carcinomas develop in pre-existing adenomas, instead developing by a ‘fast track’ mechanism with no premalignant adenoma stage.

The adenoma-carcinoma sequence was incorporated in the model of colorectal tumorigenesis proposed by Fearon and Vogelstein (1990). In this, the progression from normal mucosa through adenoma to carcinoma was associated with the stepwise accumulation of discrete genetic lesions. This model has been sufficiently robust to support the recent improvements in our knowledge of the molecular genetic basis of colorectal cancer which are discussed in detail below.
1.5 The genetic basis to colorectal carcinogenesis

1.5.1 Evidence for the genetic basis to colorectal carcinogenesis

Tumour suppressor genes

Early work involving cell fusion studies indicated that healthy cells contained factors which counteracted malignant behaviour in tumour cells (Harris et al., 1969). Cellular hybrids resulting from fusion of mouse fibroblasts and the tumorigenic HeLa cell line displayed normal growth and differentiation, indicating that these cancer genes conferred recessive malignant behaviour that was corrected in the presence of normal DNA. The malignant phenotype returned as the cell hybrids expelled certain chromosomes. In the HeLa cell line fusions the expulsion of chromosome 11, demonstrated by loss of polymorphic chromosome 11 markers (Stanbridge, 1981) was specifically associated with the return of malignant phenotype. Later studies demonstrated that tumorigenic behaviour in the HeLa-human fibroblast cell line was eliminated by the introduction of chromosome 11 which suppressed recessive malignant behaviour (Saxon et al., 1986). Cytogenetic studies had frequently identified chromosome 11 aberrations in Wilm's tumour and it was believed to arise from two genetic events at the same locus in sporadic and familial disease. The introduction of chromosome 11 into a tumorigenic cell line derived from Wilm's tumour resulted in loss of malignant phenotype, providing further evidence that a gene critical to the development of Wilm's tumour was situated on chromosome 11. Studies on interspecies hybrids have suggested the presence of tumour suppressor loci on many chromosomes including chromosomes 1, 2, 4, 11, 13, 17 and 20 (Stanbridge, 1988). Each of these indicates the likely position of genes which block tumour growth.

The theoretical basis of tumour suppressor genes was described by Knudson (1985; 1989) on the basis of the observed incidence of retinoblastoma, a rare tumour of the eye which strikes children. Knudson developed a mathematical model that postulated a 'two-hit' hypothesis of carcinogenesis. According to this model, in sporadic disease a single retinal cell would have to acquire somatic mutations in both alleles of a critical gene. However, in the familial form of the disease one mutated allele is inherited and only one somatic mutation would be required to remove gene function. Although this theory pre-dated our knowledge of tumour suppressor gene function, Knudson's hypothesis accommodates the subsequent mutation data in retinoblastoma (Rb-1) gene (Franks and Teich, 1990). Rb-1 was cloned through linkage analysis and detailed mapping of regions of deletions in chromosome 13. Mutation analysis in a panel of retinoblastoma tumours confirmed the presence of discrete genetic lesions resulting in gene inactivation. Suppressor activity of the normal gene product was demonstrated with its
introduction into a retinoblastoma cell line using a retroviral vector, resulting in loss of malignant behaviour and leading to exciting prospects for the manipulation of tumour cells in vivo (Huang et al., 1989).

Tumour cytogenetics has provided valuable evidence on the existence and localisation of cancer genes. Cytogenetic analysis identified an interstitial deletion on the long arm of chromosome 5 identified in an individual with FAP and mental retardation and provided preliminary information on the localisation of the APC gene (Herrera et al., 1986). Analysis of cancer DNA can also be undertaken and the recognition of non-random cytogenetic deletions in colorectal cancer was important in suggesting the presence of cancers genes on chromosomes 17p and 18q. In all cases these events were confirmed and extended through loss of heterozygosity studies. Loss of heterozygosity studies utilise the presence of polymorphic sites throughout the genome where an individual’s maternal and paternal chromosomes can be distinguished by variation in DNA repeat sequence or restriction enzyme sites between paired chromosomes. Examination of heterozygous loci in DNA from cancers with genetic losses or rearrangements allows fine mapping of the genetic lesions and the derivation of a region of common loss. Such a region is likely to contain at least part of a tumour suppressor locus and these strategies were paramount in the identification of p53 and DCC genes (Baker et al., 1989; Fearon et al., 1990).

Oncogenes

A comprehensive review of oncogenes is outwith the scope of this thesis and the following section is intended to provide a background account. Oncogenes are genetic elements capable of inducing and/or maintaining a transformed phenotype in a cell, and are thus said to act dominantly. Genes capable of inducing neoplasia were first isolated from acutely transforming retroviruses. These retroviruses contain viral oncogenes which are derived from human DNA sequence. In Rous sarcoma virus (RSV) the viral oncogene (v-src) has a cellular homologue (c-src), a protein with tyrosine kinase activity and transforming ability in infected cells.

Several studies in tumours have identified oncogenes. Transfection of tumorigenic DNA into immortalised fibroblasts (NIH 3T3) was particularly important in identifying the ras family of oncogenes (Cooper 1982; Bishop 1985). Studies of insertion of retroviral DNA into the host genome showed that this could result in upregulation of an adjacent cellular gene (‘downstream promotion’). The first example of this phenomenon was in B-cell tumours induced in chickens by avian leukaemia virus (ALV), leading to increased levels of myc transcription. Similarly, erb-B can also be activated by promoter insertion by other ALV strains, inducing erythroleukaemia (Franks and Teich, 1990).

Karyotype analysis with metaphase chromosomes have identified numerous non-random chromosomal abnormalities in many tumours which potentially affect oncogene function. Burkitt's lymphoma is a human B-cell lymphoma in which both Epstein-Barr virus (EBV) and malaria are known cofactors. Karyotype analysis revealed frequent translocation of the long arm
of chromosome 8 to another chromosome, usually (in 90% of cases) chromosome 14 (Rowley et al., 1990). This moves the myc locus into close proximity to the immunoglobulin enhancer sequences. Similarly, a very common translocation between chromosomes 22 and 9, the 'Philadelphia Chromosome' occurs in chronic myeloid leukaemia (CML) places the abl oncogene under the control of the bcr gene elements, greatly increasing abl activity (Bernards et al., 1987). Additionally, amplification of discrete regions of the genome is frequently observed in tumours. Amplified regions are often large (100-1000 kilobases) spanning many genes, and form either homogenously staining regions (HSR) or double minute (DM) chromosomes.

Cellular, or proto-oncogenes are involved in normal cell growth and development, but are expressed abnormally in many tumours due to alterations in regulation by mutation, gene amplification or chromosomal translocation. The physiological functions of oncogenes are diverse. Many are growth factor-related (sis, int-2, hst and fgf), growth factor receptor-related (erb-B), protein kinases (src) or are proteins with GTPase activity (ras). Other oncogene products, such as myc, myb, fos are localised in the nucleus. Thus, abnormalities of oncogene expression result in disruption of cell receptor-growth factor recognition pathways, regulation of transcription and control of cellular growth and differentiation.

Colorectal cancer is associated with the accumulation of genetic defects

Vogelstein et al. (1988) applied these concepts of tumour suppressor and oncogene dysfunction to the analysis of adenomas and carcinomas, studying activation of the Kirsten ras oncogene and loss of heterozygosity at multiple sites. This lead to the early concept that the progression from normal mucosa through the adenoma to carcinoma and metastatic disease is associated with and probably caused by the sequential accumulation of genetic defects. Ultimately, the progression is dependent on the number of genetic defects rather than the sequence. However, it is clear that certain events occur at specific points. Inactivation of APC is one of the earliest events, present in the majority of adenomas (Powell et al., 1992); mutational activation of Kirsten ras is found predominantly in larger, dysplastic adenomas (Vogelstein et al., 1988) whereas mutation of p53 gene is a feature of carcinoma (Baker et al., 1990). Genetic changes may function independently, however, it is apparent that certain genetic events, such as the inactivation of p53 and defective mismatch repair are likely to undermine the fidelity of DNA replication, potentially inducing molecular changes in other cancer genes. The following section describes the roles of those molecular events known to be important in the process of colorectal carcinogenesis.

1.5.2 The APC gene in FAP and sporadic colorectal cancer

Elucidation of the genetic basis of familial adenomatous polyposis (FAP) was central to improved understanding of the mechanisms in sporadic colorectal carcinogenesis. It has facilitated a more logical approach in management of individuals with, or at risk of, FAP.
Moreover, the lessons from FAP can be applied to sporadic colorectal cancer and other solid tumours. This section describes the background to understanding the molecular basis of FAP as well as clinical aspects of this important syndrome.

FAP is an autosomal dominant disorder with an annual incidence of around 1/7000 births. Formal clinical diagnostic criteria include the development of more than one hundred adenomatous polyps (usually many more) in the colon and rectum with the inevitable development of malignancy unless prophylactic colectomy is undertaken. The syndrome is also associated with a number of extracolonic features such as epidermoid cysts, retinal pigmentation, osteomata, particularly in the mandible, desmoid tumours and gastroduodenal polyposis. Although colorectal cancer is the outstanding risk, there is also increased incidence of upper GI malignancy including ampullary carcinoma, cholangiocarcinoma and hepatoblastomas (Parks 1990).

FAP results from germline mutations in the APC gene. Family studies suggested a single gene defect with dominant inheritance and initial localisation of the APC gene was suggested by cytogenetic studies in a mentally retarded man with adenomatous polyposis coli demonstrated to have a constitutional deletion on the long arm of chromosome 5 (Herrera et al., 1986). Further mapping by genetic linkage analysis localised the gene to chromosome 5q21 and several candidate genes were eventually cloned from this region. Mutations in one candidate gene showed germline transmission to affected offspring in FAP families (Groden et al., 1991; Kinzler et al., 1991) and led to the definitive identification of APC. Carcinomas that occur in FAP patients lose the residual, previously normal APC allele as a clonal event. Significantly, APC inactivation by mutation, deletion or both, is likely to be a ubiquitous event in sporadic colorectal neoplasia (Nagase and Nakamura, 1993). Hence APC conforms to the pattern of inactivation predicted for an oncosuppressor gene by Knudson's hypothesis.

APC is a large gene consisting of 15 exons encoding a 2843 amino acid polypeptide. Exons 1-14 are small and exon 15 is large, accounting for 77% of the coding region. The majority of APC mutations create premature stop codons resulting in a truncated protein product. Single nucleotide substitutions (most commonly C to T transitions) account for around 40% of germline mutations already identified, the remainder being deletions or insertions of short sequences, suggestive of errors of replication rather than the action of mutagens (Nagase and Nakamura, 1993; Polakis, 1995). 97% of both germline and somatic mutations are spread throughout the 5' half of the gene. Deletions at two codons (1061 and 1309) account for up to 35% of germline mutations (Polakis, 1995). In non-FAP cancers 65% of mutations occur within the mutation cluster region of exon 15 covering less than 10% of the coding region (Miyoshi et al., 1992) and typically resulting in premature stop codons.

In some FAP patients, mutations in APC have not yet been identified despite extensive analysis (Prosser et al., 1994). It is likely that such mutations will ultimately be shown to affect
transcription, occurring within promoter regions, splice sites or introns. Somatic mutations have been detected in the earliest adenomas of FAP (Levy et al., 1994) and even dysplastic aberrant crypt foci (Jen et al., 1994) believed to be adenoma precursors (Smith et al., 1994). This suggests that initiation of even the earliest phases of adenoma formation in FAP requires inactivation of both copies of APC.

**APC protein: structure and function**

Antibodies to the APC protein have identified a 300 KDa cytoplasmic protein expressed in mature epithelial cells (Smith et al., 1993). Although the exact function is unknown, several regions of functional significance have been identified. The first 900 amino acids are predicted to form a highly organised coiled coil, and the first 50 amino acids have been demonstrated to be essential for dimer formation. APC protein has been shown to associate directly with β- and γ-catenins through β catenin binding sites in the central region of the molecule. The catenins are known to bind to the cell surface molecule E-cadherin and are essential for its role in cellular adhesion. It is proposed that APC protein may affect the interaction between the catenins and E-cadherin, thus influencing cellular adhesion and possibly intercellular communication (Rubinfeld et al., 1993; Su et al., 1993a) possibly through the down-regulation of β-catenin (Polakis, 1995). In addition to the β-catenin binding sites, there are several groups of 20 amino acid repeats which can independently bind and induce down-regulation of β-catenin.

Further subcellular localisation has demonstrated that wild type, but not mutant, APC localises to microtubules and may be important in their formation by inducing polymerisation of tubulin (Munemitsu et al., 1994; Smith et al., 1994). This latter function is attributed to the basic domain within the carboxy-terminal region of the protein and certainly truncated APC product, which lacks the carboxyl terminal due to premature truncation, does not bind microtubules. The organisation and structure of microtubules are vital to cell division and there is speculation that the association of APC with the catenins, and hence cadherin, may afford a direct intracellular line of communication from cell surface to microtubule formation. In view of the central role in the development of colorectal cancer, further study to elucidate APC function holds great potential for understanding of the fundamental basis of tumour formation.

**Genotype-phenotype correlations in FAP**

The location of mutations within the APC gene have clinical implications in terms of the colonic and extracolonic manifestations of FAP. In time this may lead to invaluable information on the functional significance of APC protein domains. Normal APC molecules display optimal function when dimeric and mutant APC protein molecules are believed to be capable of forming heterodimers possibly impairing function in a dominant-negative manner (Su et al., 1993b). The extent to which normal APC function is impaired may be related to the structure and residual ability of the mutant protein to form dimers. Mutations occurring 5' to codon 157 are associated
with late onset disease with fewer polyps, whereas, those 3' to codon 168 display a more aggressive phenotype (Spirio et al., 1993). Similarly, there is evidence that mutations in the region between codons 1250 and 1464 result in a more extreme phenotype with profuse polyps in the colon and rectum (Nagase et al., 1992) and specifically the 5bp deletion at 1309 is associated with early onset of aggressive GI disease (Caspari et al., 1994). Such findings support the notion of a dominant-negative effect of truncating APC mutations where more 3' mutations allow translation of a protein structure which permits heterodimer formation resulting in impaired APC function.

The phenotypic effects of mutations at different locations in APC gene is also demonstrated in the extent of extracolonic manifestations in FAP. Congenital hypertrophy of retinal pigment epithelium (CHRPE) is a common clinical sign in FAP (Chapman et al., 1989). Olschwang et al. (1993) defined a critical boundary in exon 9 with mutations 3' to this region associated with CHRPE lesions, whereas mutations 5' of exon 9 are associated with a normal retina. More recently, evidence has suggested that mutations between codon 1445 and 1578 in exon 15 are associated with normal retinas (Caspari et al., 1995). Furthermore, the majority of individuals with mutations between codon 1445 and 1578, showing no CHRPE lesions had aggressive desmoid tumours which often predated symptomatic colorectal disease and, in some cases, resulted directly in death (Caspari et al., 1995).

These genotype-phenotype correlations may be important determinants in the approach to FAP. The presence or absence of CHRPE lesions may assist in localising the site of the mutation, thereby improving mutation detection within affected families. Antenatal diagnosis and surgical management may also be influenced. The presence of desmoid disease can be devastating to the quality of life in patients with FAP. In those families with a mutation known to be associated with severe desmoid disease this information could be considered during antenatal counselling and may affect the decision to have children. Restorative proctocolectomy with ileal pouch, if considered at all, should be performed as a primary procedure in those at high risk of developing severe desmoid disease, as subsequent dissection may be hampered by pelvic and abdominal desmoid tumours. In the longer term, as the number of reported mutations increases and the quality of clinical data improves, more subtle clinical effects of different APC mutations may well be determined, yielding details of APC gene function and ultimately means by which therapeutic intervention may succeed.

Modifying FAP phenotype

Other genetic and environmental phenotypic modifiers are likely to be important. This is suggested by the fact that identical APC mutations have been associated with diverse FAP phenotypes (Paul et al., 1993; Groden et al., 1993). It is clear that environmental modulation does occur since a reduction in number and size of colorectal polyps can be achieved following treatment with the non-steroidal anti-inflammatory agent sulindac (Giardello et al., 1993). In
addition, Nicholls et al. (1988) described regression in rectal polyps after colectomy and ileorectal anastomosis presumably due to changes in the local environment.

Genetic modification has been described in a mouse model of FAP (Moser et al., 1990) with a germline nonsense mutation in exon 15 of APC which displays multiple intestinal neoplasia (Min). The phenotypic expression in Min mouse is modified by a locus on mouse chromosome 4 (Dietrich et al., 1993) corresponding to human chromosome region 1p35-36. Interestingly, this region shows frequent allele loss in sporadic colorectal tumours, suggesting the presence of a significant colorectal cancer gene. Characterisation of such a modifier gene may allow pharmacological modulation of the FAP phenotype and even processes involved in sporadic colorectal carcinogenesis.

Presymptomatic screening and diagnosis

The initial hopes of dramatically improving presymptomatic diagnosis in FAP have been realised. Mutation analysis provides a reliable approach for presymptomatic screening in FAP with reduction in the requirement for intensive colonoscopic screening. In families where the causative APC mutation is identified, those who are demonstrated not to have the mutation may be discharged from follow-up thereby reducing patient distress and health care expenditure. Those with the mutation are offered on-going counselling and colonoscopic surveillance with a view to optimising the timing of surgery, to accommodate life events of adolescence and early adulthood as well as disease progression.

Linkage analysis has been of proven value in FAP (Dunlop et al., 1991). However, despite the identification of highly polymorphic microsatellite repeat markers, it lacks the ultimate accuracy and reliability of mutation analysis. It is largely reserved for those families in which the APC mutation has yet to be identified and excluding linkage to APC gene in other familial cancer syndromes.
1.5.3 DNA mismatch repair in colorectal carcinogenesis and susceptibility

**Hereditary non-polyposis colorectal cancer**

The identification of the APC gene was aided by the dominant, fully penetrant nature of FAP. However, more common heritable forms of colorectal cancer are described, which lack any pre-cancerous phenotype. Of this group, hereditary non-polyposis colorectal cancer (HNPCC) is the most well defined. HNPCC is an autosomal dominant disorder which can be inherited as a site-specific colorectal cancer susceptibility trait (Lynch Type 1) or associated with breast, ovarian, uterine and other malignancies (Lynch Type 2 or cancer family syndrome). Adenomatous polyps are found in HNPCC patients but polyposis is not a feature and, in general, polyp numbers are similar to that in the general population with colorectal cancer (Burt et al., 1992). There is a propensity for both adenomas and carcinomas to develop in the proximal part of the colon and the age of onset is much earlier than in the general population (Lothe et al., 1993).

2.5% colorectal cancer patients fulfil the criteria for HNPCC as detailed by the International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer (ICG-HNPCC) (Vasen, 1991). These require: three or more relatives with histologically proven colorectal cancer, one being a first degree relative of the other two; two or more generations affected and at least one family member affected before age 50 years. However, these stringent criteria were designed for research purposes and not intended for routine use in clinical diagnosis. They will only identify families where the gene defect is highly penetrant and the families are of sufficient size to allow the appropriate number of cases to arise and, therefore, many small families will be excluded inappropriately. Thus, application of ICG-HNPCC criteria has almost certainly resulted in an underestimate of the true prevalence of HNPCC predisposition. For example, two sibs affected by colorectal cancer before the age of 40 with no other family history of cancer would not satisfy the ICG-HNPCC criteria, but few would doubt an inherited predisposition. Hence diagnosis on the basis of the ICG-HNPCC criteria alone is liable to underestimate the true incidence and overestimate the penetrance of HNPCC. To date, four different genes have been implicated in the aetiology of HNPCC and their penetrance is uncertain. The presence of multiple genes, incomplete penetrance and the lack of strong preneoplastic phenotype make HNPCC an elusive problem for researcher and clinician.

Studies on repeat sequences in genomic DNA from cancers in HNPCC identified widespread instability in short repetitive microsatellite sequences, suggestive of defective DNA mismatch repair (Aaltonen et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993). A similar pattern of DNA microsatellite instability occurred in _E coli_ (Grilley et al., 1990) and yeast (Strand et al., 1993; Prolla et al., 1994a; Prolla et al., 1994b) when mutations were induced experimentally in mismatch repair (MMR) genes. This group became strong candidate genes and
subsequent studies determined that HNPCC arises from germline mutations in human homologues of bacterial and yeast MMR genes. The characteristic microsatellite instability is a manifestation of defective mismatch repair. This defect is likely to result in the accumulation of mutations throughout the genome (including tumour suppressor genes and oncogenes) at a higher than expected frequency due to a lack of ‘proof-reading’ in the repair system.

Identification of the causative genes in HNPCC

Two groups simultaneously isolated the first human MMR gene, hMSH2, responsible for HNPCC (Fishel et al., 1993; Leach et al., 1993). Using DNA sequence from yeast MSH gene, Fishel et al. (1993) exploited inter-species homology to identify the human gene, hMSH2, localising it to chromosome 2p. Leach et al. (1993) employed a combination of positional cloning and candidate gene strategies, generating multiple markers within a 25 cM region defined by earlier linkage analysis. In these studies, 2 large HNPCC pedigrees were examined for linkage at 345 polymorphic sites throughout the genome and the disease co-segregated with an anonymous marker (D2S123) on chromosome 2p (Peltomaki et al., 1993a). Detailed linkage analysis within chromosome 2p revealed recombination events which designated a 0.8 Mb region containing the locus of interest. Candidate genes mapping to this 0.8 Mb region were screened for the presence of germline mutations in HNPCC kindreds and causative mutations were demonstrated in hMSH2.

Linkage analyses (Peltomaki et al., 1993a) found significant genetic heterogeneity in HNPCC as one third of families examined were not linked with D2S123. A subsequent report established linkage to a marker on chromosome 3p in one HNPCC family (Lindblom et al., 1993) and further investigations found causative mutations in MMR gene hMLH1 on chromosome 3p (Bronner et al., 1994; Papadopoulos et al., 1994). Further studies of human homologues of MMR genes determined hPMS1 and hPMS2, on chromosomes 2q and 7q respectively, to be responsible for a proportion of remaining HNPCC families (Nicolaides et al., 1994).

More recently, a further gene, GTBP or p160, has been identified which is a member of the same family of genes as hMSH2. GTBP protein forms a complex with hMSH2, and this heterodimer, known as hMutSa, is essential for correcting subtle mismatch errors (Drummond et al., 1995; Palombo et al., 1995; Papadopoulos et al., 1995). Mutations in GTBP are associated with defective mismatch repair in cell lines derived from sporadic colorectal cancers, but, as yet, no germline mutations have been demonstrated in HNPCC (Papadopoulos et al., 1995).

Mutation analyses of the hMSH2 and hMLH1 genes in HNPCC families has provided information of significance for potential screening programmes. The vast majority of hMSH2 mutations result in a truncated gene product (Liu et al., 1994; Wijnen et al., 1995; Liu et al., 1996), thus allowing effective detection by means of straightforward in vitro protein synthesis techniques. This type of mutation detection method has already been exploited to screen for MMR gene mutations (Papadopoulos et al., 1994; Nicolaides et al., 1994). The types of
mutation occurring within hMLH1 show a wide variation across different populations (Liu et al., 1996; Tannergård et al., 1995; Han et al., 1995; Papadopoulos et al., 1994) and the efficacy of such techniques remains to be established in this case.

**DNA instability in HNPCC and ‘sporadic’ colorectal cancers**

The characteristic microsatellite instability seen in HNPCC (Peltomaki et al., 1993a; Peltomaki et al., 1993b; Aaltonen et al., 1994) is also detectable in around 15% of apparently sporadic colorectal cancers (Aaltonen et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993; Lothe et al., 1993; Bubb et al., 1996). Sporadic cancers with DNA microsatellite instability tend to be right sided (Ionov et al., 1993; Thibodeau et al., 1993; Lothe et al., 1993; Aaltonen et al., 1993; Kim et al., 1994), exhibit extracellular mucin production (Kim et al., 1994), be poorly differentiated (Ionov et al., 1993; Kim et al., 1994; Lothe et al., 1993) and have an inverse relationship with p53 protein stabilisation (Ionov et al., 1993; Kim et al., 1994), features shared with HNPCC. There is no significant association between the presence of microsatellite instability and patient age (Ionov et al., 1993; Lothe et al., 1993). Although this group will contain some occult cases of HNPCC, most patients do not have germline mutations in mismatch repair genes and microsatellite instability is presumed to arise as a somatic event (Liu et al., 1995a; Borresen et al., 1995). The absence of microsatellite instability in sporadic adenomas would certainly be consistent with a lack of germline-line mutation (Young et al., 1993).

In cancers selected from patients less than 35 years of age, 60% have been shown to have tumour DNA instability (Liu et al., 1995b) and preliminary mutation analysis suggests that germline mutations in mismatch repair genes may be responsible for over 40%. It is likely that cancers arising in this age-group are predominantly associated with constitutional predisposition, the exact nature of which is yet to be determined. In sporadic cancer it remains to be determined whether microsatellite instability may be a viable means of targeting molecular and clinical screening for the sub-group of patients with hereditary predisposition. However, at present it is appropriate to concentrate research and clinical screening activities on early onset cases and large cancer families.

In both sporadic and hereditary disease, an inverse relationship has been noted between microsatellite instability and p53 mutation, which in turn is associated with aneuploidy (Carder et al., 1993). This is suggestive that at least two mechanisms of genomic instability can lead to in colorectal cancer, one involving defects in mismatch repair in predominantly diploid cells and the other p53 mutation and the development of aneuploidy.

**Mismatch repair gene defects in sporadic colorectal cancer**

Liu et al (1995a) found that although 15% of sporadic cancers displayed DNA instability, mutations in hMSH2 and hMLH1 occurred in only 10% of these, a similar low frequency to that in the general colon cancer population. Thus, the genes which cause the
majority cases of HNPCC do not appear to underlie microsatellite instability in sporadic tumours with clinicopathological features similar to those of HNPCC. Further studies have reached similar conclusions (Borresen et al., 1995; Bubb et al., 1996), indicating that the molecular mechanisms associated with infidelity of DNA replication are different in hereditary and sporadic cancers displaying microsatellite instability.

In time, it is likely that somatic mutations in other genes will be shown to be responsible for defective mismatch repair in sporadic cancer with microsatellite instability. Indeed, the recently described gene, GTBP/p160, has already been shown to be mutated in several cases of sporadic colorectal cancer and may be proved to be of importance (Drummond et al., 1995; Palombo et al., 1995; Papadopoulos et al., 1995). In addition, defects in other mismatch repair systems may be involved. One such candidate is a nucleotide-specific mismatch repair system which recognises G-T mismatches (Wiebauer and Jiricny, 1990) resulting in a C to T transition if allowed to progress through mitosis. As previously discussed, such transitions are frequently seen in the APC gene in both FAP and sporadic colorectal tumours. Again, this suggests the intriguing possibility that many of the somatic changes that are involved in the very genesis of colorectal cancer and indeed of FAP are caused by defects in DNA repair systems.

Infidelity of DNA replication may result from inherited defects in DNA polymerases such as polymerase δ. Recent reports in colorectal cancer cell lines have identified microsatellite instability associated with polymerase δ in the absence of mismatch repair gene mutations (da Costa et al., 1995). These findings support the existence of considerable genetic heterogeneity in colorectal cancer development and predisposition, particularly in relation to infidelity in DNA replication.

**Mismatch repair defects in humans and the adenoma-carcinoma sequence**

Early *in vitro* studies suggested that a heterozygous mutation in mismatch repair gene resulted in a limited functional defect in a lymphoblastoid cell line from a patient with HNPCC (Parsons et al., 1993). Subsequently, a subset of patients have been identified with germline mismatch repair gene mutations who have an increased mutation rate in their normal tissues (Parsons et al., 1995). This is of considerable importance because such individuals have mutations developing in many tissues, but develop relatively few tumours. It may be that mutations have to occur in critical 'gateway' genes, such as *APC*, before a cancer can develop and it seems likely that environmental influences must have a major involvement in this process.

Colorectal cancer cell lines exhibiting microsatellite instability have a continuing defect in mismatch repair, which persists through passage, leading to progressive alterations in repetitive DNA sequences (Parsons et al., 1993; Shibata et al., 1994; Bhattacharyya et al., 1994). It seems reasonable to speculate that in human cancers the functional defect in mismatch repair will also persist, leading to further mutations.
Predictive testing in HNPCC

Accurate predictive testing has revolutionised clinical management and counselling in FAP, but, HNPCC presents a more difficult problem. Uncertainty over penetrance of mismatch repair gene mutation potentially undermines the benefits of pre-symptomatic diagnosis while lack of a definite pre-cancerous phenotype may cause problems in counselling and compliance with management in affected families. As noted above, it is likely that the frequency of mismatch repair gene mutation may be substantially higher than suggested from studies based on families selected through the ICG criteria: it has been calculated at around 1:200 in the general population (Lynch et al., 1992). In families where an mismatch repair gene mutation is identified, members must be offered screening for that mutation. Those carrying the mutation are at a high risk of developing colorectal cancer and should be submitted to intensive screening. Presently, this is recommended every three years, however, recent indications in high-risk individuals would support an intensive approach with colonoscopy every 1-2 years (Jarvinen et al., 1995; Vasen et al., 1995). As such screening may be required for up to 50 years, the burden in terms cost and patient morbidity could be considerable and these factors may only be addressed by the use of prophylactic measures.
1.5.4 p53 gene in colorectal carcinogenesis

Somatic abnormalities of p53 are the commonest genetic events associated with human cancers (Hollstein et al., 1991). There has been considerable impetus to understand its normal cellular functions and how these may be altered in carcinogenesis. p53 is a phosphoprotein which enters the nucleus during DNA synthesis, exerting control over the transcription of other genes and regulating the onset of DNA replication at the G1-S boundary (Vogelstein and Kinzler, 1992). Elevated levels of stabilised p53 protein are detected in many cell types on exposure to DNA-damaging agents such as ionising radiation. Under these conditions, p53 either blocks cell growth allowing DNA repair prior to cell division or alternatively induces apoptosis. The apoptosis response to ionising radiation and etoposide has been demonstrated to be p53 dependent in thymocytes (Clarke et al., 1993; Lowe et al., 1993a). This may have important clinical implications as apoptotic tumour cell death in response to radiation and chemotherapy is likely to be reduced in the absence of p53 function and such cancers may be more resistant to treatment (Lowe et al., 1993b).

Loss of function of wildtype p53 appears to be associated with chromosome instability and aneuploidy (Carder et al., 1993). This is demonstrated in fibroblast cell lines from Li-Fraumeni patients, which have constitutional p53 mutations. These cell lines readily become aneuploid and acquire immortality in vitro (Bischoff et al., 1990). The exact significance of this instability is uncertain, but it may favour the appearance of neoplastic clones.

The p53 mutational spectrum exhibits tissue specificity with the majority of mutations in colorectal cancer found in codons 175, 248, 278 and 282. These are most commonly single base transitions in CpG nucleotides causing mis-sense mutations (Prives and Manfredi, 1993). Such mutations result in a full length protein with altered conformation and abnormal function. However, the exact relationship between the type of p53 mutation and the effects on cellular function is undetermined. Under normal conditions p53 functions as a tumour suppressor gene. However, it appears that certain types of mutation may act as dominant oncogenes showing a gain of function rather than simply loss of tumour suppressor function (Halvey et al., 1990). This could occur either by binding of mutated protein to wild-type protein thereby preventing access to its receptor molecules, by altering its overall conformation, or the mutated protein may have a higher binding affinity for the receptor. Mutant p53 protein is stabilized within the nucleus, having a prolonged half life, allowing its detection by immunohistochemistry.

p53 inactivation is rare in colorectal adenomas indicating that it occurs late in the process of tumorigenesis (Baker et al., 1990; Carder et al., 1993). Constitutional p53 mutations have been shown to be responsible for the rare, autosomal dominant Li-Fraumeni syndrome (Halvey et al., 1990) which is characterised by predisposition to a variety of cancers including brain tumours, breast carcinomas, soft tissue sarcomas, leukaemia, osteosarcomas and
adrenocortical carcinomas. However, colorectal cancer is very rare in Li Fraumeni gene carriers and despite an extensive search, constitutional p53 mutations are not involved in the development of colorectal cancer in patients who succumb to the disease at an early age (Bhagirath et al., 1993).

1.5.5 Deleted in colon cancer (DCC) gene

Cytogenetic studies in colorectal cancer demonstrate frequent deletions involving the long arm of chromosome 18 and molecular genetic studies also detect frequent allele loss on 18q21-ter in cancer DNA (Vogelstein et al., 1988). Chromosome walking revealed a large gene in the region of a chromosome 18q rearrangement in a single cancer which was cloned and named DCC (deleted in colon cancer). DCC comprises a 12.5kb transcript and has features of a tumour suppressor gene. It is expressed at low level in normal colonic mucosa but expression is reduced or absent in colorectal cancer tissue (Fearon et al., 1990). In addition, both copies of the gene are inactivated in a proportion of colorectal cancers by mutation, deletion or rearrangement as detected by loss of heterozygosity (Tanaka et al., 1991). Loss of heterozygosity involving DCC gene is found in large adenomas as well as carcinomas indicating that inactivation of this gene is associated with the intermediate stages of colorectal tumorigenesis (Vogelstein et al., 1988).

Preliminary data suggested that DCC may be involved in colorectal cancer susceptibility. This was based on the description of a family which exhibited genetic linkage of the Kidd blood group to a dominant non-polyplosis trait developing colorectal cancer at an early age (Lynch et al., 1985). The Kidd blood group has been shown to map to a region very close to DCC on chromosome 18q. However, published data have not detected any evidence for linkage of intragenic DCC markers in a number of HNPCC kindreds (Peltomaki et al., 1991). As genetic heterogeneity in HNPCC is well documented it is possible that DCC is a colorectal cancer susceptibility locus in a minority of families.

1.5.6 The ras genes

Activated proto-oncogenes have been shown to occur at moderately high frequency in many human cancers. The ras family are cytoplasmic proto-oncogenes with signal transduction functions. Activating mutations in codons 12 and 13 of Kirsten ras occur in 50% of colorectal cancers and adenomas greater than 1 cm in diameter (Vogelstein et al., 1988). The frequency of Kirsten ras mutations is much lower in adenomas <1cm which suggests that this oncogene is involved in the progression of adenoma to carcinoma (Shibata et al., 1993). Kirsten ras expression, although high in primary carcinomas, tends to be lower in clinically advanced cases, indicating that when the tumour progresses to a certain stage, then ras activation is no longer required (Gallick et al., 1985).
There is some evidence to suggest that the ras genes may be involved in colorectal cancer susceptibility. A metaanalysis of all published studies identified rare Harvey ras alleles as likely predisposition factors in cancer and the authors propose from their findings that 1 in 11 colorectal cancers occur as a result of inheritance of a predisposing H-ras allele (Krontiris et al., 1989). It is possible that inheritance of a rare H-ras allele may indicate an increased risk of colorectal cancer and may interact with predisposition due to inheritance of a hMSH2 or other DNA repair gene mutation. Alternatively, these findings may have arisen through linkage between H-ras allele and as yet undetermined predisposition genes.

1.6 Other genetic changes in colorectal carcinogenesis

1.6.1 DNA methylation in colorectal cancer

DNA methylation is a covalent modification of DNA which occurs at cytosine-guanine dinucleotide (CpG) sites in the mammalian genome. In normal cells, approximately 70% of cytosine residues are methylated and a reduction in methylation appears to be associated with gene activation (Razin and Riggs, 1980). Goelz et al. (1985a) identified a reduction in methylation status of colorectal cancers and adenomas, however, this did not affect all genes suggesting that selective gene activation may occur. Overall, the effects of hypomethylation could be expected to increase expression of proto-oncogenes favouring cellular growth. Although neoplastic change is associated with generalised hypomethylation, specific areas of hypermethylation can occur (Silverman et al., 1989) possibly causing reduced expression of tumour suppressor genes, again contributing to increased cellular growth.

Reduced methylation also contributes to genomic instability (e.g. strand breaks and chromosome loss or gain), through derangement of chromatin condensation during mitosis (Laird and Jaenisch, 1994). This is possibly a consequence of reduced facility for binding methyl-specific proteins making some regions more accessible to oxidant and enzyme induced DNA breaks.

Changes in DNA methylation status appears to be an early event in the carcinogenic process, however, the exact significance is uncertain and it is possible that some effects may simply be secondary to the carcinogenic process.

1.6.2 DNA ploidy in colorectal carcinogenesis

The analysis of total DNA content is undertaken by flow cytometry. This determines the overall quantity of DNA in a population of cells and distinguishes two groups: those which are predominantly diploid and those with predominantly aneuploid population. The development of aneuploidy is associated with the later events of carcinogenesis, being more frequent in
carcinomas than adenomas (Quirke et al., 1986b). Furthermore the presence of aneuploidy is suggested to be associated with tumour aggression. A significant correlation between aneuploidy and reduced survival has been described (Armitage et al., 1985), however, this has not been confirmed in other series although aneuploidy is associated with increased local recurrence after resection in rectal cancers (Jones et al., 1989).

Aneuploidy is associated with stabilised p53 (Carder et al., 1993). p53 dysfunction is associated with increased frequency of inappropriate recombination events including amplification. and it is also possible that inactivation of p53 facilitates the generation of aneuploidy through impaired DNA repair at the G1-S boundary. The exact significance of changes in ploidy is uncertain. It is possible that it simply reflects a non-specific loss of control of DNA replication in malignant cells rather than indicating particular biological events or defects.

1.7 Evidence for the existence of an oncosuppressor locus on chromosome 8p

As outlined in section 1.5.1, the presence of a tumour suppressor locus is suggested by several experimental methods. These include the presence of non-random genetic lesions such as deletion or rearrangement of DNA and evidence suggesting that replacement of the chromosome may result in reversal of malignant behaviour at a cellular level. When present in combination, they provide powerful evidence for the presence of a putative tumour suppressor locus.

Non-random genetic events in colorectal cancer were detected by cytogenetic studies in chromosome 8 as well as chromosomes 17 and 18, (Muleris et al., 1985) 1, 13, 17 and 18 (Reichmann et al., 1981). Important tumour suppressor genes have subsequently been identified on all of these chromosomes. Cytogenetic studies have also demonstrated a stable 8p:12 translocation present in colorectal cancer tissue and passed on to the patient's offspring one of whom developed a rare testicular malignancy (Pathak et al., 1986).

Allelotype studies (Vogelstein et al., 1988) provided support for a locus on chromosome 8p when loss of heterozygosity was detected in 50% of colorectal cancers examined at two loci on the short arm of chromosome 8p. Subsequently these findings have been confirmed and expanded by many groups as discussed in Chapter 3.

Ichikawa et al. (1994) has described the introduction of human chromosome 8 in rat cell lines derived from prostatic cancer metastases. This provides collaborative evidence for a human chromosome 8 tumour suppressor locus, which is likely to be important in prostatic cancer.
1.8 Aims of the project

It is apparent from the foregoing description that colorectal carcinogenesis is associated with different types of genomic change. This thesis addresses the frequency and association (clinicopathological and molecular) of two important genetic lesions: the appearance of LOH in 8p and microsatellite instability. The data on 8p LOH (chapters 3 and 4) led to positional cloning exercise that delineated 2 distinct regions on which the critical oncosuppressor genes must lie. Study of microsatellite instability demonstrated a distinctive prognosis associated with this type of molecular lesion. The methods used are described in the next chapter.
Chapter 2

Materials and Methods

2.1 Tissue specimens

The provision of colorectal cancer and normal tissue from patients was facilitated by the Cancer Research Campaign Colorectal Cancer Study conducted in South East Scotland under the auspices of the Departments of Surgery and Pathology in the University of Edinburgh. This has permitted the formation of a bank of DNA from colorectal cancer and normal tissue, either blood or histologically normal colorectal mucosa. This DNA bank is a resource for many groups in the Cancer Research Campaign Laboratories, Department of Pathology and provides a rich source of material on which comprehensive clinical, molecular and pathological data are available. A total of 119 cancers with corresponding normal tissue were employed in studies localising the putative chromosome 8p tumour suppressor gene. Forty-seven sporadic colorectal adenomas were examined for loss of heterozygosity at chromosome 8p markers. In addition to the original 119 cancers, a further 126 were examined for evidence of microsatellite instability using (CA)$_n$ repeat markers.

2.1.1 Collection of sporadic colorectal cancers

All cancers were obtained at the time of colectomy. Specimens were removed from the operating theatre and delivered to the Department of Pathology at the University of Edinburgh. A record of the macroscopic examination was made by a pathologist and samples were obtained from the tumour and normal mucosa at a point distant from the lesion. Removed tissue was placed in 1.5ml capacity screw-capped vials, snap frozen in liquid nitrogen and stored at -70°C awaiting DNA extraction. Formal histological assessment on the resected lesion was performed by a pathologist and all histopathological data referred to in this work, e.g. Dukes' stage, is derived from this report.

2.1.2 Collection of sporadic colorectal adenomas

A total of 56 sporadic colorectal adenomas were obtained from two sources. Forty adenomas were collected from fresh colorectal specimens resected for malignant disease. The remainder were removed from cancer-free bowel as part of the Nottingham asymptomatic colorectal cancer screening programme (Hardcastle et al., 1989). In the latter group all normal DNA was leukocyte derived, whereas in the former, normal DNA was obtained from either
leukocytes or histologically normal colonic mucosa. Tissue was treated in the same manner as in section 2.1.1.

2.2 DNA purification protocols

DNA was extracted using an SDS-lysis and proteinase K digestion method adapted from Goelz et al. (1985b).

2.2.1 Purification of DNA from frozen tumours and normal colonic mucosa

All tissue handling was performed in a class 1 microbiological safety cabinet. Approximately 0.5 cm³ of tissue was obtained from -70°C storage and finely diced in a 0.5 ml solution of TE-9 SDS using a single-use scalpel blade. 2.5 mg proteinase K was added and incubated at 48°C for 24 hours with occasional gentle agitation. An equal volume of water-saturated phenol was added, mixed thoroughly and centrifuged at 500 x g for 20 minutes at 4°C. The aqueous layer was removed to a clean 1.5 ml tube and an equal volume of PC-9 added, mixed thoroughly and centrifuged at 500 x g for 20 minutes at 4°C. The aqueous layer was removed and added to an equal volume of chloroform:iso-amyl alcohol (24:1), mixed thoroughly and centrifuged at 500 x g for 20 minutes at 4°C. The aqueous layer was added to 2.5 ml of 7.5 M ammonium acetate and 30 ml of cold absolute ethanol solution. This solution was left for 24 hours at -20°C then centrifuged at 500 x g for 20 minutes. The alcohol was removed and the inverted tube left for 20 minutes. The pellet was resuspended in TE buffer. The optical density was measured at 260 nm on a Pye-Unicam spectrophotometer and the concentration adjusted to 1 mg/ml. The solution of DNA was divided into equal volumes for storage at 4°C and -20°C.

<table>
<thead>
<tr>
<th>TE-9 SDS:</th>
<th>PC-9:</th>
<th>TE buffer:</th>
</tr>
</thead>
<tbody>
<tr>
<td>500mM Tris pH8</td>
<td>480 ml phenol</td>
<td>See Appendix 1</td>
</tr>
<tr>
<td>20 mM EDTA</td>
<td>640 ml chloroform</td>
<td></td>
</tr>
<tr>
<td>10 mM NaCl</td>
<td>320 ml TE-9</td>
<td></td>
</tr>
<tr>
<td>1% SDS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.2.2 Purification of Peripheral Blood Leukocyte DNA

10 ml of whole blood were added to 10 ml of lysis buffer and left at room temperature for 30 minutes. An equal volume of water-saturated phenol was added, mixed and centrifuged at 1500 x g for 20 minutes at 4°C. The aqueous layer was removed, added to 5 ml of 7.5 M ammonium acetate and 30 ml of cold isopropanol and mixed thoroughly. This was stored at -20°C for 24 hours then centrifuged at 1500 x g for 20 minutes, the alcohol discarded and the
pellet of DNA dried and resuspended in TE buffer. The final concentration was adjusted to 1mg/ml.

Lysis buffer: TE buffer:
0.1M Tris pH8 See Appendix 1
20mM NaCl
1mM EDTA
4% SDS

2.2.3 Estimation of DNA concentration

DNA concentrations were measured using a Pye-Unicam spectrophotometer with readings taken at 260nm and 280nm. An optical density ratio (OD 260/280) of 1.8 was taken as optimum purification. DNA solutions were adjusted to a concentration of 1mg/ml for use in restriction enzyme digests. Subsequent dilutions to 25 µg/ml were performed for use as template in polymerase chain reactions.

2.3 DNA analysis with restriction fragment length polymorphisms

2.3.1 DNA digestion with restriction endonuclease

Genomic DNA was digested with appropriate enzymes according to manufacturers' instructions (Boehringer-Mannheim or NBL Gene Science Ltd). Each digest was performed in a final volume of 30µl. A 25µl aliquot was taken from a master mix of sterile distilled water, restriction enzyme buffer, restriction enzyme and spermidine to which the test DNA was added. This solution was prepared and maintained on ice giving final volumes in each 30µl digest of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x restriction enzyme buffer</td>
<td>3µl</td>
</tr>
<tr>
<td>restriction enzyme (4 units per µg of test DNA)</td>
<td>2µl</td>
</tr>
<tr>
<td>spermidine (100mM)</td>
<td>1µl</td>
</tr>
<tr>
<td>sterile distilled water</td>
<td>19µl</td>
</tr>
<tr>
<td>test DNA (1mg/ml)</td>
<td>5µl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>30µl</td>
</tr>
</tbody>
</table>

Volume of restriction enzyme varied depending on concentration (usually 10 units/µl) and if necessary this was accommodated by changes in volume of sterile distilled water.
Spermidine was used routinely in restriction digests as it can optimise enzyme activity by chelating inhibitory salts. After addition of test DNA the solution was mixed gently and incubated overnight at the recommended temperature, which in all cases was 37°C. Following overnight digestion, a 1µl aliquot of the same restriction endonuclease was added and the solution incubated for a further 3 hours, thus maximising digestion.

Restriction enzyme buffers (final concentrations):

<table>
<thead>
<tr>
<th>Buffer</th>
<th>HindIII</th>
<th>HinfI</th>
<th>EcoRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl</td>
<td>6mM</td>
<td>6mM</td>
<td>9mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>6mM</td>
<td>6mM</td>
<td>10mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>100mM</td>
<td>50mM</td>
<td>50mM</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>1mM</td>
<td>1mM</td>
<td>pH7.5</td>
</tr>
<tr>
<td>pH</td>
<td>pH7.5</td>
<td>pH7.5</td>
<td>pH7.5</td>
</tr>
</tbody>
</table>

2.3.2 Separation of restriction endonuclease products by horizontal gel electrophoresis

Restriction digest products were separated by agarose gel electrophoresis. The concentration of agarose varied according to size of fragments for separation. 0.8% agarose was employed for fragments between 2 and 10kb and 1.5% for 500bp to 2kb. Electrophoresis was performed using 20cm x 20cm horizontal gel electrophoresis tank with 1xTBE running buffer.

Digestion products were pulse-centrifuged for 1-2 seconds, 3µl loading buffer added and samples loaded onto the gel. 1 kilobase ladder or HindIII-digested lambda phage was run alongside products as a size marker (see appendix 3 for marker sizes). After loading, the gels were run for 16 hours at 60mA at 4°C to prevent overheating.

Loading buffer: TBE buffer:
30% glycerol See Appendix 1
0.25% bromophenol blue
0.25% xylene cyanol FF

2.3.3 Southern transfer of fractionated DNA

After electrophoresis the gels were transferred to a solution of ethidium bromide (0.5µg/ml), stained for 20 minutes then destained for 10 minutes in distilled water. This dye intercalates with DNA and fluoresces under ultra-violet light. The gels were assessed and photographed under ultraviolet light generating a record of the test DNA concentration and proficiency of digestion. The gel was then transferred to denaturation buffer for 45 minutes to
produce single stranded DNA and then neutralisation solution for 45 minutes prior to Southern transfer.

Transfer of the single-stranded DNA to a nylon membrane was performed by Southern blot (Southern, 1975). A 20cm x 40cm wick (Whatman number 17 chromatography paper) was suspended over a perspex plate with each end immersed in a tank of transfer solution. The gel was placed onto the paper wick and an identical size of Hybond N+ nylon membrane placed on top of the gel. All air bubbles were removed and the Hybond N+ was marked with pencil to ensure orientation. A stack was then made with two sheets of Whatman number 17 paper and 10cm of dry paper towels, topped with a 20cm x 20cm rigid perspex plate and a 1Kg weight to compress the stack and optimise uniform flow of transfer solution through the gel and membrane. The DNA fragments thus transferred were retained in the membrane. Evaporation losses of transfer solution were minimised by covering open areas of the tank with cling film. Transfer occurred overnight, after which the Hybond N+ membrane was placed in neutralising solution for 45 minutes and then air dried.

Denaturation buffer/transfer solution: Neutralising solution:
1.5M NaCl 3M NaCl
0.5M NaOH 0.5M Tris HCl pH 5.5

2.3.4 32Phosphorus labelling of DNA probes

DNA probes were labelled by the random primer method (Feinberg and Vogelstein, 1983) using a commercial 'Megaprime labelling kit' (Amersham International Plc.). Probe DNA was maintained in recombinant plasmid vectors from which it was excised using appropriate restriction endonuclease in a 20μl solution:

probe DNA 5μg (usually in concentration of 1mg/ml) 5μl
10x restriction enzyme buffer 2μl
restriction enzyme (10 units) 2μl
sterile distilled water 11μl
Total volume 20μl

Variations in concentrations of probe DNA or restriction enzyme were accommodated by changes in volume of sterile distilled water. The mix was incubated at recommended temperature for 12 hours. The products of digestion were separated by horizontal electrophoresis in 1% low melting point agarose and the products identified by comparison with kilobase ladder as described earlier. DNA concentration was calculated assuming complete digestion. Probe DNA was
removed in a block of agarose using a fresh scalpel blade, the agarose warmed to melting and sterile distilled water added such that 10μl aliquots contained 50ng of probe DNA. These aliquots were then used directly with the Megaprime labelling kit.

32P-labelling was performed with appropriate precautions in a dedicated laboratory. 20ng primer mix was added to an aliquot of probe DNA (50ng) and denatured by heating to 100°C for 2 minutes, then placed at 37°C for 5 minutes. The following labelling reaction was then performed:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>nucleotide mix (5mM each dNTP)</td>
<td>10μl</td>
</tr>
<tr>
<td>32Pα dCTP (50μCi)</td>
<td>5μl</td>
</tr>
<tr>
<td>Klenow enzyme (used from -20°C) (4U/μl)</td>
<td>2μl</td>
</tr>
<tr>
<td>distilled sterile water</td>
<td>17μl</td>
</tr>
<tr>
<td>dithiothreitol</td>
<td>2μl</td>
</tr>
<tr>
<td>10x random primer buffer</td>
<td>14μl</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>50μl</strong></td>
</tr>
</tbody>
</table>

This was incubated at 37°C for 30 minutes. The labelled probe DNA was then purified using a G50 sephadex nick column (Pharmacia Biotechnologies Ltd) according to instructions, allowing precipitation of the labelled probe and removal of unincorporated isotope. The purified probe was eluted in a 400μl volume and held on ice until required. Immediately before hybridisation it was denatured by heating to 100°C for 2 minutes, after the addition of 0.5ml (5mg/ml) of sonicated salmon sperm DNA. This reduces non-specific binding of probe to DNA, thereby improving autoradiograph definition.

10x random primer buffer: Restriction enzyme buffer:
900mM HEPES pH6.6 See 2.3.1
100mM MgCl₂

2.3.5 DNA hybridisation and autoradiography

Prehybridisations and hybridisations were performed within a hybridisation oven (Hybaid Ltd, UK) at 65°C using cylindrical bottles secured horizontally on a rotating drum. The Hybond N+ filters were sandwiched between two flat sheets of nylon mesh membranes, loosely rolled and placed in hybridisation cylinder with 20ml of hybridisation buffer for 3 hours prior to hybridisation. DNA probe was denatured by heating to 100°C for 2 minutes in the presence of sonicated salmon sperm DNA, then added to the hybridisation solution and placed in the oven overnight.
Following hybridisation, the solution was discarded and filters treated with a series of washes of increasing stringency. A 2xSSC wash was performed for 10 minutes at 65°C and repeated, followed by 2xSSC/1% SDS (wash 2) and 0.5xSSC/1% SDS (wash 3) under the same conditions. The probed filter was then removed, carefully wrapped in cling film and placed in an autoradiograph cassette with Kodak X-OMAT AR film at -70°C for 24 hours before development. If the signal was poor, the filter was exposed for up to 2 weeks.

When autoradiography was complete, membranes were stripped in 0.1 x SSC at 100°C for 20 minutes and repeatedly hybridised if necessary.

Hybridisation buffer:  
SSC:  
10% dextran sulphate  
6 x SSC  
1% SDS

2.3.6 Restriction fragment polymorphic markers

The RFLP markers used in this study were kind gifts of other workers: pGLPL35 (Dr. Michael C. Schotz); pUC92F (Dr. Sam Wilson) pKSR2 (Dr. Kiyoshi Kaneko) and pMS502 (Prof A. J. Jeffreys) or obtained through American Tissue Culture Collection (ATCC). Data relating to these probes are presented in Table 2.1. All probes were obtained as whole plasmids which were grown in Eschericia Coli providing adequate quantities of probe material. Handling of bacteria was performed in a class 1 safety cabinet.
Table 2.1
Details of restriction fragment length polymorphic markers employed in chromosome 8 analysis

<table>
<thead>
<tr>
<th>Probe (Locus)</th>
<th>Vector</th>
<th>Cloning site</th>
<th>Insert size</th>
<th>Location (PIC)*</th>
<th>Enzyme</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKSR2 (D8S163)</td>
<td>pUC 19</td>
<td>EcoRI/PstI</td>
<td>507 bp</td>
<td>8pter-8p22</td>
<td>EcoRI</td>
<td>Kaneko et al., 1991</td>
</tr>
<tr>
<td>pG2LPL35 (LPL)</td>
<td>pGEM2</td>
<td>Sal I</td>
<td>2.4kb</td>
<td>8p22</td>
<td>Hind III</td>
<td>Heinze et al., 1987</td>
</tr>
<tr>
<td>pUC9-2F (POLβ)</td>
<td>pUC9</td>
<td>EcoRI</td>
<td>560bp</td>
<td>8p12-8p11</td>
<td>EcoRI</td>
<td>SenGupta et al., 1986</td>
</tr>
<tr>
<td>pMS502 (D8S162)</td>
<td>pUC13</td>
<td>EcoRI/BamHI</td>
<td>450bp</td>
<td>8q</td>
<td>Hinf I</td>
<td>Armour and Jeffries et al., 1991</td>
</tr>
</tbody>
</table>

PIC: Proportion of informative cases with each restriction digest.

2.4 Probe plasmid preparation

2.4.1 Preparation of competent E. Coli

5mls of L-broth were inoculated with 10µl of frozen DH5α E. Coli from a competent stock and allowed to grow at 37°C overnight in an orbital shaker. This culture was then added to 500 ml of L-broth and left shaking at 37°C until OD₆₀₀ = 0.2 (approximately 3 hours). The culture was equally divided between two centrifuge bottles and allowed to cool on ice for 10 minutes. Samples were then centrifuged at 4000 x g for 5 minutes. The supernatant was discarded with appropriate precautions and each of the pellets resuspended in 10ml of freshly made TSB. After standing on ice for 10 minutes, 100µl aliquots were placed in 1.5ml tubes and snap frozen in liquid nitrogen prior to storage at minus 70°C. Efficiency was tested by transforming with a range of concentrations of whole plasmid as in section 2.4.2.
L-broth:
10g/l Bacto-tryptone
10g/l NaCl
5g/l yeast extract
Autoclave before use

TSB:
L-broth containing:
10% polyethylene glycol
10mM MgCl₂
10mM MgSO₄
5% dimethyl sulphoxide (DMSO)

2.4.2 Transformation of competent *E. Coli*

25ng of whole plasmid was added to 100μl of competent DH5α *E Coli* on ice for 30 minutes. Samples were heat-shocked by placing in a water bath at 42°C for 4 minutes. 500μl of pre-heated L-broth was added and the culture maintained at 37°C in an orbital shaker for 1 hour. 20μl and 100μl volumes were then spread onto L-amp plates and incubated overnight at 37°C. A negative control was included, where 100μl of DH5α bacteria were subject to the same protocol with the omission of plasmid. Three discrete colonies were picked using sterile toothpicks and each transferred to 10ml of L-broth in 50ml polypropylene tubes. These were grown overnight at 37°C in an orbital shaker. The contents of one 50ml tube were added to 500ml of L-broth for continued culture at 37°C until OD₆₀₀ = 2.0 when the culture was suitable for large-scale expansion (maxi-preparation). The remaining tubes were stored at -20°C.

Maxi-preparation was performed using a Qiagen Maxi-preparation kit according to manufacturer's instructions. This is a simple and rapid technique designed to perform Maxi-preparation on 500μg of DNA in a maximum of 500ml cultures of *E. coli*. 500ml culture was divided between two centrifuge tubes with careful balancing. Solutions were spun at 15000 x g for 10 minutes and each pellet resuspended in 10ml of buffer P1. 10ml of buffer P2 was added and incubated at room temperature for 5 minutes. 10ml of buffer P3 was added and the solution centrifuged at 15000 x g for 30 minutes. The supernatant was removed promptly and centrifuged again, such that a particle-free lysate was obtained. This lysate was then processed using the Qiagen-tip 500 as directed. DNA was precipitated from the eluted solution using 0.7x volumes of isopropanol and centrifuged at 15000 x g for 30 minutes. The DNA was washed with 70% alcohol, partially air-dried and resuspended in TE. Concentration was determined using optical density and adjusted as appropriate (usually 1mg/ml).
L-amp plates: Buffer P1: Buffer P2:  
L-broth containing: 100mg/ml RNase A 200mM NaOH  
100µg/ml ampicillin 50 mM Tris/HCl 1% SDS  
12g/l Bacto-agar 10 mM EDTA pH 8.0  
Buffer P1:  
lOOmg/ml RNase A  
50 mM Tris/HCl  
Buffer P2:  
200mM NaOH 1% SDS  
1% SDS  
Buffer P3: TE buffer:  
2.55 M KAc See 2.4.1  
pH 4.8 See Appendix 1  
(Buffers P1, P2 and P3 supplied by Qiagen)

2.4.3 Exceptions to the above protocols

pMS502 was kindly provided by Mr Malcolm Dunlop in sufficient quantities that maxi-preparation was not required.

2.4.4 HindIII polymorphism detected by PCR

The HindIII polymorphic site in intron 8 of the lipoprotein lipase gene was exploited by means of polymerase chain reaction amplification and subsequent HindIII digest of products. This produced two alleles, of 1200 bp and 600 bp. In this series the polymorphism was informative in 25% of cases. A 1.2 kb fragment was amplified under conditions similar to those described by Bruin et al. (1991). Polymerase chain reactions consisted of 25µl volumes with 200µM of each nucleotide, 0.625units of Taq polymerase enzyme, 2.5µl of buffer, 50ng of each primer and 50 ng of template DNA. The reaction constituents were made in a master mix from which 23µl were aliquoted into 96 well PCR plates and 2µl of template DNA (25 µg/ml) was added. The 25µl reaction mixes were then overlaid with 25µl of paraffin oil. Precautions were taken to minimise chances of cross contamination. PCR reactions were carried out in a Hybaid Omnigene thermal cycler comprising 30 cycles of: denaturation at 95°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 1 minute. PCR products were digested in the same plates without removal of the overlying paraffin oil. 10µl volume from a master mix of HindIII was pipetted through the paraffin oil such that each reaction received HindIII restriction enzyme (0.8µl, 8units), HindIII buffer solution (3.5µl) sterile distilled water (5.5µl) and spermidine (0.2µl of 100mM stock). Digests were left overnight at 37°C. 5µl of running buffer as described above were added and digest products separated by horizontal agarose gel electrophoresis. 10µl volumes were loaded in 15cm x 15cm horizontal gel electrophoresis tanks.
with 2% agarose in 1xTBE. Electrophoresis was performed at 60 mA for 2 hours. The gel was stained in ethidium bromide and visualised under ultra-violet light. Alleles were readily identified against the kilobase ladder which was run simultaneously.

HindIII buffer (final concentration)
6mM Tris-HCl
6mM MgCl₂
100mM NaCl
1mM dithiothreitol
pH 7.5

10x *Taq* polymerase buffer
200mM (NH₄)₂SO₄
750mM Tris-HCl pH 9 at 25°C
0.1% Tween

TBE buffer:
See Appendix 1
2.5 Analysis of LOH using (CA)$_n$ repeat polymorphisms

2.5.1 Polymerase chain reactions

PCR reactions were carried out in a Hybaid Omnigene thermal cycler using 96 well plates. The reactions were labelled with $\gamma$-[$^{32}$P]ATP and the products separated by vertical electrophoresis in 6% denaturing polyacrylamide gel. The same conditions were used for all reactions, details of which are given below.

2.5.2 Preparation of oligonucleotide primer

All oligonucleotide primers were synthesised by Oswel DNA. These sequences were diluted with sterile distilled water before use, giving a final concentration of 50ng/µl. Details of loci employed are given in Table 2.2.

2.5.3 Radioisotope labelling of oligonucleotide primer

All handling of radioactive material was performed in a dedicated area with routine precautions. The (CA)$_n$ primer strand in each reaction was 5' end-labelled with $\gamma^{32}$P ATP using T4 polynucleotide kinase. Labelling was performed prior to each PCR and sufficient primer produced for up to 250 PCR reactions. 250ng of oligonucleotide (50ng/ml) was added to 5µl of 5x polynucleotide kinase buffer, 10µl of sterile distilled water, 3µl of $\gamma^{32}$P ATP (equivalent to 30µCi) and placed on ice. 3µl (30U) of T4 polynucleotide kinase was then added giving total volume of 24µl and the reaction placed in a water bath at 37°C for one hour. This amount was sufficient for up to 250 PCR samples. The final volume was adjusted by the addition of sterile distilled water, giving a final labelled oligonucleotide concentration of 1ng/10µl, which was a convenient volume for addition to each reaction.

T4 polynucleotide kinase buffer:
350mM Tris-HCl pH 7.6
50mM MgCl$_2$
500mM KCl
5mM $\beta$-mercaptoethanol

38
Table 2.2
Details of polymorphic microsatellite markers employed in chromosome 8 analysis

<table>
<thead>
<tr>
<th>Loci</th>
<th>Clone name</th>
<th>Primers</th>
<th>Allele size</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D8S201</td>
<td>Mfd199</td>
<td>TGGCTAACACGGTGAAACCA ATCAGACCAATAACCCCGAGG</td>
<td>170-285</td>
<td>8p22</td>
<td>Tomfohrde et al., 1992</td>
</tr>
<tr>
<td>LPL</td>
<td>LPL3gt</td>
<td>ATCTGGGTATGATATCATATGTGATCTGCTCTGACGCTCTCA</td>
<td>118-142</td>
<td>8p22</td>
<td>Tomfohrde et al., 1992</td>
</tr>
<tr>
<td>D8S 298</td>
<td>AFM234y10</td>
<td>AGGCTTCAACCCATGGACC ACGAGCACACACACATCATT</td>
<td>155-167</td>
<td>8p</td>
<td>Weissenbach et al., 1992</td>
</tr>
<tr>
<td>D8S 133</td>
<td>D8S 133CA/GT</td>
<td>CAGGTGGGAAAACCTGA ATCTGCTGTAACATATT</td>
<td>94-112</td>
<td>8p21.3-q11.1</td>
<td>Wood and Schertzer, 1992</td>
</tr>
<tr>
<td>D8S 136</td>
<td>D8S 136CA/GT</td>
<td>GCCCAAAGAGGAGAATAAAGGTGGGAAAACTGA</td>
<td>71-89</td>
<td>8p</td>
<td>Gyapay et al., 1994</td>
</tr>
<tr>
<td>NEFL</td>
<td>c171B10</td>
<td>GCAGTAGTGGCCGAGTTTCA TGCAATTCATTCCTCCTCTTCTT</td>
<td>137-147</td>
<td>8p21</td>
<td>Rogaev et al., 1992</td>
</tr>
<tr>
<td>D8S 137</td>
<td>Mfd39CA/GT</td>
<td>GATCACGAGACTCATCATA GTGAACTCATACCCAA</td>
<td>152-162</td>
<td>8p21.3-q11.1</td>
<td>Gyapay et al., 1994</td>
</tr>
<tr>
<td>D8S 87</td>
<td>Mfd39CA/GT</td>
<td>GGGTGGTGTGAATTAAACAC TGTAAAATTTTCAGACAG</td>
<td>145-149</td>
<td>8p12</td>
<td>Gyapay et al., 1994</td>
</tr>
<tr>
<td>ANK1</td>
<td></td>
<td>TCCAGATCGCTCTCATGAGGTTTGTGCCAG</td>
<td>107-115</td>
<td>8p21.1-p11.2</td>
<td>Polymeropoulos et al., 1992</td>
</tr>
</tbody>
</table>
2.5.4 Preparation of polymerase chain reactions

Polymerase chain reaction constituents, excluding radiolabelled oligonucleotide and template, were made as a master mix for each set of reactions (i.e. up to 96/Hybaid omniplate) such that 14μl aliquots contained 200μM of each nucleotide, 0.625 units of Taq polymerase enzyme, 2.5 μl of 10x Taq polymerase buffer and 50ng of each primer. 50ng (1μl) of template DNA was added followed by 1ng of labelled (CA)-strand primer (in 10μl volume) giving a final volume of 25μl. This was overlaid with 25μl of liquid paraffin.

The (CA)_n repeat microsatellite loci described above were amplified under conditions comprising 29 cycles of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute and extension at 72° for 2 minutes. 10 μl of loading buffer was added to each reaction and 10μl aliquots electrophoresed on 6% denaturing polyacrylamide gels.

10x Taq polymerase buffer: Loading buffer:
200mM (NH₄)₂SO₄ 95% deionised formamide
750mM Tris-HCl pH9 at 25°C 10mM Na₂ EDTA
0.1% Tween 0.1% xylene cyanol

2.5.5 Preparation of polyacrylamide gel

6% denaturing polyacrylamide gel was used in vertical sequencing gel apparatus with TBE buffer. Stock acrylamide was made in volumes of 500ml with 75ml of 40% 19:1 acrylamide:bis acrylamide ('Instagel', Severn Biotech Ltd), 50ml of 10x TBE, 230g of urea and the volume made up to 500ml with distilled water. This was protected from light and maintained at 4°C.

All gels were prepared in 35cm x 50cm vertical DNA sequencing electrophoresis plates (Bio-Rad Laboratories, UK). Plates were thoroughly cleaned with 'Decon' detergent, rinsed with sterile water and finally cleared of dust, using lint free wipes in 70% alcohol. One plate of each pair was siliconised after three uses by wiping with 3ml Gel Slick (Hoefer Scientific Instruments, Ltd) air drying and removing excess with water.

Plates were assembled into the buffer tank and a seal formed with 10ml of 6% acrylamide, 50μl of 25% ammonium persulphate and 50μl of N,N,N',N'−tetramethylmethylenediamine (TEMED). 50μl of 25% ammonium persulphate and 50μl of TEMED were added to 50ml of stock 6% acrylamide and mixed gently, avoiding excessive aeration. This was drawn into a 50ml syringe and run between the plates which were supported approximately 30° above the horizontal forming the 0.4mm gel. The comb was inserted and the gel allowed to polymerise for at least 2 hours. The apparatus was covered in cling film to
prevent excessive drying. Once formed, the gel apparatus was constructed with buffer chambers filled with 1xTBE and the gel pre-run for 30 mins at 100W to reach 55°C.

10μl of combined PCR product, prepared as in 2.5.4, and loading buffer were added to the wells after rinsing well with TBE to disperse urea. The gel was then run at 100W for 4-8 hours or until bromophenol blue approached the lower third of the electrophoresis plate. At this point the apparatus was dismantled, leaving the gel exposed, adherent to one electrophoresis plate. The gel was then fixed for 10 minutes in a solution of 10% methanol and 10% acetic acid and transferred to Whatman No 17 paper. The gel was then covered in cling film, leaving the paper back exposed, and dried on a vacuum gel drier. Dried gel was exposed to Kodak X-OMAT AR film for 1-3 days. Autoradiographs were assessed visually for loss of heterozygosity.

TBE buffer:
See Appendix 1

2.6 Assessment of allele loss at RFLP and (CA)n microsatellite loci

The same principles were employed for both RFLP and (CA)n microsatellite loci. Allele loss was assessed independently by at least two observers. Those markers that were homozygous (ie. two identical allelic bands, the maternal and paternal chromosomes are indistinguishable) were scored as 'non-informative' as deductions on allelic imbalance could not be made. Those markers that were heterozygous were regarded as 'informative'. The absence or marked reduction in intensity of an allelic band in tumour DNA was scored as 'loss of heterozygosity' whereas if both alleles were unchanged in the tumour DNA the case was scored as 'retained heterozygosity'. Any cases proving to be ambiguous were repeated and, if doubt remained, scored as non-informative. The same principle was used for RFLP and (CA)n microsatellite repeat markers. Although some groups have employed laser densitometry to quantify loss of heterozygosity (Emi et al., 1992a) visual assessment of loss of heterozygosity has been proven in other studies (Baker et al., 1990; Ashton-Rickardt et al., 1991; Miki et al., 1991). Two examples of loss of heterozygosity are presented in Figure 2.1. In both cases the 'lost' allele can be identified by a faint residual band. This represents contaminating stromal tissue or inflammatory cell infiltrate.
Figure 2.1
Interpretation of autoradiographs

A. Case 1 Case 2 Case 3
   N  T  N  T  N  T

   17Kb  12Kb

B. Case 4 Case 5 Case 6
   N  T  N  T  N  T

   280b  275b  180b

Results from 6 different cases:

A. Restriction fragment length polymorphism (pKSR2)
   B. (CA)n repeat polymorphism (D8S201) on chromosome 8p.

Each case consists of normal (N) and tumour (T) DNA sample. Allele sizes are indicated. In cases 1 and 4 both loci show retained heterozygosity in tumour DNA. Cases 2 and 5 are homozygous. Cases 3 and 6 show loss of heterozygosity of the 12Kb allele at pKSR2 and the 280 base allele in D8S201, respectively.
2.7 Assessment of microsatellite loci for evidence of replication errors (RER)

As described in chapter 1, microsatellite instability has been noted in colorectal cancers arising as part of the syndrome of hereditary non-polyposis colorectal cancer (HNPCC). The presence of microsatellite instability was determined in the original 119 cancers used in the series of chromosome 8p loss of heterozygosity and in a further group of 126 cancers from the Department of Pathology, University Medical School, Edinburgh. These cancers were assessed by two methods of proven value in detecting replication errors in microsatellite loci: silver staining of unlabelled PCR products separated by polyacrylamide gel electrophoresis and fluorescein-labelled PCR with the use of the automated laser fluorescence (ALF) DNA sequencer (Pharmacia Biotechnologies Ltd). Methods employing silver staining were performed by Dr Jill Bubb in the Department of Pathology, University Medical School, Edinburgh.

2.7.1 Polymerase chain reaction with $\gamma^{32}P$ ATP labelling and autoradiography

The technique described for analysis of loss of heterozygosity was also employed in the assessment of microsatellite loci for evidence of replication errors. Those microsatellite loci used in loss of heterozygosity studies were routinely examined for evidence of microsatellite instability in cancer DNA.

2.7.2 Microsatellite analysis with Silver staining of (CA)$_n$ gels

Evidence of microsatellite instability was sought at 4 or more of the following loci: D2S119, D3S1293, D8S282, D13S160, D15S132, D17S849 (Gyapay et al., 1994). Polymerase chain reaction methods were identical to those in 2.5 except that there was no labelling of the oligonucleotide primers.

Five to 15µl of PCR product was denatured at 95°C for 5 minutes in 0.5X volume short tandem repeat (STR) loading buffer and then placed on ice. Electrophoresis was carried out on 0.4mm 6% denaturing acrylamide gel (19:1 acrylamide:bis acrylamide) containing 7M urea. To facilitate staining the gel was bound to the large glass plate by first treating the plate with 5µl γ-methacyryloxypropyltrimethoxysilane in 1ml 95% ethanol, 0.05% acetic acid for 5 minutes and cleaning twice with 95% ethanol before pouring the gel. Electrophoresis buffer was 0.5x TBE and gels were electrophoresed at constant power (70W) after pre-heating by running for 1 hour.

Gels were stained on a rocking platform according to the method of Bassam et al. (1991), as detailed in Promega’s DNA Silver Staining System protocol. The gel was placed in 10% glacial acetic acid for 20 minutes, washed 3 times in distilled water distilled water for 2 minutes and placed in 1g/l silver nitrate, 1.5ml 37% formaldehyde staining solution for 30
minutes. The gel was then washed for 10 seconds in distilled water and placed in chilled developer solution until bands appeared. The reaction was stopped with 10% glacial acetic acid for 5 minutes, the gel washed for 2 minutes in distilled water and then interpreted and photographed on a light box. For re-use, plates were soaked in 5M sodium hydroxide for one hour and cleaned with detergent and 70% ethanol.

STR loading buffer: Developer solution:
10mM NaOH 81g sodium carbonate (decahydrate)
95% formamide 1.5ml 37% formaldehyde
0.05% bromophenol blue 200µl 10mg/ml sodium thiosulphate
0.05% xylene cyanol FF Made to 1 litre with DDW

TBE buffer:
See Appendix 1

2.7.3 Microsatellite analysis with automated laser fluorescence (ALF) DNA sequencer

An automated laser fluorescence DNA sequencer was also used in assessment of microsatellite instability in tumour DNA. This technique is routinely employed in automated genotyping and linkage analyses (Mansfield et al., 1994). It permits the rapid assessment of microsatellite loci for evidence of instability in cancers, which may be applicable to large scale use.

Preparation of oligonucleotide primers

Oligonucleotide primers were synthesized by Oswel DNA, Southampton. For each primer pair, the oligonucleotide synthesising the CA-rich DNA strand was fluorescein isothiocyanate (FITC)-labelled by the manufacturer. These sequences could be used directly and were diluted with sterile distilled water, giving a final concentration of 50µg/ml.

Polymerase chain reaction

PCR mixtures consisted of diluted template DNA (50 to 75ng), 200µM dNTP's, 10mM Tris HCl pH9 (at 25°C), 1.0mM MgCl2 50mM KCl, 0.1% Triton X-100, 400nM primers (one of which was FITC-labelled), 1.5 units Taq polymerase in a 25µl volume, overlaid with paraffin oil. Polymerase chain reactions were performed in 96-well plates on a thermal cycler according to cycling conditions described in 2.5.4. After completion of reaction the samples were stored at -20°C until used. 1 µl of PCR solution was added to 4µl of stop solution with appropriate internal standards. The 5µl volume was then heat denatured and loaded onto ALF apparatus gel, which was run at a constant 2000V, 70mA, 55W and 50°C.
Stop solution:
95% formamide
0.8% dextran blue
25mM EDTA pH 9

Internal standards
Internal standards were prepared by amplification from single stranded DNA of M13mp18 vector using a variety of 20-24-mer primers and the above reaction conditions. These generated standard fragments between 65 and 313 nucleotides, and appropriate standards were added to microsatellite PCR products prior to electrophoresis. These were kindly prepared and supplied by Mr Stewart Morris, at the MRC Human Genetics Unit, Edinburgh.

Electrophoresis and analysis of raw data
Electrophoresis was performed in an automated laser fluorescence (ALF) DNA sequencer. Glass plates were thoroughly cleaned with 'Decon' detergent, rinsed with sterile water and finally cleared of dust using lint free wipes in 70% alcohol. Plates were assembled with 0.4mm spacers on a flat surface. 50μl of 25% ammonium persulphate and 50μl of TEMED were added to 6% Hydrolink MDE gel containing 7M urea and 0.6xTBE. This was poured carefully between the plates ensuring that no air bubbles formed within the substance of the gel. Combs were introduced between the electrophoresis plates and secured while the gel was left to polymerise at room temperature for at least 30 minutes. Following this, combs were removed, the plates and gel were installed in the automated laser fluorescence analyser and 0.6xTBE buffer solution added to the tanks. After rinsing the wells with buffer solution (0.6xTBE), the prepared samples with internal controls were added and gels run at constant power (55W) and temperature (50°C). The automated DNA sequencer generates a single trace corresponding to each DNA well. The internal standards can be readily recognised and the peaks generated by each allele subsequently identified. Evidence of microsatellite instability or loss of heterozygosity in cancer DNA can be determined by the 'shift' of allele peak to a new position or reduction in magnitude of allele peak, respectively. In both circumstances there is a residual peak due to the presence of contaminating normal DNA (from stroma or lymphocytes) in the cancer DNA sample.

TBE buffer:
See Appendix 1
2.8 Analysis of chromosomes 5q and 17p

LOH was examined in 5q by Southern blotting using the markers p227, L5.71, EF5.44 and YN5.48, within 5q21-22 (Ashton-Rickardt et al., 1989 and 1991) and in 17p using YNZ.22 and MCT35.1 (Barker et al., 1987; Nakamura et al., 1987). In some tumours PCR-amplified regions bearing polymorphic sites within APC and MCC in 5q (Heighway et al., 1991 and 1992) and within p53 or the subtelomeric YNZ.22 locus in 17p (Horn et al., 1989; McDaniel et al., 1991) were also examined. This work was performed by Mr Robert Morris and Dr Colin Purdie in the Department of Pathology, University of Edinburgh Medical School.

2.9 Hybrid panel mapping

Human/hamster somatic cell hybrids were obtained by courtesy of Dr Michael Wagner (Wagner et al., 1991). 2μg of DNA from clones 1HL 12 and 20xPO435-2 were digested with Taq I restriction enzyme (NBL Gene Science Ltd) according to the manufacturer's instructions. Southern hybridisations were performed as in 2.3, except that filters were washed to a stringency of 0.5 x SSC at 65°C. On repeated analysis, probe pMS502 did not hybridise with either of these clones but consistently hybridised to digested genomic DNA which acted as a positive control. Clones 1HL 12 and 20xPO435-2 encompass the whole of chromosome 8p, therefore pMS502, which has been localised to chromosome 8 (Armour and Jeffreys, 1991) is not localised to 8p.

TaqI buffer (final concentration)  
6mM Tris-HCl  
6mM MgCl₂  
100mM NaCl  
1mM dithiothreitol  
pH7.5

2.10 Flow cytometry

Flow cytometry was performed on a subgroup of cancers examined in this series. Flow cytometry was performed by Dr CA Purdie and Miss S White of Department of Pathology, University Medical School, Edinburgh. Single nuclei suspensions were prepared according to Vindelov et al. (1983). Nuclei were stained with 0.62M propidium iodide and analysed in a
Coulter Epics CS flow cytometer at a wavelength of 488nm. Chicken red blood cells were used as controls, producing a peak equivalent to around 35% of normal human diploid DNA content. Aneuploidy was considered present when two distinct G0/G1 peaks were visible and a DNA index calculated for each tumour (1.0 being diploid).

2.11 p53 immunohistochemistry

p53 immunohistochemical staining was performed by Dr Colin Purdie and Miss Susan White of the University Department of Pathology, Edinburgh using the monoclonal anti-p53 antibody, Pab 1801 on sections of tumour fixed in periodate-lysine-paraformaldehyde-dichromate (PLPD) and embedded in paraffin wax. Those samples with nuclear staining identified by at least 2 observers were scored as positive. The method is described fully in Purdie et al. (1991).
Chapter 3

Chromosome 8p allele loss and deletion analysis

in sporadic colorectal cancer

3.1 Introduction

3.1.1 Loss of heterozygosity studies in the localisation of tumour suppressor genes

Within an individual, maternal and paternal alleles may be distinguished by the use of polymorphic DNA sequences, commonly arising in non-transcribed regions of the genome. Polymorphisms may be identified in the length of repeat DNA sequences, e.g. variable number tandem repeats (VNTR) including minisatellites and microsatellites. Alternatively, single or multiple base substitutions, deletions and insertions may be exploited through resultant changes in restriction enzyme cleavage sites.

Individuals in whom maternal and paternal alleles can be distinguished through such polymorphisms are considered heterozygous at that locus. Genetic changes in tumour DNA may be displayed by a loss of the heterozygous state in tumour compared to normal DNA from the same individual. Such loss of heterozygosity in tumour DNA markers indicates that a genetic alteration has occurred at that locus during the development of that cancer. When loss of heterozygosity at one locus is found at high frequency in a particular cancer it is powerful evidence for a tumour suppressor locus in the region of the marker. However, any evidence supporting the presence of putative tumour suppressor gene must have considerable substantiating data as loss of heterozygosity at a given locus may be a random event in up to 13% of cases (Ashton-Rickardt et al., 1991).

Loss of heterozygosity occurs as a result of deletion or recombination events and may affect all or part of the chromosome arm. In a proportion of cases, a discrete, interstitial region of loss occurs where the region is flanked by polymorphic markers showing retained heterozygosity. Comparison of losses between cancers of the same type, allows derivation of a region of common loss of heterozygosity, which is likely to contain at least part of the tumour suppressor locus. This approach is an essential tool in localisation of tumour suppressor loci. Its power depends on identifying a large number of polymorphic markers linked closely to the tumour suppressor locus. Once the region of common loss of heterozygosity is defined to a
sufficiently small region, identification of this region in libraries can lead to 'positional cloning' of the candidate oncosuppressor genes at the site.

Preliminary work in colorectal cancer (Fearon and Vogelstein, 1990) described an overall loss of heterozygosity in 50% of tumours at two restriction fragment length polymorphic markers on the short arm of chromosome 8p. This work was part of an allelotype study, where 56 colorectal cancers were examined for loss of heterozygosity at one or two polymorphic markers on each chromosome providing little information on subchromosomal localisation of putative tumour suppressor locus. Prior to undertaking the work of this thesis there were no other reports on chromosome 8p loss of heterozygosity in colorectal cancer. The aim of this project was to establish the frequency of loss of heterozygosity affecting chromosome 8p in 119 colorectal cancers selected at random. The analysis was performed at up to 14 polymorphic loci, allowing the creation of a deletion map and localisation of two putative tumour suppressor loci.

3.2 Methods

One hundred and nineteen sporadic colorectal cancers, from different individuals, were analysed for loss of heterozygosity at up to 13 chromosome 8p markers and one chromosome 8q marker. Control tissue comprised either blood or histologically normal colorectal mucosa. Details of tissue collection and DNA extraction are described in sections 2.1 and 2.2. Details of Southern hybridisation and analysis at microsatellite loci are present in 2.3 and 2.5, respectively. Loss of heterozygosity was assessed visually as described in 2.6.

3.2.1 Assessment of allele loss at RFLP and (CA)n microsatellite loci

Allele loss was assessed independently by at least two observers as detailed in section 2.6.

Complete details of the polymorphic markers employed are provided in Tables 2.1 and 2.2. The relative positions of these markers is shown in Figure 3.1. These had been derived from linkage studies and physical mapping using chromosome 8p hybrid panels and fluorescence in situ hybridisation. This map has been derived from published work of NIH/CEPH Collaborative Mapping Group, 1992; Tomforde et al., 1992; and Wood et al., 1993. The assignment of pMS502 to chromosome 8q was derived from the use of hybrid panel as detailed in section 2.9.
Figure 3.1
Position of loci on chromosome 8.
3.3 Results

3.3.1 Analysis of loss of heterozygosity with (CA)n microsatellite markers

Interpretation of \((\text{CA})_n\) microsatellite markers was occasionally hampered by two phenomena. First, these markers display 'stutter bands' associated with each principal allele. The stutter bands are believed to be an artefact of the polymerase chain reaction (Weber & May, 1989) resulting from slippage of the polymerase enzyme. Despite manipulation of polymerase chain reaction conditions, this could not be eradicated and conclusions regarding loss of heterozygosity were made only when both alleles were distinguishable. As a consequence, the effective percentage of informative cases was less than predictions from original publications (Tomforde et al., 1992; Polymeropoulos et al., 1991). Secondly, certain cancers displayed instability at microsatellite loci, which results from errors in mismatch repair in the host or tumour DNA. This subject, described in 1.5.3, will be discussed in Chapter 5. In those cancers displaying microsatellite instability, tumour suppressor localisation, based on \((\text{CA})_n\) loss of heterozygosity, was disregarded as it could not be guaranteed that apparent loss of heterozygosity was not due to microsatellite instability.

3.3.2 Frequency of loss of heterozygosity on chromosome 8p

One hundred and nineteen cancers were analysed at up to 14 loci. Ninety nine were informative at one or more loci on chromosome 8p. The informative cancers were obtained from patients with an average age of 69.8 years and a range of 21-95 years. 59 of this group (59.6%) showed loss of heterozygosity at one or more loci on chromosome 8p. Table 3.1 presents clinicopathological data available on those cases which were informative at chromosome 8p markers. Chromosome 8p loss of heterozygosity was independent of patient age and sex and site and Dukes’ stage of cancer.
Table 3.1
Clinicopathological features of informative cases

A. Association between 8p loss of heterozygosity and patient sex and site and stage of cancer.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>LOH</th>
<th>%LOH</th>
<th>Yates corrected Chi-squares</th>
<th>p-value</th>
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<td><strong>Sex</strong></td>
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<td>Male</td>
<td>46</td>
<td>32</td>
<td>70%</td>
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<td>0.0934</td>
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<td>Female</td>
<td>53</td>
<td>27</td>
<td>51%</td>
<td>0.46</td>
<td>0.4974</td>
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<tr>
<td><strong>Site</strong>(^a)</td>
<td></td>
<td></td>
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<tr>
<td>Proximal</td>
<td>35</td>
<td>18</td>
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<tr>
<td>Distal</td>
<td>64</td>
<td>41</td>
<td>64%</td>
<td>1.02</td>
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<tr>
<td><strong>Dukes' stage</strong>(^b)</td>
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<tr>
<td>A</td>
<td>10</td>
<td>6</td>
<td>60%</td>
<td></td>
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<tr>
<td>B</td>
<td>45</td>
<td>26</td>
<td>58%</td>
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<tr>
<td>C</td>
<td>44</td>
<td>27</td>
<td>61%</td>
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B. Association between 8p loss of heterozygosity and patient age

<table>
<thead>
<tr>
<th></th>
<th>LOH</th>
<th>RH</th>
<th>Test of means</th>
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<tbody>
<tr>
<td>Mean Age</td>
<td>67.8</td>
<td>72.7</td>
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<tr>
<td>Age range</td>
<td>28-95</td>
<td>21-87</td>
<td>p=0.056</td>
</tr>
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</table>

\(^a\)Site of cancer defined as proximal or distal to splenic flexure  
\(^b\)Dukes' stages A and B were combined for the analysis.

LOH Number of cancers displaying loss of heterozygosity  
%LOH Percentage of informative cancers displaying loss of heterozygosity  
RH Retained heterozygosity
3.3.3 Localisation of putative tumour suppressor locus

The percentage loss of heterozygosity at each of the polymorphic markers is presented in Table 3.2. Although one might expect a higher frequency of loss in those markers close to the tumour suppressor locus, the individual frequencies are of limited value as certain markers (D8S87, D8S137, NEFL, D8S136, D8S298, D8S133) were employed in a 'second wave' of loss of heterozygosity studies which excluded those cancers demonstrated to have lost all, or most, of the chromosome arm as they would not provide further information on localisation. Those cancers already demonstrated to have limited or no evidence of loss of heterozygosity were targeted in a search for small interstitial regions of loss of heterozygosity.

Table 3.2
Details of loss of heterozygosity at fourteen chromosome 8 loci in colorectal cancer

<table>
<thead>
<tr>
<th>Marker</th>
<th>%LOH</th>
<th>LOH/INF</th>
<th>Total</th>
<th>%IC</th>
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<tr>
<td>TELOMERE</td>
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<tr>
<td>pKSR2</td>
<td>37.5</td>
<td>12/32</td>
<td>94</td>
<td>34.0</td>
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<td>D8S201</td>
<td>57.5</td>
<td>23/40</td>
<td>63</td>
<td>63.5</td>
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<td>LPLHdIII</td>
<td>37</td>
<td>10/27</td>
<td>98</td>
<td>27.5</td>
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<tr>
<td>LPL3GT</td>
<td>46.2</td>
<td>24/52</td>
<td>87</td>
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</tr>
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<td>LPL35</td>
<td>47.4</td>
<td>18/38</td>
<td>98</td>
<td>38.8</td>
</tr>
<tr>
<td>D8S298</td>
<td>35.5</td>
<td>11/31</td>
<td>41</td>
<td>75.6</td>
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<td>D8S133</td>
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<td>D8S136</td>
<td>37.8</td>
<td>14/37</td>
<td>58</td>
<td>63.8</td>
</tr>
<tr>
<td>NEFL</td>
<td>18.4</td>
<td>7/38</td>
<td>58</td>
<td>65.5</td>
</tr>
<tr>
<td>D8S137</td>
<td>24.4</td>
<td>10/41</td>
<td>72</td>
<td>56.9</td>
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<tr>
<td>D8S87</td>
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<td>1/5</td>
<td>8</td>
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<td>ANK1</td>
<td>30.8</td>
<td>12/39</td>
<td>102</td>
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<td>pUC92F</td>
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<td>5/14</td>
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<td>16.9</td>
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<td>pMS502</td>
<td>22</td>
<td>9/41</td>
<td>54</td>
<td>75.9</td>
</tr>
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</table>

%LOH      Percentage loss of heterozygosity
LOH/INF   Fraction of informative case (INF) which displayed loss of heterozygosity
Total     Total number of cases examined
%IC       Percentage of cases examined which were informative
Fifty-nine cancers displayed loss of heterozygosity at one or more chromosome 8p marker. In 40 of these, loss of heterozygosity was demonstrated at all informative markers, however, in 19, loss affected part of the chromosome arm and in 10 of these, discrete losses were identified which provided useful data on localisation of the putative tumour suppressor loci. These cancers are the essential tools allowing the further mapping of the putative tumour suppressor locus. A deletion map of these 10 important cases is presented in Figure 3.2.

These data support the existence of at least two putative tumour suppressor loci on chromosome 8p. Cases 181, 195, 145, 113 and 115 demonstrate interstitial losses which provide localisation of one tumour suppressor locus. The telomeric limit of these regions are markers within the LPL gene. The centromeric limit of this region is D8S133, as shown in cases 181, 195, 19 and 113. In case 145 the centromeric limit of the region of deletion is D8S136. These cases provide powerful evidence supporting a tumour suppressor locus within the region D8S133 and LPL. Case 115 also supports a telomeric limit at LPL gene, and on repeated testing the breakpoint appeared to occur within the LPL gene: both alleles were retained at pGLPL35 and allele A1 was lost at LPL3GT. Autoradiographs of each of these cases at the relevant loci are presented in Figure 3.3. This region of common loss of heterozygosity is within chromosome 8p22-p21.3. The genetic distance involved in this region is uncertain, however, linkage analysis (NIH/CEPH collaborative mapping group, 1992) suggests it may as little as 4 cM.

A more centromeric site is demonstrated by cases 117, 17, 213, and 75. Case 117 delineates a region between D8S137 and D8S136, encompassing the NEFL gene. This alone provides impressive evidence of a more centromeric site and offers a localisation to a region of approximately 17 cM within 8p22-p11.2 (NIH/CEPH collaborative mapping group, 1992). Cases 17, 213 and 75 provide a telomeric limit which supports the localisation derived from case 117, i.e. centromeric to D8S133 and therefore likely to be distinct from the localisation supported by cases, 181, 195, 145, 19 and 113. However, no other cases were helpful in supporting the centromeric limit of D8S137 suggested by case 117. The autoradiographs generated from these cases are presented in Figure 3.3.
These ten cancers (case numbers 181 through to 75) delineate two regions of common loss of heterozygosity on chromosome 8p. Cases 181, 195, 145, 19, 113 and 115 define a region of loss of heterozygosity from LPL to D8S133. Cases 117, 17, 213 and 75 define a more centromeric region of loss around NEFL from D8S136 to D8S137. Positions of loci are indicated.
### Figure 3.3

**Autoradiographs of critical regions of loss of heterozygosity on chromosome 8**

<table>
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<tr>
<th></th>
<th>181</th>
<th>195</th>
<th>145</th>
<th>19</th>
<th>113</th>
<th>115</th>
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Autoradiographs supporting the localisation of 2 tumour suppressor loci on chromosome 8p in ten patients, each consisting of normal (N) and tumour (T) DNA. Cases 181, 195, 145, 19, 113 and 115 support a telomeric site in chromosome region 8p22 - p21.3. Cases 117, 17, 213 and 75 support a centromeric site within 8p21 - p11. Alleles shown in red indicate a region of loss of heterozygosity.
3.4 Discussion

This work demonstrates that chromosome 8p contains putative tumour suppressor loci which may be implicated in over 59% of colorectal cancers. Two sites have been determined which are likely to contain tumour suppressor genes: a 4cM region on chromosome 8p22-21.3, delineated by loci D8S133 and LPL, and a larger 17cM region between D8S136 and D8S137 on 8p21-11.2. The presence of loss of heterozygosity on chromosome 8p is independent of patient age and sex and the Dukes' stage and site of the cancer.

3.4.1 Sub-localisation of tumour suppressor loci in colorectal and other cancers

Chromosome 8p loss of heterozygosity has been described in colorectal cancer (Vogelstein et al., 1989; Emi et al., 1992a, Fujiwara et al., 1993; Chang et al., 1994a; Fujiwara et al., 1994; Kelemen et al., 1994; Yaremko et al., 1994), prostate cancer (Bergenheim et al., 1991; Kunimi et al., 1991; Bova et al., 1993; Macoska et al., 1994, Matsuyama et al., 1994; MacGrogan et al., 1994; Trapman et al., 1994), non-small cell lung cancer (Fujiwara et al., 1994), bladder carcinoma (Knowles et al., 1993), collecting duct carcinoma of the kidney (Schoenberg et al., 1995), squamous carcinoma of the head and neck (Li et al., 1994) and breast cancer (Yaremko et al., 1995; Kerangueven et al., 1995).

Despite considerable efforts in establishing loss of heterozygosity, many groups have generated rather disappointing localisation data, often with conflicting conclusions. This confusion has probably arisen from a variety of researchers using different sets of polymorphic markers which are frequently poorly localised. It is compounded by the fact that a spectrum of primary cancers has been employed and direct comparisons between cancers of different origin may not be legitimate. Furthermore the presence of more than one putative tumour suppressor locus has also contributed to imprecision. Principal localisations of putative tumour suppressor loci in colorectal and other cancers are presented in Table 3.3 and discussed in detail below.
### Table 3.3
Summary of localisation of putative tumour suppressor in colorectal, prostate, hepatocellular, lung and bladder cancers and malignant fibrous histiocytoma.

<table>
<thead>
<tr>
<th>Source</th>
<th>Tumour origin</th>
<th>Proximal Site</th>
<th>Distal Site</th>
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<tr>
<td>This thesis</td>
<td>Colorectal</td>
<td>D8S136 - D8S137</td>
<td>LPL - D8S133</td>
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<tr>
<td>Emi et al., 1992</td>
<td>Colorectal</td>
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<td>C18-266 - pSVL-LPL</td>
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<tr>
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<td>C18-319 - C18494</td>
<td>C18-245 - C18-2644</td>
</tr>
<tr>
<td>Fujiwara et al., 1994</td>
<td>Colorectal</td>
<td>C18-266 - pSVL-LPL</td>
<td>C18-245 - C18-2644</td>
</tr>
<tr>
<td><strong>Chang et al., 1994a</strong></td>
<td>Hepatocellular</td>
<td>LPL - NEFL</td>
<td>D8S201 - LPLb</td>
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<tr>
<td>Yaremko et al., 1994</td>
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<td>LPL - NEFL</td>
<td>D8S201 - LPLb</td>
</tr>
<tr>
<td><strong>Fujiwara et al., 1994</strong></td>
<td>Lungc</td>
<td>LPL - NEFL</td>
<td>D8S201 - LPLb</td>
</tr>
<tr>
<td>Chang et al., 1994a</td>
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<td>D8S206 - D8S259</td>
</tr>
<tr>
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<td>D8S206 - D8S259</td>
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<tr>
<td>McGrogan et al., 1994</td>
<td>Prostate</td>
<td>D8S137 - D8S85</td>
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<td>D8S137 - D8S85</td>
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<td>Chang et al., 1994a</td>
<td>MFHd</td>
<td>D8S136 - ANK1</td>
<td>D8S264 - D8S133</td>
</tr>
<tr>
<td>Takle and Knowles, 1996</td>
<td>Bladder</td>
<td>D8S505 - D8S135</td>
<td>D8S264 - D8S133</td>
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</table>

aMarkers which flank region of common loss of heterozygosity showing retained heterozygosity in tumour DNA unless otherwise stated.
bLPL marker retained, telomeric markers lost.
cNon small cell lung cancer.
dMalignant fibrous histiocytoma.
eTransitional cell carcinoma of bladder.
Localisation of putative tumour suppressor loci in colorectal cancer

Work in colorectal cancer has been predominantly performed by Nakamura's group who have generated a prodigious number of polymorphic markers on chromosome 8 (Emi et al., 1992b). Emi analysed loss of heterozygosity in 88 informative colorectal cancers at five polymorphic sites along the short arm (Emi et al., 1992a). They found an overall frequency of loss of heterozygosity of 40.2%, with a peak of 47.5% occurring at the macrophage scavenging receptor (MSR) on 8p22. A derived region of common deletion lay between markers D8S238 and D8S220, covering chromosome regions 8p23.1-p21.3 and including the MSR marker on chromosome 8p22. In the same report, Emi describes the identical region of common deletion in a study of 97 informative cases of hepatocellular carcinoma.

This work was extended in a report from the same research group (Fujiwara et al., 1993) where 131 informative colorectal cancers were examined at 20 RFLP sites on chromosome 8, detecting an overall frequency of loss of heterozygosity of 44%. This work derived two regions of common deletion, but as the authors employed new markers and made little reference to those markers used to derive the region of common deletion in earlier work (Emi et al., 1992a) there is substantial difficulty in aligning these observations with those of others, or even the earlier work from the same group. One region was delineated by markers C18-266 and pSVL-LPL at 8p23.2-p22. The other, more centromeric region, was delineated by C18-319 and C18-494 at 8p21.3-p11.22. The genetic distances covering each of these regions were 28 and 18cM respectively. From observations in hepatocellular cancer they concluded that the more centromeric locus is specific to colorectal cancer and the telomeric common to both. Markers generated and used by this group were mapped physically using FISH.

Fujiwara subsequently created a high density deletion map of the more telomeric region, common to both colorectal cancer and hepatocellular cancer (Fujiwara et al., 1994). They derived a 1.2Mb region flanked by markers C18-245 and C18-2644, found in both colorectal and hepatocellular cancer, which they believed likely to contain the putative tumour suppressor gene. It is important to note that the region of this more telomeric deletion was significantly different from the earlier publication by this group (Fujiwara et al., 1993), indeed it extended proximally into the 8p21.3 region which was, in fact, within the more centromeric region noted in Fujiwara et al., 1993. Furthermore, they derived a 300Kb region which is consistently deleted in non-small cell lung cancer flanked by markers C18-1051 and C18-2644, which lies within the 1.2 Mb region. From this region, Fujiwara has subsequently produced a candidate tumour suppressor gene which is homologous to the platelet-derived growth factor (PDGF) receptor β and has been designated PRLTS, for PDGF receptor β-like tumour suppressor (Fujiwara et al., 1995). PRLTS codes for 375 amino acids and contains 7 exons. Although this appears a reasonable candidate gene, mutation analysis provides only weak supporting data. Single stranded conformational polymorphism (SSCP) analysis was performed on the seven exons of PRLTS,
with sequence analysis in those cancers displaying abnormal bands. Examination of 123 colorectal cancers and 102 hepatocellular cancers revealed only one frameshift and two missense mutations, while somatic rearrangement was detected in one of 70 non-small cell lung cancers, analysed by Southern-blot hybridisation.

Taken as a whole, the work of Emi and Fujiwara support the existence of at least two tumour suppressor loci. The more telomeric locus is mapped between markers C18-245 and C18-2644 on 8p22-p21.3 and from this region PRLTS has been proposed as a candidate tumour suppressor gene. The centromeric locus is less well defined but is delineated by C18-319 and C18-494 at 8p21.3-p11.2 (Fujiwara et al., 1993).

Further limited localisations have been offered by other workers. Chang et al. (1994a) studied 8 colorectal cancers and 8 cases of dysplasia arising in ulcerative colitis for loss of heterozygosity at 3 microsatellite loci on chromosome 8p, D8S201, LPL3GT, D8S87. They established loss of heterozygosity in 4 cases of carcinoma and 3 cases of dysplasia. They suggest the putative tumour suppressor locus lies telomeric to LPL as in 2 cases loss of heterozygosity occurred at the terminal marker D8S201 with retained heterozygosity at the LPL loci. In view of the limited number of cases and markers, the significance of their observations is uncertain. The loss at D8S201 may represent telomeric shortening. In addition, the presence of chromosome 8p loss of heterozygosity in premalignant lesions contrasts with our own findings (Chapter 4) raising the possibility of an alternative mechanism of carcinogenesis in ulcerative colitis.

Two regions of localisation were reported in a study of colorectal cancer with five RFLP markers: D8S7, LPL, SFTP2, NEFL, POLB. Overall, these demonstrated a loss of heterozygosity in 33% of 75 informative colorectal cancers and outlined two regions of interest (Yaremko et al., 1994). One of these, proximal to LPL, has a centromeric limit at the 8p21 loci, NEFL and SFTP2. The more telomeric region, distal to LPL was only identified by loss of D8S7 marker, with little supporting detail.

van der Bosch et al. (1992) examined 30 colorectal cancer cell lines and tumour tissue in a combined cytogenetic and molecular approach. Thirteen cell lines showed chromosome 8p deletion over large regions. Three showed terminal deletion, however, one of these also showed loss at 8p21.3-8p21.1. Molecular analysis with three polymorphic markers (LPL35, pSW50 and pBS8.9) demonstrated a loss of heterozygosity in 87% (13/15) of informative cases and 40% (4/10) of original tumour DNA samples examined. Data were too limited to offer reasonable localisation, but six cell lines which did not show cytogenetic deletions showed loss of heterozygosity at RFLP markers, indicating small regions of loss. The approach of this group is potentially useful in that they use cytogenetic assessment to rule out those cancers with large deletions and thereby allow efforts to be concentrated on mapping of small interstitial lesions. However, the use of cancer cell lines must be of limited value as they may have undergone further mutation and genetic recombination in culture, particularly affecting the telomeres.
Several reports have indicated the presence of possible chromosome 8p tumour suppressor locus telomeric to LPL in colorectal, hepatocellular, lung (Fujiiwara et al., 1993) and prostate (Bova et al., 1993) cancer. In this series we have only identified three cases in which D8S201 (8pter-22) shows a loss of heterozygosity in the presence of more centromeric loci showing retained heterozygosity. These cases may be simply the result of telomeric shortening, which is a well described phenomenon in carcinogenesis and ageing and is known to occur in colorectal cancer (Hastie et al., 1990). Alternatively, the fact that this thesis concentrated on the region of chromosome more centromeric to LPL may make any conclusions regarding a telomeric site invalid due to a relative dearth of substantiated data when compared to that supporting the more centromeric sites detailed above.

Sublocalisation of tumour suppressor loci in carcinoma of prostate

Considerable efforts have been made in the localisation of the chromosome 8p tumour suppressor loci in prostate cancer since early investigations revealed a high frequency of loss of heterozygosity. Bergerheim et al. (1991) examined local and metastatic malignancy for loss of heterozygosity at six chromosome 8p markers: D8S7, LPL, NEFL, PLAT, D8S39 and TG. Seventeen cancers were informative at one or more loci and 11 cancers (65%) displayed loss of heterozygosity. Localisation of the critical region was imprecise, extending from PLAT to 8pter which is effectively the whole of the short arm. Chang et al. (1994a) in a similar investigation to that described for colorectal cancer, analysed 19 primary cancers and 7 lymph node metastases from prostate at markers, D8S201, LPL3GT, D8S87. Overall, 13/26 (50%) demonstrated loss of heterozygosity and again, localisation to a terminal site was ascribed on the basis of D8S201 loss of heterozygosity but supporting data were limited.

Subsequent studies provided deletion maps with finer localisation and supported the presence of at least two tumour suppressor loci. Bova et al. (1993) found 32 out of 51 informative prostatic cancers (63%) displayed loss of heterozygosity at chromosome 8p. They identified loci within 8p22-p21.2 as showing most frequent loss of heterozygosity, but deletion mapping revealed a region of overlap delineated by LPL gene centromerically and D8S163 (pKSR2) telomERICALLY which corresponds to 8pter-p22. In addition they found a homozygous deletion which supported this region of common loss. Substantiating evidence was weak however, because of the dearth of informative markers over the critical region.

Matsuyama et al. (1994) employed cosmids from LPL (8p22) and D8S7 (8p22-pter) in double target fluorescence in situ hybridisation and detected deletions in 71% (30/41) of cancers. They offered little in the way of localisation, save to indicate that 8p22 is likely to be near the tumour suppressor loci. They describe cases where the two sites were lost independently and suggest this may indicate two tumour suppressor loci, however, it is impossible to prove that such changes are not simply related to the random genetic derangement accompanying malignant
change. This analysis used a novel approach in assessing chromosome losses in carcinogenesis. The power of the localisation data provided by FISH has still to be determined.

McGrogan et al. (1994) presented a comprehensive study of 63 prostatic cancers at 28 markers, 27 of which are microsatellite polymorphisms based on dinucleotide repeats. 29/63 (46%) demonstrated LOH and 15 of these showed interstitial losses with a further three providing evidence for two discrete regions of loss, consistent with the idea of two putative tumour suppressor loci. This work provided strong support for a gene close to chromosome band 8p22, delineated by markers D8S206 and D8S259 covering chromosome bands 8p23.1-p21.1. Evidence was presented for another more proximal locus (8p21-p12) which is supported by only three cancers all of which also have losses over the 8p23.1-p21.1 bands. McGrogan et al. were careful to emphasise the pitfalls of information derived from deletion mapping when there is sub-optimal informative data from adjacent loci. It is salutary to remember that even in the region of a tumour suppressor locus there is likely to be a significant frequency of loss of heterozygosity occurring as a 'background' genetic abnormality concomitant with malignant behaviour (Ashton-Rickardt et al., 1991).

Trapman et al. (1994) analysed 44 prostatic cancers at 14 loci on chromosome 8p. 59% of cancers showed loss of heterozygosity at 3 or more chromosome 8p loci and the region of common loss was delineated by markers D8S133-D8S87 (8p21-p12) a region estimated to be 17cM. This derived region concurs with the centromeric region described in this thesis.

**Chromosome 8p loss of heterozygosity in other cancers**

Knowles et al. (1993) examined 110 transitional cell tumours of urinary bladder at up to 16 RFLP and microsatellite loci and recently extended the analysis with a further 83 bladder cancers and 30 microsatellite markers (Takle and Knowles, 1996). They identified loss of heterozygosity on chromosome 8p in 22% (43/193) of cases and provided localisation data on two putative oncosuppressor sites. One site encompasses the telomeric localisation provided in this thesis, delineated by loci D8S264 and D8S133 covering chromosome bands 8pter-8p21.1. The other, more centromeric site, is delineated by loci D8S505 and D8S135 within 8p12-p11.2 and supported by fine deletion mapping. The interval over this centromeric site has been estimated at 4cM and appears to be centromeric to the centromeric localisation proposed in this thesis. In bladder cancer, the centromeric site has so far only been lost with the telomeric site and the authors suggest there may be an association between the two sites which does not appear to be the case in colorectal cancer.

In addition to studies of colorectal and prostate cancer, Chang et al. (1994a) also described an interesting finding in malignant fibrous histiocytomas. Loss of heterozygosity was detected in 4/12 (33%) cases. With the use of six more chromosome 8p markers (D8S201, LPL3GT, D8S87, D8S136, D8S137, ANK1) they delineated a region of loss between D8S136 and ANK, around D8S137. This provided more convincing localisation data and derived a
region which coincides with the centromeric loss noted in this thesis. However, one must be cautious of comparisons between such diverse tissue types. It is also of interest that sarcomas (of which malignant fibrous histiocytoma is a type) are a feature of Werner syndrome which has recently been mapped to band 8p12 between markers ANK1 and D8S137 (Goto et al., 1992; Thomas et al., 1993) thereby raising the possibility of a common gene.

Chromosome 8p loss of heterozygosity has been identified in several other cancers although the derived localisations are less helpful. Li et al. (1994) found a 31% (11/36) frequency of loss of heterozygosity in 56 squamous carcinomas of the aerodigestive tract using a single marker D8S201 (8p22-pter). Despite the failure of one group (Emi et al., 1992a) to identify loss of heterozygosity in chromosome 8p in cancer of the breast, Pykett et al. (1994) using markers in the region 8p21-p12, found loss of heterozygosity 6/8 breast cancer cell lines. This has recently been supported by work in tumour tissue, although significant localisation of a putative gene was not provided (Yaremko et al., 1995; Kerangueven et al., 1995).

As noted above, Fujiwara localised a 300Kb region in non-small cell lung cancer flanked by markers C18-1051 and C18-2644, as likely to contain the putative tumour suppressor locus (Fujiwara et al., 1994) and subsequently cloned a candidate gene (PRLTS) in this region. Although this is an exciting advance, the low frequency of mutation of the retained gene in the tumours does not provide satisfactory evidence that PRLTS is an oncosuppressor gene.

3.4.2 Pitfalls in tumour suppressor gene localisation

It is clear that massive efforts have been spent in determining the frequency of chromosome 8p loss of heterozygosity in a selection of cancers with a view to cloning and identifying the putative tumour suppressor gene. The localisations offered in this thesis are supported by the subsequent work of others and, indeed, a candidate gene has been proposed in the telomeric locus 8p22-p21.3.

The apparent confusion which arose from multiple localisations is of some importance and demonstrates the potential for error in tumour suppressor gene localisation studies. Any loss of heterozygosity, even occurring in the region of a tumour suppressor gene, may be a random event and this may have accounted for the changing localisations offered by Nakamura's group. It is essential that derived regions of common loss of heterozygosity should be supported by several informative loci. This point has also been appreciated and discussed by McGrogan et al. (1994).

A further source of weakness in the localisations of the chromosome 8p tumour suppressor loci has arisen from uncertainty in the exact location of several markers employed. This is particularly the case with pKSR2 (D8S163) and D8S201 mapping to the telomeric 8p23 band (Trapman et al., 1994; Bova et al., 1994; Yaremko et al., 1994; Chang et al., 1994a). The
exact location of these markers is uncertain and the high frequency of ill-defined loss of heterozygosity associated with them, may be a consequence of loss through telomeric shortening (Hastie et al., 1990). Many of these studies equate the regions of loss of heterozygosity associated with these telomeric markers with the 'telomeric' region in Fujiwara et al. (1993). However, as has been demonstrated, further mapping of this region, in fact, confirmed it to be localised to the 8p22-p21.3 region.

The presence of microsatellite instability in cancer DNA has been extensively described (Ionov et al., 1993; Aaltonen et al., 1993; Thibodeau et al., 1993; Lothe et al., 1993) and it was noted in 15.9% (19/119) of cancers in this series (these data are discussed in Chapter 5). The presence of this instability can lead to incorrect assessment of loss of heterozygosity since shift in the electrophoretic mobility of DNA fragments can be attributed to either mechanisms. For this reason, those cancers displaying instability were excluded from critical localisation data described in this chapter. Microsatellite markers were employed by numerous groups (McGrogan et al., 1994; Trapman et al., 1994; Bova et al., 1994; Yaremko et al., 1994; Chang et al., 1994a; Takle and Knowles, 1996) and it is surprising that no account was taken of this phenomenon. This fact could account for rogue localisations as a consequence of false positive results in loss of heterozygosity analyses. Again, it clearly indicates the requirement for reliable substantiating data.

The work presented in this chapter offers a fine localisation of the telomeric tumour suppressor locus in colorectal cancer, which is consistent with the findings of others and includes the site of a proposed candidate gene (PRLTS). Such a localisation may be employed in the cloning and isolation of other putative tumour suppressor genes. Furthermore, this work offers a detailed localisation to 17cM of a more centromeric site, which may contain a further gene important in the development of colorectal and other cancers.
Chapter 4

Implications of chromosome 8p loss of heterozygosity in the development and behaviour of sporadic colorectal cancer

4.1 Introduction

The development of colorectal cancer incorporates several discrete genetic events as detailed in Chapter 1. The prevalence of each genetic lesion is related frequently to the size and dysplasia of adenomas and the presence of frank malignancy and late stage disease (Powell et al., 1992; Fearon and Vogelstein 1990). These observations have led to interpretations of the role of the genetic abnormalities in permitting evolution along a supposed sequence from normal cell through adenoma to carcinoma and then metastasis. For example, inactivation of tumour suppressor genes APC and DCC by mutation or loss occurs in the majority of both colorectal adenomas and carcinomas (Powell et al., 1992; Fearon & Vogelstein, 1990). Such lesions presumably facilitate the expansion of neoplastic populations without directly conferring a malignant phenotype. In contrast, the p53 gene is inactivated in 75% of colorectal cancers but is normal in adenomas except those displaying features of severe dysplasia. Inactivation of this gene would appear to have a major role initiating malignant behaviour (Baker et al., 1990; Kikuchi-Yanoshita et al., 1992; Carder et al., 1993) suggesting the existence of genes involved in the critical transition from benign to malignant growth. Data presented in Chapter 3 establishes chromosome 8p loss of heterozygosity as a frequent event in sporadic colorectal cancer. In this section the significance of chromosome 8p abnormalities in the development of colorectal cancer was determined through the analysis of loss of heterozygosity in 56 sporadic colorectal adenomas. The use of microsatellite markers also allowed tentative deductions regarding the prevalence of microsatellite instability in colorectal adenomas, although this was not a primary aim in this analysis.

Certain genetic abnormalities appear to determine or at least be associated with tumour behaviour and as such may convey prognostic information. Microsatellite instability, which is present in up to 15% of carcinomas is suggested to favour long term survival after surgery (Lothe et al., 1993; Thibodeau et al., 1993). In contrast, p53 mutations (Hamelin et al., 1994), and aneuploidy (Wolley et al., 1982) have been claimed to show association with poor prognosis, although these findings are still controversial. There is some evidence that chromosome 8p loss of heterozygosity has been associated with advanced clinicopathological stage (Fujiwara et al., 1993) and the presence of microinvasion in colorectal cancer (Keleman et al., 1994). Analyses in
hepatocellular carcinoma (Emi et al., 1993a) transitional cell carcinoma of bladder (Knowles et al., 1993) prostate cancer (McGrogan et al., 1994) and squamous carcinoma of the aero-digestive tract (Li et al., 1994) suggest that loss of heterozygosity on chromosome 8p is prevalent in advanced disease. However, the exact significance of these studies has been hampered by the use of small populations and formal correlations with survival have not been sought.

In this chapter the implication of chromosome 8p loss of heterozygosity for tumour behaviour was measured by patient survival. Cox proportional hazard model was employed in a prospectively collected series of 90 patients with survival data for up to 1968 days.

4.2 Methods

4.2.1 Adenoma source and extraction of DNA

A total of 56 sporadic colorectal adenomas and matched normal tissue, either blood or histologically normal colonic mucosa were obtained from 49 individuals. Thirty three adenomas were collected from fresh colorectal specimens resected for malignant disease. Of the remainder, sixteen were removed endoscopically from cancer-free bowel as part of a presymptomatic screening programme. I am grateful to Professor JD. Hardcastle and Dr. D. Jenkins for providing these 'screen-detected' adenomas.

DNA was purified from 56 sporadic colorectal adenomas and normal tissue by protocols described in section 2.2.

4.2.2 Chromosome 8p loss of heterozygosity studies

Loss of heterozygosity at chromosome 8 markers in survival analysis

Details of this analysis are provided in Chapter 3. 119 cancers were examined for evidence of chromosome 8 loss of heterozygosity, of which 99 were informative at one or more loci. Survival data were available on 90 of these cases.

Analysis of loss of heterozygosity in colorectal adenomas

Three microsatellite (CA)$_n$ repeats markers were employed which showed a high frequency of loss of heterozygosity in colorectal cancers (Chapter 3). ANK1 (Polymeropoulos et al., 1991) maps to 8p21-p11.2, LPL3GT (Tomforhde et al., 1992) maps to 8p22 and D8S137 (Tomforhde et al., 1992) to 8p21-p12. Polymerase chain reactions, polyacrylamide gel electrophoresis, autoradiography and assessment of loss of heterozygosity were performed as described in sections 2.5 i.e. identical methods to those performed with DNA derived from cancer tissue.
HindIII polymorphism in intron 8 of the lipoprotein lipase gene (8p22) was demonstrated by PCR under conditions described by Bruin et al... (1991). PCR products were digested with HindIII and separated by electrophoresis on 2% agarose gels stained with ethidium bromide and visualised under ultra-violet light as detailed in section 2.4.4.

4.2.3 Microsatellite instability in sporadic colorectal adenomas

Evidence of microsatellite instability was assessed for markers ANK1, D8S137 and LPL3GT.

4.2.4 p53 immunohistochemistry

p53 immunohistochemical staining was performed by Dr Colin Purdie and Miss Susan White of the University Department of Pathology, Edinburgh. Results were available on 84 cancers using the monoclonal anti-p53 antibody, Pab 1801. The method is presented in section 2.11.

4.2.5 DNA ploidy analysis

Flow cytometry was performed on a subgroup of 32 cancers examined in this series for chromosome 8p loss of heterozygosity. This work was undertaken largely by Dr Colin Purdie and Miss Susan White of the University Department of Pathology, Edinburgh. Method is described in Section 2.10.

4.2.6 Collection of survival data

Survival data were collected prospectively through the Cancer Research Campaign colorectal cancer project in Lothian Region, Scotland. Survival was measured from the date of operation to the date of death, from any cause, or the last known date alive prior to censoring on 31 December 1994. All deaths were notified to the project by the Scottish Cancer Deaths Registry. Information on those patients surviving was obtained from family practitioners, colorectal cancer follow-up clinics or through direct contact with patients. Follow-up data were available on 90 patients of the original 99 patients who were informative at one or more chromosome 8p marker, as detailed in Chapter 3. Of the 9 cases with no survival follow up, two had moved abroad and the family of general practitioner of seven could not be contacted by the date of censoring.

4.2.7 Statistics

Statistical analyses were undertaken by Dr Andrew Carothers of the Medical Research Council Human Genetics Unit, Edinburgh. Cox proportional hazard method using both forward and backward selection was employed. This permitted analysis of the effects on survival of
several factors simultaneously using Cox Regression Routines of SPSS for Windows Release 6.0 (SPSS for Windows). The model included patient age and sex; site (proximal or distal to splenic flexure) and Dukes' stage of tumour. Nuclear ploidy and p53 immunohistochemistry, available on 32 and 84 patients respectively, were also included in the model. Follow up was available for a median of 696 days with a range of 8 to 1968 days.

4.3 Results

4.3.1 Chromosome 8p loss of heterozygosity in sporadic colorectal adenomas

The four polymorphic markers used in this analysis have displayed a high frequency of loss of heterozygosity in sporadic colorectal cancers, described in detail in section 3.3.2. Of the 56 adenomas, 51 were informative at one or more marker and, of these, only 5 (9.8%) showed loss of heterozygosity. In contrast, when employed in the analysis of colorectal cancers these four markers detected loss of heterozygosity in 31 out of 79 informative cases (39.2%) a difference which is highly significant ($\chi^2 = 11.98; \ p=0.0005$). Loss of heterozygosity at each locus in colorectal adenomas and carcinomas are presented in Table 4.1 and graphic comparison of percentage loss of heterozygosity in adenomas and carcinomas is shown in Figure 4.1.
Table 4.1
Comparison of loss of heterozygosity at four chromosome 8p polymorphic markers (individually and cumulatively) in colorectal adenomas and carcinomas

<table>
<thead>
<tr>
<th>Marker</th>
<th>Adenomas LOH/INF</th>
<th>%LOH</th>
<th>Carcinomas LOH/INF</th>
<th>%LOH</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANK1</td>
<td>1/31</td>
<td>3.2</td>
<td>9/41</td>
<td>21.9</td>
<td>0.04</td>
</tr>
<tr>
<td>D8S137</td>
<td>2/31</td>
<td>6.4</td>
<td>10/41</td>
<td>24.4</td>
<td>0.09</td>
</tr>
<tr>
<td>LPL3GT</td>
<td>2/27</td>
<td>7.4</td>
<td>24/52</td>
<td>46.2</td>
<td>0.001</td>
</tr>
<tr>
<td>LPLHdIII</td>
<td>0/12</td>
<td>0</td>
<td>10/27</td>
<td>37</td>
<td>0.017</td>
</tr>
<tr>
<td>Cumulative</td>
<td>5/51</td>
<td>9.8</td>
<td>31/79</td>
<td>39.2</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

LOH/INF Number of cases displaying loss of heterozygosity/number of informative cases.
%LOH Percentage of cases displaying loss of heterozygosity.

4.3.2 Adenoma morphology and chromosome 8p loss of heterozygosity

The adenomas consisted of 35 tubulovillous lesions (mean size: 27 mm; range: 6-80 mm); 20 tubular lesions (mean size: 14 mm; range: 5-20 mm) and 1 villous lesion (15 mm). The five shown to have loss of heterozygosity at chromosome 8p were tubulovillous adenomas of 10 mm or more in diameter. Chromosome 8p loss of heterozygosity was not detected in any of the 10 adenomas less than 10 mm in diameter, 7 of which were tubular adenomas and 3 tubulovillous lesions.

4.3.3 Microsatellite instability in sporadic colorectal adenomas

No evidence of microsatellite instability was detected in 56 sporadic colorectal adenomas examined at 3 microsatellite loci. When these markers were employed in a similar analysis of colorectal carcinomas, microsatellite instability was detected at ANK1 in 9.8% (10/102); D8S137 in 5.5% (4/72) and LPL3GT in 8.7% (10/87). Further details of these analyses are presented in chapter 6.
Figure 4.1
Loss of heterozygosity at chromosome 8p loci in colorectal adenomas and carcinomas.
4.3.4 Clinicopathological associations of chromosome 8p loss of heterozygosity

Survival data were available on 90 cancers which were informative for markers on chromosome 8p. 52 (57.8%) displayed loss of heterozygosity at one or more chromosome 8p marker. The remaining 38 cancers (42.2%) showed no loss of heterozygosity. This population is smaller than that described in Chapter 3 and the clinicopathological features are presented in Table 4.2.

The cancers were obtained from patients with an average age of 69.8 years and a range of 21-95 years. Those displaying loss of heterozygosity on chromosome 8p had an average age of 68.8 years compared to an average age of 72.55 years in those showing retained heterozygosity, which was significant on statistical analysis with Student T-test of means (p=0.05).

As in the larger group described in Chapter 3, chromosome 8p loss of heterozygosity tends to be more prevalent in cancers arising in males and in the distal colon although, again, this failed to reach statistical significance. An inverse trend was noted between positive p53 nuclear immunohistochemical staining and loss of heterozygosity affecting chromosome 8p. 51% (25/49) p53 positive cancers displayed chromosome 8p loss of heterozygosity compared to 71% (25/35) of p53 negative cancers. However, this trend was not statistically significant (p=0.0983). Loss of heterozygosity on chromosome 8p was independent of Dukes' stage of cancer and DNA ploidy.
Table 4.2
Association between 8p loss of heterozygosity and patient sex and site and stage of cancer DNA ploidy and p53 staining.

<table>
<thead>
<tr>
<th></th>
<th>LOH(%)</th>
<th>RH (%)</th>
<th>Yates corrected Chi-squares</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>27 (52%)</td>
<td>13 (34%)</td>
<td></td>
<td>0.1455</td>
</tr>
<tr>
<td>Female</td>
<td>25 (48%)</td>
<td>25 (66%)</td>
<td>2.12</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Site</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>15 (29%)</td>
<td>16 (42%)</td>
<td></td>
<td>0.2789</td>
</tr>
<tr>
<td>Distal</td>
<td>37 (71%)</td>
<td>22 (58%)</td>
<td>1.17</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dukes' stage</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>5 (9%)</td>
<td>4 (11%)</td>
<td></td>
<td>0.9885</td>
</tr>
<tr>
<td>B</td>
<td>24 (46%)</td>
<td>18 (47%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>23 (45%)</td>
<td>16 (42%)</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DNA ploidy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diploid</td>
<td>5 (29%)</td>
<td>5 (33%)</td>
<td></td>
<td>0.8860</td>
</tr>
<tr>
<td>Aneuploid</td>
<td>12 (71%)</td>
<td>10 (67%)</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>p53 staining</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>25 (51%)</td>
<td>25 (71%)</td>
<td></td>
<td>0.0983</td>
</tr>
<tr>
<td>Negative</td>
<td>24 (49%)</td>
<td>10 (29%)</td>
<td>2.73</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>49</td>
<td>35</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Site of cancer defined as proximal or distal to splenic flexure

<sup>b</sup>Dukes' stages A and B were combined for the analysis.

LOH  loss of heterozygosity (percentage value in parentheses)

RH  retained heterozygosity (percentage value in parentheses)
4.3.5 Analysis of survival

Overall survival for the ninety informative patients is 37% at 3 years, similar to that obtained in other studies of survival following resection of colorectal cancer (Black et al., 1993). Cox survival analysis was first carried out with the following variables as predictors: age, sex, site, Dukes', ploidy, p53 immunohistochemistry and chromosome 8p loss of heterozygosity under both backward and forward selection.

Chromosome 8p loss of heterozygosity and survival

Using this method, survival was determined to be independent of chromosome 8p loss of heterozygosity. The survival curves for those with and without chromosome 8p loss of heterozygosity are presented in Figure 4.2. Cox multivariate analysis demonstrates that chromosome 8p loss of heterozygosity has no measurable independent effect on survival.

Other variables as predictors of survival

Of the parameters considered, Dukes' stage was the only significant predictor of outcome in this group of 90 colorectal cancer patients ($\chi^2 = 30.65, p<0.0001$). 95% of Dukes' stage A were alive at 3 years, compared to 62% of Dukes' stage B and 26% of Dukes' stage C ($p<0.0001$). This analysis included deaths from all causes and patient age was, naturally, an important variable with increasing age associated with poorer survival. Site of cancer, staining for p53 and ploidy were independent of patient outcome in this analysis.
Figure 4.2

8p status and survival in sporadic colorectal cancer

Cox proportional hazard analysis of survival in 90 sporadic colorectal cancer patients with 8p retention or loss of heterozygosity. No association between chromosome 8p loss of heterozygosity and survival was demonstrated.
4.4 Discussion

Abnormalities of chromosome 8p are a common event in carcinogenesis and this chapter presents valuable information on the implications of 8p loss of heterozygosity in the development and behaviour of colorectal cancer. In this analysis, loss of heterozygosity was detected in fewer than 10% (5/51) of adenomas examined using four polymorphic markers demonstrated previously to display a high frequency of loss of heterozygosity in colorectal cancers. Indeed, when these loci are considered in the series of 119 colorectal cancers described in Chapter 3, loss of heterozygosity was demonstrated in 31 out of 79 (39.2%) informative cancers. As detailed in section 4.3.2, this was a mixed group of adenomas in terms of size and histological types. The five lesions showing loss of heterozygosity were all tubulovillous adenomas, 10mm or more in diameter. Although the numbers are small, chromosome 8p loss of heterozygosity was only detected in this study in the subgroup of adenomas with features associated with a greater malignant potential.

There are no other accounts of chromosome 8p loss of heterozygosity in sporadic adenomas, but, a report of 37 adenomas arising in 2 individuals with FAP found no 8p losses (Ichii et al., 1993). The 56 sporadic adenomas examined in this thesis are more likely to have included a higher proportion of tubulovillous lesions than this group of FAP adenomas. This may account for the presence, albeit rare, of chromosome 8p loss of heterozygosity in the sporadic lesions reported here. The only other report of chromosome 8p loss of heterozygosity in premalignant colorectal lesions comes from Chang et al. (1994a). As noted in 3.4.1, they identified chromosome 8p loss of heterozygosity in 3 out of 8 cases of dysplasia arising in ulcerative colitis suggesting that the chromosome 8p loci may have an early role in carcinogenesis in ulcerative colitis.

As noted in 1.5.1 and discussed in detail in Chapter 5, considerable attention has focused on the roles of mismatch repair genes in hereditary and sporadic colorectal carcinogenesis. Constitutional mutations in hMSH2 and hMLH1 account for more than 90% of cancers arising within HNPCC and microsatellite instability has been identified in up to 26% of sporadic colorectal cancers suggesting a role in sporadic disease (Thibodeau et al., 1993). Adenomas arising in individuals affected by HNPCC display microsatellite instability (Ionov et al., 1993), indicating that the underlying defect is expressed in the earliest stages of tumorigenesis. However, in sporadic disease microsatellite instability appears to arise only in carcinomas. Young et al. (1993) failed to identify microsatellite instability in 46 adenomas analysed at a minimum of 5 loci, and, although not an initial aim of our study of sporadic colorectal adenomas, it is noteworthy that no adenomas displayed microsatellite instability at any of the three loci examined for loss of heterozygosity on chromosome 8p. Overall, it appears that genomic
instability arising from mismatch repair gene mutation is important in the later stages of sporadic colorectal tumorigenesis but rare in adenomas.

These data suggest that the chromosome 8p tumour suppressor genes are likely to be associated with the later stages of sporadic tumorigenesis in colorectal epithelium. This pattern in sporadic colorectal tumorigenesis is similar to that noted for inactivation of p53 gene (Baker et al., 1990; Kikuchi-Yanoshita et al., 1992) and induction of microsatellite instability, as a consequence of defective mismatch repair (Young et al., 1993). Genetic abnormalities in both p53 and mismatch repair are associated with genomic instability. In the case of mismatch repair this is manifested by errors in short sequences of repetitive DNA sequence and in p53 by chromosomal instability resulting in aneuploidy. It is interesting to speculate that the chromosome 8p gene may have a similar role in maintaining fidelity of DNA, which may assist in identifying candidate genes involved in such processes.

There is evidence suggesting loss of heterozygosity on chromosome 8p is associated with more advanced malignant disease in a spectrum of cancers. In colorectal cancer, chromosome 8p loss of heterozygosity has been associated with advanced clinicopathological stage in 58 patients (Fujiwara et al., 1993) and the presence of tumour microinvasion in a small series of 14 (Keleman et al., 1994). In carcinoma of the prostate, chromosome 8p loss of heterozygosity was associated with high Gleason score (McGrogan et al., 1994) and the presence of local invasion and lymph node metastases (Trapman et al., 1994). Bladder cancers displaying chromosome 8p loss of heterozygosity displayed significantly higher tumour grade and stage (Knowles et al., 1993) and in hepatocellular cancer chromosome 8p loss of heterozygosity was associated with poor histopathological grade (Emi et al., 1993).

From these reports there would appear to be an association between chromosome 8p loss of heterozygosity and pathological factors which are known to predict a poor prognosis. However, there have been no reports exploring the relationship between chromosome 8p loss of heterozygosity and patient survival. In this survival analysis of 90 cases of colorectal cancer, chromosome 8p loss of heterozygosity was of no prognostic value in colorectal cancer being independent of patient outcome. This is consistent with our findings that chromosome 8p loss of heterozygosity was independent of Dukes' stage which is confirmed to be the most reliable predictor of outcome. Therefore, although the putative 8p oncosuppressors are involved in the later stages of carcinogenesis they do not appear to be determinants of aggressive disease in this population. Frequency of loss of heterozygosity is an underestimate of actual inactivation of the 8p oncosuppressors genes: it simply reflects one mode of inactivation rather than its presence. The true frequency of chromosome 8p inactivation can only be resolved when the 8p gene(s) are cloned and mutation frequency established fully. In APC, 50% loss of heterozygosity has equated with apparently ubiquitous gene inactivation in the development of colorectal carcinogenesis. It
is feasible that similar involvement could be found with the 8p oncosuppressors. If this were the case the prognostic value of inactivation of 8p oncosuppressor could be reassessed in finer detail.

In section 3.2, loss of heterozygosity affecting chromosome 8p appeared to occur in a younger population, but this failed to reach significance at the 95% level. However, in this smaller population of 90 cancers, this age difference was significant at this level (mean age 68.8 years compared to 72.55 years in those showing retained heterozygosity, p=0.05). This is most likely a spurious result. No previous reports have identified any correlation with age and there are no reports suggesting a role for the putative chromosome 8p genes in colorectal predisposition, which may be expected to be associated with a younger group of patients. Age represented an important predictor of outcome in this series, however, using Cox proportional hazard analysis, we are confident that the age difference between these showing loss and those showing retained heterozygosity is not concealing a prognostic effect of chromosome 8p loss of heterozygosity.

In this series, survival is independent of both immunohistochemistry for p53 and DNA ploidy. There have been conflicting reports on the prognostic significance of each of these. It is likely that the presence of mutated p53 is associated with a survival disadvantage (Hamelin et al., 1994) and immunohistochemistry for p53 is likely to have a poor correlation with mutated p53 (Cripps et al., 1995). Aneuploid cancers have been associated with poorer prognosis in some reports, however, it is likely that this a marginal effect, which is perhaps most significant in identifying a sub-population of Dukes' B colorectal cancers which have poorer survival associated with the presence of aneuploidy (Armitage et al., 1985; Jones et al., 1988).

It is clear from the high frequency of chromosome 8p loss of heterozygosity in colorectal cancer that the 8p oncosuppressors are likely to have a significant role in carcinogenesis. The additional analyses in this chapter have demonstrated some of the important clinicopathological implications of chromosome 8p loss of heterozygosity.
Chapter 5
Mutator phenotype in sporadic colorectal cancer

5.1 Introduction

The previous chapters have described the incidence of one type of genomic change found in colorectal cancer: the major chromosomal event that results in deletion of part or all of a chromosome arm and is detected through loss of heterozygosity. The factors underlying occurrence of such events and the conditions which permit survival of the cell that undergoes them, are poorly understood but undoubtedly complex. Interstitial deletion, for example, requires two DNA double strand breaks with an inappropriate recombination event between them. Mitotic recombination, the other major mechanism for loss of heterozygosity (Ashton-Rickart et al., 1991) requires a recombination event between double strand breaks in homologous chromosomes. In all cases the DNA breakage is thought to have resulted from interaction with an exogenous carcinogen. This chapter discusses a different type of chromosomal event, which is also clonally expanded in neoplasms: microsatellite instability.

The presence of microsatellite instability is characteristic of cancers arising as part of the syndrome of hereditary non-polyposis colorectal cancer (HNPCC). This phenotype results from heritable defects in DNA mismatch repair (Peltomaki et al., 1993; Aaltonen et al., 1993; Aaltonen et al., 1994). The identification and nature of mismatch repair genes are detailed in Chapter 1. To date, four human homologues of yeast and bacterial DNA repair genes have been identified in which germline mutations result in HNPCC. hMSH2 on chromosome 2p (Fishel et al., 1993, Leach et al., 1993), hMLH1 on chromosome 3p (Bronner et al., 1994, Papadopoulos et al., 1994), hPMS1 on chromosome 2q (Nicolaides et al., 1994) and hPMS2 on chromosome 7q (Nicolaides et al., 1994). More recently, GTBP, a member of the same family of genes as hMSH2, has also been identified as important in human DNA mismatch repair. GTBP forms a complex with hMSH2, known as hMutSa and this appears to be important in recognition and subsequent correction of replication errors involving single base mismatches or single loops (Drummond et al., 1995; Palombo et al., 1995; Papadopoulos et al., 1995). Mutations in GTBP are associated with defective mismatch repair in cell lines derived from sporadic colorectal cancer, however, no germline mutations have been demonstrated in HNPCC (Papadopoulos et al., 1995).

Microsatellite instability has also been demonstrated in up to 28% of sporadic colorectal cancers (Ionov et al., 1993, Aaltonen et al., 1993, Thibodeau et al., 1993, Lothe et al., 1993).
In common with HNPCC, sporadic cancers displaying microsatellite instability are reported to occur more frequently in the proximal colon, maintain DNA diploidy and show an inverse relationship with p53 mutation. However, no age difference has been identified between patients with tumours exhibiting microsatellite instability and those that do not (Ionov et al., 1993; Lothe et al., 1993). Some cancers displaying microsatellite instability may represent occult cases of HNPCC, where familial inheritance is less obvious due to poor gene penetrance and/or family size, however, the exact significance of this sub-group is yet to be determined.

Microsatellite instability is defined by the presence of replication errors in tumour DNA demonstrated at mononucleotide runs, tri- tetra- and, most commonly, dinucleotide repeats. During the course of chromosome 8p loss of heterozygosity studies (Chapter 3) a proportion of cancers was found to display instability at microsatellite loci. This analysis was extended to include a further 135 cancers at up to six loci on chromosomes 2, 3, 8, 13, 15 and 17 by at least one of three methods detailed below.

As described in Chapter 1, defective mismatch repair is associated with a 'mutator phenotype'. Di- or trinucleotide repeat sequences within the exons of many genes may be targets for mismatch repair error. This could provide a mechanism for the accumulation of mutations in 'cancer genes' in colorectal cancer, entirely different from that envisaged as underlying the major chromosomal disruptions responsible for loss of heterozygosity. In particular, mutations that result from mismatch repair do not require the presence of external carcinogens, but merely the event of replication, occurring in circumstances in which mismatch detection and repair are deficient. Such radically different mechanisms of mutation, even if their target sites lie within the same set of 'colon cancer genes', may well produce tumours of different phenotype and behaviour.

Accordingly, the association between microsatellite instability and certain clinicopathological factors was assessed in a population of sporadic colorectal cancers. These included patient age and sex; site and Dukes' stage of cancer and the presence of mucinous pathology. In addition tumour DNA ploidy, p53 immunohistochemistry and loss of heterozygosity at polymorphic markers on chromosomes 17p and 5q were known in a proportion of cases. Finally, survival data were available on 169 of the 245 patients in whom tumour microsatellite instability was determined.

5.2 Methods

5.2.1 Assessment of microsatellite loci in chromosome 8p in loss of heterozygosity studies

119 colorectal cancers were analysed for evidence of loss of heterozygosity on chromosome 8p as detailed in Chapter 3. These cancers were routinely assessed at polymorphic microsatellite loci for evidence of microsatellite instability.
5.2.2 Assessment of microsatellite instability at loci on chromosomes 2, 3, 8, 13, 15 and 17

A further 135 colorectal cancers were selected at random from the colorectal cancer tissue bank in the Department of Pathology, University Medical School, Edinburgh. DNA was extracted from these and matched normal tissue according to the protocol in section 2.2. Analysis was performed at up to six microsatellite loci on chromosomes 2, 3, 8, 13, 15 and 17 for evidence of instability. Details of these loci are presented in Table 5.1. Three methods were employed in the analysis and multiple cancers were assessed by more than one means to ensure consistency.

Automated laser fluorescence DNA sequencer

Microsatellite instability was assessed at loci on chromosomes 2, 3, 8, 13, 15 and 17 using automated laser fluorescence (ALF) DNA sequencer (section 2.7.3). These loci were amplified under the same condition as in section 2.5.4, except polymerase chain reactions involved 30 cycles of denaturation at 95°C for 1 min, various primer-specific annealing temperatures (indicated in Table 5.1) for 1 min and extension at 72°C for 2 min. Magnesium concentration are indicated in Table 5.1. The CA-strand oligonucleotide primer was obtained already labelled with fluorescein (FITC) (Oswel DNA services, Edinburgh). 1 µl of PCR solution was added to 4 µl of loading buffer with appropriate internal standards. The 5 µl volume was then heat denatured and loaded onto ALF apparatus gel, which was run at a constant 2000 V, 70 mA, 55 W and 50°C. Data were analysed as described in 2.7.3.

Silver staining of acrylamide gel

These analyses were performed by Dr Jill Bubb in the Department of Pathology, University Medical School, Edinburgh. Details are provided in 2.7.2. In brief, loci on chromosomes 2, 3, 8, 13, 15 and 17 were amplified by PCR as described above. Loading buffer was added and a 15 µl aliquot run on 6% denaturing polyacrylamide gel and finally stained using BioRad silver stain kit. The presence of microsatellite instability was detected by 'shift' in position of allelic bands in cancer DNA compared to normal.
Table 5.1

Details of loci employed in microsatellite analysis

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sequence</th>
<th>[Mg2+]</th>
<th>Size range</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2S119</td>
<td>CTTGGGAACAGAGGTCATT, GAGAATCCCTCAATTTCTTTGGA</td>
<td>2.0mM</td>
<td>214-232</td>
<td>Gyapay et al., 1994</td>
</tr>
<tr>
<td>D3S1293</td>
<td>ACTCACAGAGCCCTTCACA, CATGGAAATAGAACAGG</td>
<td>2.0mM</td>
<td>116-144</td>
<td>Gyapay et al., 1994</td>
</tr>
<tr>
<td>D8S282</td>
<td>GGCAACAGGCAAGTTGT, GGCTGCAATTCTGAAGGTTA</td>
<td>1.5mM</td>
<td>260-272</td>
<td>Gyapay et al., 1994</td>
</tr>
<tr>
<td>D13S160</td>
<td>CAGGGAATCTAGGCTTCTA, GGCAAGGCTGAGGCAAAAA</td>
<td>1.5mM</td>
<td>229-241</td>
<td>Gyapay et al., 1994</td>
</tr>
<tr>
<td>D15S132</td>
<td>CTGATAATAAACCAGGAAGACAC, TATGGGCTGAAAGTG</td>
<td>1mM</td>
<td>69-83</td>
<td>Gyapay et al., 1994</td>
</tr>
<tr>
<td>D17S849</td>
<td>CAATCTGTTCTTAAGATTATTTTTG, CTCTGGCTGAGGAGGC</td>
<td>1mM</td>
<td>251-261</td>
<td>Gyapay et al., 1994</td>
</tr>
</tbody>
</table>

Locus name, primer sequence, magnesium concentration, allele size range and source for each of the microsatellite loci employed in the analysis of instability.

5.2.3 Collection of survival data

Survival data were collected as detailed in 4.2.6. The survival period was calculated from the date of operation to the known date of death or point of censoring on 31 December 1994.

5.2.4 Survival analysis

Survival analysis was performed in 169 patients, 35 (20.7%) of which displayed instability at one or more microsatellite loci. Follow up was available for a median of 776.5 days with a range of 8-1968 days. Cox proportional hazard method was employed by Dr Andrew Carothers as described in 4.2.7 and included the following features.

1. microsatellite instability at one or more loci
2. site of cancer (proximal or distal to splenic flexure)
3. age of patient
4. Dukes’ stage
5. p53 immunohistochemical staining (available in 84 patients)
6. tumour DNA ploidy (available in 32 patients).
5.2.5 Determination of DNA ploidy

Data on DNA ploidy were provided by Dr C. Purdie and Miss S. White as outlined in section 2.10.

5.2.5 Determination of p53 immunohistochemical staining

Data on p53 immunohistochemical staining were provided by Dr C. Purdie and Miss S. White as described in section 2.11.

5.3 Results

5.3.1 Microsatellite instability in sporadic colorectal cancers

In the original series of 119 cancers, 19 (15.9%) displayed evidence of microsatellite instability at one or more chromosome 8 loci. This group was extended to include a total of 245 cancers and matched normal tissue which were analysed at an average of 4.8 microsatellite loci (range, 3 to 8) by at least one of the above techniques, i.e. autoradiography with $^{32}$P-labelled PCR, 'ALF' DNA sequencer or silver staining of unlabelled PCR products. Evidence of replication errors at one or more microsatellite loci was present in 41 cancers, i.e. 16.7%. Fourteen cancers (5.7%) displayed microsatellite instability at 2 or more loci. The three methods of microsatellite assessment were reproducible and consistent as demonstrated in Figure 5.1.

5.3.2 Microsatellite instability and relationship to clinicopathological factors

There is a significant association between the presence of microsatellite instability and cancers originating in the proximal colon (p=0.0423) and maintaining a normal diploid DNA content (p=0.00534). However, there was no correlation between microsatellite instability and patient age, sex, p53 immunohistochemistry, loss of heterozygosity on chromosome 5q and 17p. A trend between the presence of microsatellite instability and poor differentiation was not statistically significant, however, the cancers displaying microsatellite instability were more likely to show mucinous histology (p=0.044). These data are presented in relation to microsatellite instability in Table 5.2.
Figure 5.1
Assessment of microsatellite instability

Microsatellite instability was assessed by the three methods illustrated above, automated laser fluorescence (ALF), silver staining and autoradiography. Examples are shown for three cases: A, B and C at locus D13S160. For ALF, each peak represents one allele. Cases A and B display microsatellite instability with the generation of shorter fragments in the tumour DNA (T) compared with the normal (N) clearly visible by all three methods. Case C shows no microsatellite instability. Alleles range from 229 to 241 bases.
Table 5.2
Relationship between microsatellite instability and clinicopathological factors.

<table>
<thead>
<tr>
<th></th>
<th>MI+ No (%)</th>
<th>MI- No (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (years)</td>
<td>71.7</td>
<td>69.8</td>
</tr>
<tr>
<td></td>
<td>Range (years)</td>
<td>37-93</td>
<td>21-95</td>
</tr>
<tr>
<td>Patient age (n=245)</td>
<td>Male</td>
<td>17 (41.5%)</td>
<td>101 (49.5%)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>24 (58.5%)</td>
<td>103 (50.5%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>41</td>
<td>204</td>
</tr>
<tr>
<td>Sex (n=245)</td>
<td>Proximal</td>
<td>22 (53.6%)</td>
<td>72 (35.3%)</td>
</tr>
<tr>
<td></td>
<td>Distal</td>
<td>19 (46.4%)</td>
<td>132 (64.7%)</td>
</tr>
<tr>
<td>Site of cancer (n=245)</td>
<td>Total</td>
<td>41</td>
<td>204</td>
</tr>
<tr>
<td>Dukes’ stage (n=245)</td>
<td>A</td>
<td>3 (7.3%)</td>
<td>23 (11.3%)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>21 (51.2%)</td>
<td>92 (45.1%)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>17 (41.5%)</td>
<td>89 (43.6%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>41</td>
<td>204</td>
</tr>
<tr>
<td>p53 staining (n=206)</td>
<td>Positive</td>
<td>19 (52.8%)</td>
<td>106 (62.4%)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>17 (47.2%)</td>
<td>64 (37.6%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>36</td>
<td>170</td>
</tr>
<tr>
<td>Chromosome 17p (n=38)</td>
<td>LOH</td>
<td>5 (71.4%)</td>
<td>26 (83.9%)</td>
</tr>
<tr>
<td></td>
<td>No LOH</td>
<td>2 (28.6%)</td>
<td>5 (16.1%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>7</td>
<td>31</td>
</tr>
<tr>
<td>Chromosome 5q (n=46)</td>
<td>LOH</td>
<td>5 (100%)</td>
<td>24 (58.5%)</td>
</tr>
<tr>
<td></td>
<td>No LOH</td>
<td>0</td>
<td>17 (41.5%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>5</td>
<td>41</td>
</tr>
<tr>
<td>DNA ploidy (n=111)</td>
<td>Diploid</td>
<td>11 (61.1%)</td>
<td>23 (24.7%)</td>
</tr>
<tr>
<td></td>
<td>Aneuploid</td>
<td>7 (38.9%)</td>
<td>70 (75.3%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>18</td>
<td>93</td>
</tr>
<tr>
<td>Differentiation (n=121)</td>
<td>Poor</td>
<td>14 (63.6%)</td>
<td>52 (52.5%)</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>8 (36.4%)</td>
<td>47 (47.5%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>22</td>
<td>99</td>
</tr>
<tr>
<td>Histopathology (n=120)</td>
<td>Mucinous</td>
<td>9 (40.1%)</td>
<td>18 (18.4%)</td>
</tr>
<tr>
<td></td>
<td>Non mucinous</td>
<td>13 (59.1%)</td>
<td>80 (81.6%)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>22</td>
<td>98</td>
</tr>
</tbody>
</table>

aDukes’ stages A and B were combined for this analysis

MI+ Presence of microsatellite instability at one or more loci (number of cases with percentage in parenthesis)

MI- Absence of microsatellite instability

n.s. Not significant
Cox survival analysis

Survival analysis was performed in 169 patients, 35 (20.7%) of whom displayed replication errors at one or more microsatellite loci in cancer DNA. Cox proportional hazard model was employed as described above. Follow up was available over a mean of 776.5 days with a range of 8-1968 days and deaths from all causes were included. Survival for the population of 169 patients on whom data were available at the completion date of the study is presented in the survival curve in Figure 5.2. Overall survival is comparable to that found in other reports, suggesting that this is a representative group (Black et al., 1993).

Those 35 patients with cancers displaying microsatellite instability at one or more loci showed a dramatically improved survival of 77% at three years, compared to 43% in cancers without microsatellite instability. This difference was highly significant, ($\chi^2 = 7.83$, 1 df; $p=0.0051$) and survival curves are presented in Figure 5.2. The hazard ratio of microsatellite instability was estimated to be 0.39 (95% CI, 0.19-0.82), that is at any given time an individual with a cancer displaying microsatellite instability is at 39% of the risk of death of an individual with no microsatellite changes.

The most significant predictor of outcome was Dukes' stage ($\chi^2 = 38.4$, $p<0.0001$) followed by increasing age ($\chi^2 = 6.35$, 1 df; $p=0.012$). Dukes' stage A was associated with an 88% three year survival compared to 68% and 36% for Dukes' stages B and C respectively and survival curves for Dukes' stage are presented in Figure 5.3. As this analysis included death from all causes, it is to be expected that the risk of death increases with age.

Those cancers arising proximal to the splenic flexure were associated with a significantly improved survival although this effect was less significant than the presence of microsatellite instability. The association between the presence of microsatellite instability and proximal location in the colon has been detailed in Table 5.2. However, it is noteworthy that in this survival analysis the effects are independent. When the analysis was repeated excluding 'site of tumour', those with microsatellite instability still had significant survival advantage (hazard ratio of 0.37, 95% CI 0.18-0.77).
Figure 5.2

Microsatellite instability and survival in sporadic colorectal cancer

Cox proportional hazard analysis of survival in 169 sporadic colorectal cancer patients with and without microsatellite instability. Presence of microsatellite instability is associated with a significant survival advantage.
Dukes' stage and survival in sporadic colorectal cancer

Cox proportional hazard analysis of survival in 169 sporadic colorectal cancer patients classified as Dukes' stage A, B and C. Dukes' stage is the most reliable predictor of survival.
5.4 Discussion

5.4.1 Methods of assessing microsatellite instability

Assessment of microsatellite instability most often employs the use of isotope-labelled polymerase chain reactions, employing autoradiography to allow direct visual comparison between normal and cancer DNA. In this present work one primer in each PCR was end-labelled, such that each allele gave rise to only one visible strand on autoradiography following polyacrylamide gel electrophoresis. Two further methods were also employed. Visualisation of PCR products by silver staining method produces a similar pattern to that found in isotope-labelling of PCR primer, however, staining of denatured PCR products means that each DNA strand comprising the alleles is visualised producing two bands per allele. Although both of these methods are proven, use of the ALF DNA sequencer could be hoped to increase the safety and speed of the process as it reduces the use of radioactive and toxic materials and allows immediate assessment of the gel, thereby avoiding delays through autoradiograph exposure. The ALF DNA sequencer has been employed with microsatellite sequence analysis in large scale semi-automated genetic linkage analyses where it is proven to be convenient and reliable (Mansfield et al., 1994). Several samples were tested by all three methods and these were shown to concur Figure 5.1

5.4.2 Relationship to clinicopathological factors

Cancers displaying microsatellite instability appear to represent a distinct biological subgroup with associated clinicopathological factors. They arise more often in proximal colon and tend to maintain a state of DNA diploidy and have a high proportion with mucin secreting architecture. In this present analysis there was no relationship between microsatellite instability and age of onset, although this has been suggested by others (Ionov et al., 1993; Kim et al., 1994).

An inverse relationship between microsatellite instability and p53 stabilisation by immunohistochemistry (Ionov et al., 1993; Kim et al., 1994) has been described and Ionov has proposed that defective mismatch repair and p53 inactivation may represent distinct pathways to cancer development. However, this thesis fails to support these findings, with no significant association between p53 immunohistochemistry and microsatellite instability. This may be partially attributed to differences in immunohistochemical assessment. In this project, the presence of any stained nuclei was scored as positive and this approach has been shown to have an incomplete correlation with p53 mutation (Cripps et al., 1995). Potential relationships between mismatch repair and p53 inactivation may only be resolved with p53 mutation analysis in a large population. A strong relationship was identified between microsatellite instability and
maintenance of near diploid state (Table 5.2, p=0.00534) which confirms other reports (Peltomaki et al., 1993; Lothe et al., 1993).

Inactivated p53 (denoted by 17p loss of heterozygosity) has been described as co-existing frequently with aneuploidy and this is confirmed with immunohistochemical analysis in this study. DNA ploidy and p53 staining data were on 110 cancers. 80.3% (49/61) of p53 positives were aneuploid compared to 55.1% (27/49) of p53 negative cancers (χ²=6.96, p=0.008). This association may be causal and it is certainly interesting to consider that loss of p53 function facilitates the development of aneuploidy, through an impaired ability to regulate cell division at the G1-S boundary. Deficient p53 function is also likely to permit the survival of cells with DNA breaks and inappropriate recombination events which are required for the formation of interstitial deletions.

5.4.3 Survival analysis

The most striking clinical relationship with microsatellite instability is demonstrated in survival analysis. In this multivariate analysis, microsatellite instability was second only to Dukes' stage as a tumour-based prognosticator. This is particularly surprising considering the tendency for cancers with microsatellite instability to have mucin-secreting architecture, traditionally associated with poor outcome (Cotran et al., 1994). There is some speculation that the presence of defective mismatch repair may be associated with the accumulation of mutations in many genes, potentially giving rise to a spectrum of mutant proteins. It is possible that such mutations may produce changes in antigenic properties of cell surface proteins, increasing immune system recognition during haematogenous phase of metastatic spread (Kim et al., 1994).

5.4.4 Significance of findings and basis for further studies on MMR mutation analysis

As described in 1.5.3, microsatellite instability is seen in over 80% of colorectal cancers arising in HNPCC. An important feature of this analysis of microsatellite instability in a population of sporadic cancers was to identify if this subgroup may include a proportion of occult HNPCC cases which had remained undetected, due to poor penetrance or small family size. Certainly, the subgroup identified share similarities with cancers arising as part of the HNPCC: proximal location in the colon, diploid state and mucinous-type architecture. However, recent mutation analysis of hMSH2 in 36 of this present series of cancers with microsatellite instability has identified no case of HNPCC with truncating germline mutations in hMSH2 which occur in approximately 40% of HNPCC cases (Bubb et al., 1996). This confirms work which found germline mismatch repair gene mutations in only one of ten lymphoblastoid cell lines established from individuals with cancers displaying instability (Lui et al., 1995a). Liu identified somatic mutations in one of the four MMR genes (hMSH2, hMLH1, hPMS1, hPMS2) in only 3 out of 7 cancers with microsatellite instability and no family history of HNPCC. These findings provide
convincing evidence that microsatellite instability in a sporadic colorectal cancer population does not represent occult cases of HNPCC and even those in which mismatch repair genes are implicated by somatic mutation, the spectrum of mutation is different to HNPCC. It is likely that further investigations will disclose other mismatch repair genes or indeed different systems of repair as described in section 1.5.3.

5.4.5 Possible use as a molecular tool in screening analysis

Initial hopes that the identification of cancers with microsatellite instability may have earmarked a group of cancers with genetic predisposition have been blighted by a failure to identify germline mutations in mismatch repair genes. However, the majority of cancers occurring in young patients (<35 years of age at diagnosis) with no history to suggest HNPCC, have microsatellite instability and mutation analysis of a group of 12 such patients identified germline mutations in mismatch repair genes in 5 (Lui et al., 1995b). Three of these were nonsense mutations in hMSH2 with one nonsense mutation in hMLH1, characteristic of HNPCC. The fifth was a missense mutation in hMLH1. The remaining seven patients may have mismatch repair mutations which escaped detection (eg in intronic or splice regions) or they may have arisen from other defects in DNA replication or repair. In any event it is likely that cancers arising in individuals at this age are driven by constitutional, heritable predisposition, even in the absence of familial malignant disease. Therefore, such individuals should be enrolled in molecular screening projects, subsequently offered presymptomatic diagnosis, counselling and treatment to their relatives. It is likely that a small proportion of sporadic cases will also have an inherited predisposition, however, paradoxically, Aaltonen has suggested that presumed sporadic cancers with microsatellite instability arising in the distal colon are more likely to be associated with genetic predisposition (Aaltonen et al., 1994). This stems from the observation that the subgroup of sporadic cancers with microsatellite instability and no evidence of predisposition either on history or mutation analysis, most often have proximal tumours, whereas HNPCC patients have a significant number of distal colon cancers.
Chapter 6

Discussion

6.1 Introduction

This thesis describes two different types of genetic defect, both of which are common, clonal events in colorectal cancer and examines their clinical and pathological associations. First, loss of heterozygosity at chromosome 8p emerges as one of the more frequent acquired genetic lesions in colorectal cancer. Data presented in this thesis strongly support the existence of critical deletion sites of oncosuppressor genes, of significance in the later stages of colorectal carcinogenesis. It seems probable that two separate loci in chromosome 8p are involved, at 8p22-p21.3 and 8p21-p11.2, at least one of which is of significance in other major cancers. Further studies will focus on the identity and function of these genes. Second, microsatellite instability was identified in 16.7% of sporadic colorectal cancers examined in this thesis. Unlike loss of heterozygosity at chromosome 8p, it is a powerful prognosticator amongst known risk factors, exceeded only by Dukes' stage. Further studies, following from this observation, relate to the basis of the instability in these tumours, the reason for their interesting phenotype, and the clinical use to which this information may be put. This discussion deals in turn with the prospect of these future studies.

6.2 Towards identifying the 8p oncosuppressor genes

As described in section 3.4, a candidate tumour suppressor gene on chromosome 8p, PRLTS, has been identified. However, substantiating data are weak (Fujiwara et al., 1995) and continued efforts must be undertaken to examine the exact contribution of PRLTS and identifying further candidate genes. To this end, the work of this thesis has been extended with creation of a yeast artificial chromosome (YAC) map over the region of 4 cM deletion at 8p22-21.3 (Farrington et al., 1996). Several genes on chromosome 8p of potential interest as candidate tumour suppressors are discussed below.

6.2.1 Chromosome 8p genes of potential importance in colorectal carcinogenesis

DNA polymerase β is a well characterised nuclear protein known to be involved in DNA repair following chemical-induced DNA damage. Wang et al., (1992) proposed that DNA polymerase β may be a tumour suppressor gene, and postulated that its inactivation would result
in a mutator phenotype, facilitating the accumulation of mutations in other colorectal cancer genes. Wang sequenced the entire coding sequence of polymerase β mRNA from six colorectal carcinomas and adjacent normal mucosa. Mutations were found in 5 out of the 6 colorectal cancers. These comprised an 87 base pair deletion in four cases, two of which also had mutations of 21 and 42 base pairs and a third which had an A-T substitution changing lysine to methionine. A fifth cancer had a 217 base pair deletion resulting in frame shift and a premature STOP codon. All of the mutations affected the carboxyl catalytic domain as opposed to the DNA-binding amino-terminal. Initially, this provided exciting evidence of a possible candidate chromosome 8p tumour suppressor gene, however, the findings of this limited report have yet to be confirmed in larger independent series. Polymerase β has been assigned to chromosome 8p11.2 (Chang et al., 1994b) and it is likely to lie centromeric to the regions of common loss of heterozygosity proposed in this thesis.

The gene responsible for Werner's syndrome has been localised to chromosome 8p by linkage studies (Goto et al., 1992). This syndrome is associated with premature ageing, and the development of sarcomas and meningiomas, but there is no increase in epithelial cancers. It is believed to result from an inherited defect in DNA repair. Localisation of the gene responsible for Werner's syndrome indicates that it is likely to lie between D8S87 and D8S131 at chromosome 8p12. This is within the region of loss involved in colorectal cancer and it is possible that the Werner's syndrome gene may be involved in colorectal carcinogenesis, either directly or as a modulator of a linked cancer gene on the same chromosome arm.

One of the genes causing retinitis pigmentosa has been localised to chromosome 8p (Blanton et al., 1991). This is a dominantly inherited condition characterised by retinal degeneration and blindness often associated with premature cataracts and mental retardation. Although there is no known association between malignant disease, the functional abnormality in retinitis pigmentosa is likely to involve defects in cellular differentiation. Thus, it is possible that such a gene could be a candidate tumour suppressor or have a role as a modifier affecting the tumour suppressor locus.

Two forms of arylamine N-acetyl transferase (NAT1, NAT2) have been localised to chromosome 8p22 (Franke et al., 1994). Some interesting work has proposed associations between certain forms of enzyme polymorphisms and carcinogenesis (Blum et al., 1990). The general population can be considered as 'fast', 'slow' or 'intermediate' acetylators according to the nature of arylamine N-acetyl transferase (NAT) activity. This activity may be measured directly (Blum et al., 1990) or inferred from NAT polymorphic genotypes (Oda et al., 1994). There are epidemiological reports suggesting that colorectal cancer is more prevalent in 'fast' acetylators (Lang et al., 1986; Ilett et al., 1987). However, more recent work examining colonic cytosol NAT activity in individuals with and without colorectal cancer failed to reveal an association between colorectal cancer and acetylator status (Kirlin et al., 1991).
which is also associated with a putative chromosome 8p tumour suppressor locus. There is compelling evidence suggesting a positive association between slow acetylator status and susceptibility, believed to be through inadequate metabolism of urinary benzidine (Blum et al., 1990; Risch et al., 1995). There are no reports relating chromosome 8p loss of heterozygosity, acetylator status and bladder cancer. As noted in section 3.4.1, Takle and Knowles (1996) provided deletion mapping of two putative tumour suppressor loci on chromosome 8p in bladder cancer. The more telomeric site (8pter-p21.1) includes the NAT1 and NAT2 loci and it would be interesting to examine the specific role of NAT genes in this disease.

The role of NAT in the activation or deactivation of arylamine carcinogens is complex and the relative contributions of hepatic and colonic metabolism is undetermined. Although this may initially appear to undermine the basis of their involvement, it is important to appreciate that there may be significant differences in environmental/dietary factors and genetic background which may be important in determining an individual's likelihood of developing malignancy. Oda et al. (1994) suggested that fast acetylation was associated with the generation of mutagens causing mutations in Kirsten ras oncogenes. In the small number of colon cancers examined, there was a preponderance towards K-ras mutations in fast acetylators but this exciting idea was not substantiated.

The potential roles for NAT genes in colorectal carcinogenesis is uncertain, although it seems likely that NAT phenotype may have a role in the pathogenesis of some colorectal cancers. Alternatively, a significant tumour suppressor locus may lie close to the NAT genes on chromosome 8p with acetylator status acting as a modifying gene or merely a marker of the underlying physical linkage of the tumour suppressor locus to NAT genes.

6.1.2 Chromosome 8p deletion syndromes

Early data supporting the localisation of the APC gene on chromosome 5q were derived from the description of an interstitial chromosome 5q deletion identified in an individual with FAP and mental retardation (Herrera et al., 1986). Chromosome 8p deletion syndrome is well described and comprises mental retardation, facial dysmorphism and congenital heart defects (Pecile et al., 1990). Although reports have indicated deletions corresponding to the putative tumour suppressor locus (Morrison et al., 1992) no incidence of increase malignant disease has been reported. Clearly, the identification of such an individual with predisposition to malignant disease would assist the localisation of the putative tumour suppressor locus.
6.3 Microsatellite instability in colorectal cancer

6.3.1 Genetic lesions resulting in microsatellite instability

In HNPCC, the genetic defect has been defined as germline mutations in mismatch repair genes, primarily hMSH2 and hMLH1 (section 1.5.3). The presence of microsatellite instability in sporadic, non-HNPCC cancers is associated with particular clinicopathological features as detailed in 5.4.2. Initial conjecture that this subgroup may represent occult cases of hereditary colorectal cancer have not been confirmed on subsequent study (Liu et al., 1995; Borresen et al., 1995). In a proportion of the cancers examined in this thesis, Dr Jill Bubb and Ms Lucy Curtis have undertaken hMSH2 mutation analysis in the Cancer Research Campaign Laboratories, Department of Pathology, University of Edinburgh. They have demonstrated no germline mutation in hMSH2 in 36 individuals with cancers displaying instability (Bubb et al., 1996). Mutation analysis of these cancers with microsatellite instability identified only one somatic exonic mutation in hMSH2: a frame-shift in exon 15 creating a downstream stop codon. One other cancer had a base transition in intron 1 of unknown function. Seven cancers had a reduction in the length of the polythymine tract in intron 1 which is predicted to be part of the splice site. Twenty two cancers, including that with the exonic mutation, showed a reduction in the length of a polyadenine tract in intron 5. Thus, a total of 61% of cancers with microsatellite instability had abnormalities in hMSH2, compared to 6% (2/32) controls with no evidence of microsatellite instability. The effects of these intronic mutations are uncertain, it is possible that they may compromise transcriptional efficiency. Alternatively, they may be incidental manifestations of mutator phenotype resulting from infidelity of DNA replication in other systems. In any event, it appears that if hMSH2 has a role in sporadic colorectal carcinogenesis it is certainly different to that in HNPCC.

The absence of microsatellite instability in the analysis of adenomas in this thesis (section 4.3.3) and Young et al. (1993) suggests that in sporadic colorectal cancer, defective mismatch repair may be more important in the later stages of tumorigenesis. The acquisition of defective mismatch repair may contribute to increased mutation frequency in genes with essential functions in cellular growth and differentiation. An example of this is shown in the gene coding for TGF-β receptor, which has been shown to be inactivated in the majority of cancers displaying microsatellite instability (Markowitz et al., 1995). The inactivation results directly from alteration in microsatellite sequences within the gene, consistent with defective mismatch repair, resulting in the reduction or abolition of the growth inhibitory function of this protein. This demonstrates one mechanism by which DNA repair defects are linked to a plausible pathway of carcinogenesis.
6.3.2 Phenotype of sporadic cancers displaying microsatellite instability

There is a powerful association between the presence of microsatellite instability in sporadic colorectal cancers and tumour phenotype, as described in Chapter 5 (Ionov et al., 1993, Lothe et al., 1995). They occur more frequently in the proximal colon, have an improved prognosis and, at a molecular level, maintain DNA diploidy and show an inverse relationship with p53 mutation. The impressive survival advantage for individuals with cancers displaying microsatellite instability phenotype compared to those showing no microsatellite instability most likely represents a reduced ability of primary cancers to undergo metastasis successfully. As noted in 5.4.3, Kim et al. (1996) proposed that this may be secondary to the increased antigenic properties of cell surface proteins mutated through DNA mismatch repair defects. The immune system is believed to have increased recognition of such cells, facilitating cytotoxicity during spread or early in implantation. Such a phenomenon would be consistent with the survival advantage seen in this analysis. Alternatively, cancer cells with microsatellite instability may acquire mutations at such high frequency that genes which are essential to survival are compromised or inactivated, thereby reducing tumour cell survival. The apparent predilection for growth in the proximal colon may be a reflection of increased incidence of cancers showing no evidence of microsatellite instability in the distal bowel. These cancers, induced by the carcinogen environment in the distal colon, would be superimposed on the background of cancers with microsatellite instability.

6.3.3 Screening for MMR mutations

Aspects of clinical and molecular screening for colorectal cancer have been discussed in Chapter 1. A great hope of advances in our understanding of the molecular basis of colorectal cancer susceptibility is to provide rational genetic screening which will allow more informed counselling and targeting of clinical screening procedures. Recent reports investigating the causative mismatch repair mutations in HNPCC (Liu et al., 1996) indicate that 31% (15/48) had mutations identified in hMSH2 gene and 33% (16/48) in hMLH1 gene with hPMS1/2 accounting for 6% (3/48) and none in GTBP. The remaining 30% of kindreds having no mutation detected. This analysis employed in vitro synthesised protein (IVSP) assay and sequencing of mutations demonstrated by truncated proteins. In those with no truncations, the coding regions of all five genes were sequenced. Such rigorous analysis will have to be applied to a wider group of individuals, including those with no personal or family history of colorectal cancer, before reliable information can be determined on the significance of missense mutations and their prevalence and penetrance. In the shorter term, it would be reasonable to undertake analysis of individuals with early onset disease and/or a family history of colorectal cancer. Such analysis as used by Liu et al. (1996), could not be undertaken by most laboratories with a service rather than research interest in colorectal cancer. However, it is prudent to note that 24 of the 26 truncated
proteins in hMSH2 and hMLH1 were detected by IVSP. Thus more than half of families affected by HNPCC could have the germline MMR defect identified by a relatively simple approach which may be undertaken with proprietary kits. Such developments herald the introduction of molecular screening at a basic service level in the presymptomatic diagnosis and management of colorectal cancer in the population. Enthusiasm must be tempered by recognition that these exciting findings should be thoroughly tested before they are made widely available for use. The use of genetic analysis in this vulnerable population must be accompanied by sound counselling and audit if the real benefits are to be realised.
Chapter 7

Bibliography


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Peltomaki P, Lothe RA, Aaltonen LA et al. Microsatellite instability is associated with tumours that characterize the hereditary non-polyposis colorectal carcinoma syndrome. *Cancer Res.* 1993; 53: 5853-5855. (b)


Trapman J, Sleedens HFBM, van der Weiden MM et al. Loss of heterozygosity of chromosome 8 microsatellite loci implicates a candidate tumor suppressor gene between the loci D8S87 and D8S133 in human prostatic cancer. Cancer Res. 54: 6061-6064.


Appendix 1

Buffer solutions

10xTBE
121.1g Tris (0.89M)
55g Boric acid (0.89M)
7.4g Na$_2$EDTA (2M) 20ml
made up to 1 litre with DDW
pH8.3

TE
10mM Tris pH8
1mM EDTA

20xSSC
175.3g NaCl
88.2g sodium citrate
made up to 1 litre with DDW
pH to 7
Appendix 2

List of suppliers

Amersham International plc Amersham Place Little Chalfont Buckinghamshire HP7 9NA Hybond N+ Klenow fragment Megaprime kit α32PdCTP γ32PdATP X-OMAT autoradiography film

Bio-Rad Laboratories Ltd Bio-Rad House Maylands Avenue Hemel Hempstead Hertfordshire HP2 7TD Sequencing apparatus used for denaturing gel electrophoresis

Boehringer-Mannheim UK (Diagnostics and Biochemicals) Ltd Various restriction enzymes and corresponding buffers Bell Lane Lewes E. Sussex BN7 1LG Proteinase K

Difco Laboratories Ltd Central Avenue East Molesey Surrey KT8 0SE Bacto-tryptone Bacto-agar Yeast extract

Fisons Scientific Equipment Bishop Meadow Road Loughborough Leicestershire Methanol Glacial acetic acid Silver nitrate Sodium carbonate (decahydrate)
LE11 ORG

Formaldehyde solution (37%)
Sodium thiosulphate
3MM/ no. 17 paper
Chloroform
Urea
Ammonium acetate
Isopropanol
Sodium dodecyl sulphate (SDS)
Ethanol
Sodium hydroxide
Dithiothreitol
Dextran blue
Triton-100
Potassium chloride
Boric acid
Sodium chloride
Tris
Formamide
EDTA (disodium salt)
Sodium citrate
Dextran sulphate
Magnesium chloride
Glycerol
Hydrochloric acid

Hybaid Ltd
111-113 Waldegrave Road
Teddington
Middlesex
TW11 8LL

Hoefer Scientific Instruments Ltd
Unit 12
Croft Road Workshops
Croft Road
Off Hempstall Lane
Newcastle under Lyme
1 kilobase DNA ladder
λ HindIII marker
T4 polynucleotide kinase + buffer
Low melting point agarose
Spermidine
Ampicillin
HEPES
Taq polymerase + buffer
Salmon sperm DNA
Agarose
Various restriction enzymes and corresponding buffers
Deoxy nucleotide triphosphates
Sephadex nick columns
Maxipreparation kit
Dorking Business Park
Dorking
Surrey
RH4 1HJ

Rathburn Chemicals Ltd
Walkerburn Scotland
EH46 6AU

Water-saturated phenol

Severn Biotech Ltd
Unit 2
Park Lane
Kidderminster
Worcestshire
DY11 6TJ

40% 19:1 Instagel
TEMED
Ammonium persulphate

Sigma-Aldrich Company Ltd
Fancy Road
Poole
Dorset
BH17 7NH

γ-methacryloxypropyltrimethoxysilane
Magnesium chloride
β-mercaptoethanol
Polyethylene glycoldimethyl sulphoxide (DMSO)
Bromophenol blue
Xylene cyanol FF
Ethidium bromide
Appendix 3

*Marker sizes*

Sizes given in base pairs

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Deletion mapping in colorectal cancer of a putative tumour suppressor gene in 8p22-p21.3

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Although previous studies of acquired loss of heterozygosity (LOH) in colorectal tumours have suggested that a tumour suppressor gene may lie within the short arm of chromosome 8, its precise localisation remains to be determined. To obtain a more accurate positional map 120 colorectal cancers were examined with eight chromosome 8 polymorphic markers comprising both restriction fragment length polymorphisms and microsatellite polymorphisms based on (CA)n repeats. 91 cases were informative and LOH was detected in 47 (51%). The markers most commonly sited within the lost region mapped to the lipoprotein lipase gene (LPL) at chromosome 8p22. From study of tumours showing break-points within 8p, a common region of deletion was established extending centromERICALLY from LPL to the ankyrin 1 gene (ANK1) which is mapped to 8p21.1-11.2. This overlaps with common deleted regions observed in other studies of colorectal tumours (8p23.1-p21.3) and bladder tumours (8p21-q11.2). Taken together, the results in colorectal cancer delineate a region in 8p22-p21.3 where the putative tumour suppressor gene must lie. The chromosome 8p deletions appear to be independent of those involving 5q and 17p in the same tumours. No relationship was found between the presence of 8p deletion and site or stage of the tumour, or the sex or age of the patient at diagnosis.

Results

Frequency of LOH on Chromosome 8p

120 colorectal cancers were examined with eight polymorphic chromosome 8 markers comprising five restriction fragment length polymorphisms (RFLP) and three microsatellite polymorphisms based on (CA)n repeats. The percentage of informative cases with each of the markers is given in Tables 1 & 2. Use of (CA)n repeat microsatellite polymorphisms was sometimes hindered by difficulties in autoradiograph interpretation. This particularly relates to the occurrence of 'stutter bands' which appear in the region spanning the expected allelic band. This is believed to be an artefact of the polymerase chain reaction (PCR) (Weber & May, 1989). Despite manipulation of PCR conditions this artefact could not be completely eradicated and conclusions regarding LOH were only made when both alleles were clearly distinguishable. As a consequence, the effective percentage of informative cases was reduced to 40-50% which is significantly less than predictions from original publications (Tomforde et al., 1992; Polymeropoulos et al., 1991). 91 colorectal cancers were informative with one or more markers and 47 (51%) of these demonstrated LOH. There was no correlation between loss at an 8p locus and site of
tumour, Duke's stage, sex or age of patient at diagnosis. Within the same tumour set, LOH was identified at the APC-MCC locus in 5q in 66% (37 of 56 informative cases) and in 17p in 79% (38 of 48 informative cases). There was no correlation between LOH in 8p and loss at other loci.

Table 1 Restriction fragment length polymorphisms used in LOH studies on chromosome 8 in colorectal cancer.

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1percentage of informative cases
2HindIII polymorphism in lipoprotein lipase gene on chromosome 8p22 was demonstrated by restriction enzyme digest of PCR products. All other polymorphisms employed Southern hybridisation techniques.

Localisation of target gene locus

Loss of heterozygosity for each marker is presented in Figure 1. We assigned pMS502 to chromosome 8q on the basis of the hybridisation pattern to DNA from a human/hamster hybrid panel (Wagner et al., 1991) with absence of a hybridisation signal from clones 1HL 12 and 20xPO435-2 which comprise chromosome 8p and a fragment of 8q.

Table 2 (CA)n repeat microsatellite polymorphisms employed in LOH studies of chromosome 8 in colorectal cancer.

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1percentage of cases informative
Tumours

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**Figure 2** Representation of critical tumours allowing derivation of a common region of deletional overlap which extends from LPL markers on chromosome 8p22 to ANK1 marker on 8p21.1-p11.2. Closed symbols, LOH; open symbols, retained heterozygosity; stippled, non-informative/not done.

Similarly, probes pKSR2 and Mfd199 are mapped to 8p23 (Kaneko K, et al., 1991; Tomforde et al., 1992) but no critical breakpoints were found between these loci and, therefore, determination of their relative positions is not crucial. The remaining markers, LPL3GT, pG2LPL35 and the PCR-amplified HindIII polymorphism, LPLHdIII, map to the LPL gene in 8p22. Overall, the peak incidence of LOH was found with the LPL gene markers suggesting that the putative tumour suppressor gene may be close to this locus. In most cases the pattern of loss suggested that all or most of the chromosome arm was deleted, but in seven, shown in Figure 2, LOH involved only part of the short arm. In these, the common region of deletion lay between LPL and ANK1 markers, a region of approximately 25-30 Mb at 8p22-8p11.2. Autoradiograph findings in cases 21 and 169 which significantly delineate this region of common deletion are presented in Figure 3.

**Discussion**

We have detected LOH of 8p markers in 51% of tumours, an incidence similar to that reported in other, generally smaller, studies of colorectal cancer (Van der Bosch, et al., 1992; Emi et al., 1992; Vogelstein et al., 1989). LOH at 5q and 17p was observed in 66% and 75%, respectively, in the same cases. These frequencies are also similar to those reported by others at these loci (Ashton-Rickardt et al., 1989; Baker et al., 1989; Miki et al., 1991) indicating the LOH on 8p determined in this study is not the result of selection of unrepresentative tumour samples for analysis. This incidence of LOH establishes the 8p gene as one of the most commonly affected loci in sporadic colorectal cancer. It is, of course, possible that inactivation of the putative 8p tumour suppressor gene by point mutation may occur at still higher frequency than heterozygous deletion, as has been recorded for APC (Powell et al., 1992). The chromosome 8p deletions appear independent of deletions involving APC in 5q or p53 in 17p, suggesting a role distinct from these other oncosuppressor genes.
Analysis of critical cases in this series has allowed the derivation of a minimum region of overlap which extends from the LPL locus on 8p22 to the ANK1 locus at 8p21.1-p11.2. Combining data from this study and those of Emi et al., (1992) from colorectal carcinomas of Japanese origin, allows the smallest region of deletional overlap to be resolved still further. Whilst the minimum region of overlap in the Japanese series is relatively large, involving 8p23.1-8p21.3, the overlap common to both series is confined to a region in 8p22-p21.3, with the LPL locus situated at its telomeric limit. This localisation is compatible with the common region of deletion (8p21-q11.2) described by Knowles et al., (1993) in bladder cancer.

Recently, attention has been drawn to a possible role for the polymerase β gene (8p12-p11) in colorectal carcinogenesis since frequent mutations within this gene were demonstrated in a small series of colorectal cancers (Wang et al., 1992). However, this gene is clearly outside the region identified in our studies and its role remains unclear. Indeed, definitive information is currently lacking on the role of any of the genes identified in this region and as yet there are no data linking the 8p oncosuppressor with familial cancer syndromes such as hereditary non-polyposis colorectal cancer. The improved localisation now available may make such family linkage studies feasible.

Materials and Methods

Tissue Specimens, DNA extraction and Southern Blotting

DNA was extracted from one hundred and twenty colorectal cancers and matched normal tissues using an SDS-lysis and proteinase K digestion method (Goelz et al. 1985). 5µg of genomic DNA were digested with appropriate enzymes according to manufacturers' instructions. Digests were electrophoresed on 0.6-0.9% agarose gels for 8-24 hours and transferred to Hybond N+ membranes (Amersham, Aylesbury, U.K.). RFLP markers used in Southern hybridisations are detailed in Table 1. Probes were labelled by the random primer method with α-[32P]dCTP using the Megaprime DNA Labelling System (Amersham). Hybridisations were performed at 65°C in 6 x SSC with 10% Dextran Sulphate. Filters were washed to a stringency of 0.1 x SSC at 65°C and exposed for 2-5 days at -70°C. Membranes were stripped in 0.1 x SSC at 100°C and repeatedly hybridised.

Analysis of LOH using (CA)ₙ repeat polymorphisms

Three (CA)ₙ repeat polymorphisms were used. The primer sequences, chromosome loci and predicted allele size are presented in Table 2. Polymerase chain reactions consisted of 25µl volumes with 200µM of each nucleotide, 0.625 units of Taq polymerase enzyme (Advanced Biotechnologies Ltd, London U.K.) 2.5µl of buffer (Advanced Biotechnologies Ltd) 50ng of each primer and 50 ng of template DNA. 10 µg of one primer in each reaction was end labelled with γ-[32P]dATP, using T4 polynucleotide kinase. PCR reactions were carried out in a Hybrid Omnigene (Hybrid, Middlesex, U.K.) using 96 well plates (Hybaid) and consisted of 29 cycles of 95°C for 1min, 55°C for 1min and 72°C for 2min. 10µl of loading buffer (95% de-ionised formamide, 10mM Na₂ EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue) were added to each reaction and 10µl aliquots were run on 6% denaturing polyacrylamide gels. Gels were dried and exposed to Kodak X-OMAT AR film for 1-3 days.

HindIII polymorphism detected by PCR

One of the polymorphic markers depended on the presence of the polymorphic HindIII site in intron 8 of the lipoprotein lipase gene. A 1.2Kb fragment was amplified under conditions described by Bruin et al. (1991). This was digested with HindIII and the products separated by electrophoresis on 2% agarose gels stained with ethidium bromide and visualised under ultra-violet light.

Analysis of chromosomes 5q and 17p

LOH was examined in 5q by Southern blotting using the markers α227, L5.71, EF5.44 and YN5.48, within 5q21-22 (Ashton-Rickardt et al., 1989 and 1991) and in 17p using YNZ.22 and MCT35.1 (Barker et al., 1987; Nakamura et al., 1987). In some tumours PCR-amplified regions bearing polymorphic sites within APC and MCC in 5q (Heighway et al., 1991 and 1992) and within p53 or the subtelomeric YNZ.22 locus in 17p (Horn et al., 1989; McDaniel et al., 1991) were also examined.

Hybrid panel mapping

Human/hamster somatic cell hybrids were obtained by courtesy of Dr Michael Wagner (Wagner et al., 1991). 2µg of DNA from clones 1HL 12 and 20xPO435-2 were digested with Taq I (Northumbria Biologicals Ltd., U.K.) according to the manufacturer's instructions. Southern hybridisations were performed as described above, except that filters were washed to a stringency of 0.5 x SSC at 65°C. On repeated analysis, probe pMS502 did not hybridise with either of these clones but consistently hybridised to digested genomic DNA which acted as a positive control.
Acknowledgements
We thank Dr James Weber for sharing information on (CA)n repeat polymorphisms prior to publication and the Lothian colorectal surgeons for their collaboration in providing colorectal tumour samples. Dr P.G. Ashton-Rickardt, Dr C.A. Purdie, Mr R.G. Morris, Mr W. Rowand, Miss H. Stephens and Miss Monica Piris provided the LOH data on chromosomes 5q and 17p, some of which has already been published.
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References
Deletion analysis of chromosome 8p in sporadic colorectal adenomas

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Summary In order to assess the stage of colorectal tumorigenesis at which chromosome 8p loss of heterozygosity (LOH) occurs, 56 sporadic adenomas were examined for LOH at four polymorphic loci which show frequent LOH in carcinomas. LOH was found in only 5 out of 51 (9.8%) informative adenomas, whereas studies with the same markers in 85 informative cancers showed a LOH of 45%. The adenomas showing LOH were all in the 'high-risk' clinicopathological category, being 10 mm or more in diameter and showing tubulovillous architecture. It is concluded that the chromosome 8p locus is involved preferentially in the development of tumourigenesis rather than adenomas.

The development of colorectal cancer incorporates several discrete genetic events. Some are largely restricted to carcinomas, whereas others are found in both adenomas and carcinomas. Inactivation of tumour-suppressor genes APC and DCC by mutation or loss occurs in the majority of both colorectal adenomas and carcinomas (Fearon & Vogelstein, 1990; Powell et al., 1992). Similarly, activating mutations in K-ras oncogene occur with nearly identical frequency in carcinomas and larger adenomas (Fearon & Vogelstein, 1990). Presumably such lesions affect the expansion of neoplastic populations without directly conferring a malignant phenotype. In contrast, the p53 gene is inactivated in 75% of colorectal cancers but is not abnormal in adenomas except those displaying features of severe dysplasia, and this gene would appear to have a major role initiating malignant behaviour (Baker et al., 1990; Kikuchi-Yanoshita et al., 1992; Carder et al., 1993). Such genetic lesions are of interest because they suggest the existence of genes involved in the critical transition from benign to malignant growth. We (Cunningham et al., 1993) and others (Fujiwara et al., 1993) have recently identified a region on chromosome 8p that exhibits frequent LOH in colorectal cancer, indicating a further putative oncosuppressor locus or loci. In this paper we assess the frequency of LOH at this region in sporadic colorectal adenomas to determine at what stage in evolution of colorectal tumours this molecular lesion exerts preferential selective advantage.

Loss of heterozygosity affecting chromosome 8p is found in over 50% of colorectal cancers (Cunningham et al., 1993). A similar frequency of chromosome 8p LOH has been determined in bladder, prostate, lung and hepatocellular cancer (Bergenhjem et al., 1991; Emi et al., 1992; Knowles et al., 1993). In bladder and hepatocellular cancer, there is a correlation between chromosome 8p LOH and advanced tumour stage and grade (Emi et al., 1993; Knowles et al., 1993). In colorectal cancer we were unable to identify a correlation between chromosome 8p LOH and tumour site or Duke's stage (Cunningham et al., 1993), although such an association with clinicopathological stage has been suggested by others (Fujiwara et al., 1993). In addition, the possibility of two separate chromosome 8p oncosuppressor loci has been proposed (Fujiwara et al., 1993). This work addresses the question of the role of the chromosome 8p locus or loci in tumorigenesis by determining the frequency of loss of heterozygosity in 56 sporadic colorectal adenomas at four chromosome 8p loci which have shown a high frequency of LOH in colorectal cancers. The adenomas were gathered from both cancer-bearing and cancer-free bowel. The results suggest preferential involvement of the chromosome 8p locus in the later stages of colorectal tumorigenesis.

Materials and methods

DNA was purified from 56 sporadic colorectal adenomas and matched normal tissue, either blood or histologically normal colonic mucosa, obtained from 49 individuals. Thirty-three adenomas were collected from fresh colorectal specimens resected for malignant disease. Of the remainder, 16 were removed endoscopically from cancer-free bowel as part of a presymptomatic screening programme.

Analysis of LOH using (CA)n repeat polymorphisms

Three microsatellite (CA) repeat markers were employed which had shown a high frequency of LOH in colorectal cancers. ANK1 (Polymeropoulos et al., 1991) maps to 8p21-p11.2, LPL3GT (Tomforde et al., 1992) maps to 8p22 and D8S137 (Tomforde et al., 1992) to 8p21-p12. Polymerase chain reactions comprised 25 μl volumes with 200 μM of each nucleotide, 0.625 units of Taq polymerase enzyme (Promega, UK) 2.5 μl of buffer (Promega, UK) 2.5 mM magnesium sulphate, 50 ng of each primer and 50 ng of template DNA. In each reaction 10 ng of one primer was end labelled with [α-32P]dATP, using T4 polynucleotide kinase. PCR reactions were carried out in microwell plates in an Omnimage thermal cycler (Hybaid, Middlesex, UK) and consisted of 29 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min. A 10 μl aliquot of loading buffer (95% deionised formamide, 10 mM disodium EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue) was added to each reaction and 4 μl aliquots were run on 6% denaturing polyacrylamide gels. Gels were dried and exposed to Kodak X-OEMAT AR film for 24 h. Autoradiographs were assessed visually by two observers.

HindIII polymorphism detected by PCR

HindIII polymorphism in intron 8 of the lipoprotein lipase gene (8p22) was demonstrated by PCR under conditions described by Bruin et al. (1991). PCR products were digested with HindIII, separated by electrophoresis on 2% agarose gels, stained with ethidium bromide and visualised under ultraviolet light.

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Results

We have previously reported LOH analysis of three of the markers used in this analysis (ANK1, LPL3GT and LPLHdIII) in 120 colorectal cancers (Cunningham et al., 1993). In this study we further analysed the same colorectal cancers at the D8S137 locus and found a frequency of LOH of 50% (27 out of 54 informative cases). The percentage LOH for each of these markers in colorectal cancers and adenomas is presented in Figure 1. Of the 56 adenomas, 51 were informative with at least one marker, and of these only five (9.8%) showed loss of heterozygosity. In contrast, when used in the study of 120 colorectal cancers, these four markers detected a LOH in 38 out of 85 informative cases (45%), a difference that is highly significant ($\chi^2 = 16.38; P < 0.0005$). The adenomas consisted of 35 tubulovillous lesions (mean size 27 mm, range 6–80 mm), 20 tubular lesions (mean size 14 mm, range 5–20 mm) and one villous lesion (15 mm). The five shown to have LOH at chromosome 8p were tubulovillous adenomas of 10 mm or more in diameter. Chromosome 8p LOH was not detected in any of the 10 adenomas less than 10 mm in diameter, seven of which were tubular adenomas and three tubulovillous lesions.

Discussion

We have detected loss of heterozygosity in less than 10% (5/51) of adenomas examined in this series, which is significantly less than the frequency of LOH (45%) in a similar analysis of 85 informative malignant tumours. As detailed above, this was a mixed group of adenomas in terms of size and histological types. The five lesions showing LOH were all tubulovillous adenomas, 10 mm or more in diameter. Although the numbers are small, chromosome 8p LOH was only detected in this study in the subgroup of adenomas which are known to carry a greater malignant potential. We are unaware of any study of chromosome 8p LOH in sporadic adenomas. However, a recent report of adenomas arising in familial adenomatous polyposis (FAP) describes no chromosome 8p LOH in 37 informative adenomas from two individuals (Ichii et al., 1993). Our series is likely to have included a higher proportion of tubulovillous lesions than this group of FAP adenomas, and this may account for the presence, albeit rare, of chromosome 8p LOH in the sporadic lesions reported here. Overall, our data suggest that the putative chromosome 8p tumour-suppressor gene is important in the later stages of tumorigenesis in the colon and rectum. This pattern is similar to that noted for the p53 gene (Baker et al., 1990; Kikuchi-Yanoshita et al., 1992) and strikingly different to that found in APC, DCC and K-ras (Fearon & Vogelstein, 1990; Powell et al., 1992).

Thus, LOH at the 8p locus appears to be one of a select group of acquired genetic lesions preferentially associated with malignant change in colorectal epithelium. Others include abnormalities of p53 and aneuploidy. In several cell lineages, including colorectal mucosa, abnormalities of p53 are known to induce instability of the genome, of which aneuploidy is an example (Livingstone et al., 1992; Carder et al., 1993). A further lesion, at the hMSH2 gene on chromosome 2p, is associated with hereditary non-polyposis colorectal cancer (HNPPC), a familial disorder characterised by the development of carcinoma without prior proliferation of benign lesions (Fishel et al., 1993; Leach et al., 1993). This also appears to involve infidelity in DNA replication, characterised by variability in the length of microsatellite repeats between normal and tumour DNA (Aaltonen et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993). Such instability has been recorded in up to 28% (Thibodeau et al., 1993) of apparently sporadic colorectal cancers but is rare in adenomas (Young et al., 1993). At the three microsatellite loci examined in this paper we detected instability in only one adenoma, an 18-mm-diameter tubulovillous lesion removed from non-cancer-bearing bowel. Published data indicate that genomic instability manifest as either aneuploidy or microsatellite instability is commonly acquired in malignant colorectal lesions. It is interesting to speculate that the defects in the putative 8p oncosuppressor may also relax the fidelity of DNA or chromosomal replication or impair DNA repair mechanisms.

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References

Familial adenomatous polyposis

Introduction

Familial adenomatous polyposis (FAP) is the most common single gene disorder resulting in the development of colorectal cancer. The incidence rate in a population based registry is 1.8 new patients/million population/year, responding to 1/6670 live births/year. The syndrome characterised by the development of 100 or more colorectal adenomas, usually during teenage years and early adulthood, with virtual certainty of the development of colorectal cancer by the third or fourth decade if prophylactic colectomy is not undertaken. First described in 1881, familial basis was noted by Cripps in 1882, and a autosomal dominant mode of transmission was elucidated by Reed. Around 25% of affected individuals have no family history of FAP, the disease arising as a result of a new mutation in the polyposis gene. These individuals present with symptoms attributable to the presence of colorectal polyps such as rectal bleeding, altered bowel habit and abdominal pain. Presymptomatic screening of at-risk individuals from polyposis kindred results in early recognition of gene carriers. The reported prevalence of colorectal cancer at diagnosis was 69% for asymptomatic non-screened individuals, whereas, only 1% of at-risk family members undergoing screening have malignancy at diagnosis. Clearly, every effort must be made to identify those at risk of FAP and to offer screening.

The gene responsible for FAP (known as APC) was identified on chromosome 5q, sequenced and causative mutations demonstrated in 1991. A new era in the understanding and clinical management of this syndrome was enabled by these discoveries.

This article reviews the clinical spectrum of the FAP phenotype and explores recent advances in the understanding of the molecular basis of the disease focusing on the clinical relevance of the molecular genetic aspects.

Clinical and pathological aspects

The polyps in the colon and rectum which characterise FAP are predominantly tubular adenomas. 1000-2000 polyps are usual but over 5000 is not uncommon. Detailed histological examination of resected colons reveals the various stages of adenoma development from single crypt lesions (Fig. 1) to large tubular and villous adenomas.

Extracolonic lesions in association with FAP were first noted by Devic and Bussey in 1912. Gardner described the triad of colonic polyposis, multiple osteomas and epidermoid cysts which was initially believed to be distinct from FAP. Mutation analysis, however, has demonstrated that it is part of the spectrum of the FAP disease phenotype. In addition to colorectal adenomatosis, the more common extracolonic manifestations of FAP include gastroduodenal and periampullary polyposis, desmoid tumours, craniofacial and upper long bone osteomas, cutaneous epidermoid cysts and pigmented lesions of the retina. Multiple osteoma or early onset epidermoid cysts should raise suspicion that the patient may carry a new mutation of the APC gene. Less common associated features include small bowel polyposis, hepatoblastoma, papillary thyroid carcinoma in women, gallbladder, bile duct and pancreatic malignancy and brain tumours.

With prevention of death from colorectal cancer, the management of upper gastrointestinal (GI) polyposis in FAP is one of the major challenges in the long-term care.
of affected individuals. In a multicentre survey of mortality from upper GI malignancy, Jagelman reported 4.5% of patients developed duodenal or peri-ampullary carcinoma at a median age of 52 years. Following colectomy and ileorectal anastomosis, it is more likely that a patient will die from upper GI malignancy than cancer in the retained rectal stump. The gastroduodenal polyposis in FAP consists of various lesions. The majority of gastric polyps are hamartomatous lesions consisting of dilatation of the fundic glands and are present in 55% of patients with FAP. Adenomas tend to be localised to the gastric antrum and to the duodenum, predominantly the second and third parts. It seems likely that this distribution is secondary to exposure to a constituent of bile since the most dense polyposis occurs in the peri-ampullary region. Over 90% of gene carriers have duodenal adenomas and approximately half of these have large and severely dysplastic lesions causing some dilemma when determining optimum management. Prophylactic pancreatic-duodenectomy is a major undertaking for patients with benign disease and further adenomas may well develop at the new site of bile influx. Open transduodenal or endoscopic polype excision may delay the disease for a time but is clearly not the answer. Some recent encouraging work using sulindac has shown a reduction in duodenal epithelial proliferation although the polyp count was not altered. Further assessment of the prophylactic use of non-steroidal anti-inflammatory drugs (NSAIDs) is required. Replacement of the defective gene with normal APC by gene therapy may have much to offer these patients in the future.

Desmoid tumours cause morbidity and mortality in patients with FAP. These neoplastic fibromatous lesions do not metastasis but exert deleterious effects by local expansion and compression of vital structures. 15% of patients with FAP develop this problem and more commonly in females. Desmoid tumours occur in two sites: involving the abdominal wall and intra-abdominal. The majority of those in the abdominal wall arise in recent surgical abdominal wounds. They usually lead a relatively uncomplicated course with the primary problem resulting from local pain. Medical management has been of little success and when lesions are tolerated by the patient they are best left alone. When complications such as intractable pain or possible intra-abdominal extension occur, adequate surgical removal should be performed with replacement of the abdominal wall with prosthetic material if required.

Intra-abdominal desmoids represent a more sinister and complicated group. Many arise in the small bowel mesentery or in the pelvic retroperitoneal space and may prevent the construction of an ileoanal pouch. Although not a prerequisite, desmoid tumour development is promoted by previous surgery. Some surgeons recommend an ileoanal pouch because of the risk of desmoid disease. Complications result from compression of ureters, small bowel and mesenteric vessels or the formation of fistulae. Management consists of surgical palliation as radical excision results in unacceptable morbidity and mortality together with likely desmoid regrowth. Treatment with teromphine, tamoxifen, sulindac, indomethacin, chemotheray and radiotherapy give only limited success. Understanding the functional deficit resulting from an inherited mutant APC allele may lead to more effective treatment approaches for patients affected by this complication.

Demarcated pigmented lesions of the retinal pigment epithelium (known as CHRPE lesions) are present in around 90% of affected individuals and the presence or absence of such lesions are a useful marker to assess the likely status of an at-risk individual. Figure 2 shows the co-inheritance of the CHRPE and polyposis phenotype in a small family.

Genetics

Following a report of a cytogenetic deletion of the long arm of chromosome 5 in a patient with FAP, genetic linkage studies in affected kindreds identified a small region containing the causative gene. Using a positional cloning strategy, a region of DNA containing the gene was isolated from chromosome 5q21. A number of candidate genes were cloned and eventually DNA sequence analysis identified mutations in one of these genes which could be demonstrated to show germline transmission to affected offspring in such families. The gene was named adenomatous polyposis coli (APC) and had features of a tumour suppressor gene. APC consists of 15 exons encoding a large 2843 amino acid
Fig. 2—Co-inheritance of CHRPE and polyposis phenotype in a small FAP family. The two children in the third generation are shown to carry the APC mutation by virtue of the presence of multiple pigmented lesions in both eyes.

Fig. 3—FAP pedigree with affected individuals denoted by black symbols. At risk children, aged 7 and 9 years, in the fourth generation denoted by half black/white symbols. The mutation was identified in an affected individual as a base substitution which changed arginine (ARG) to a STOP at codon 302 in exon 8. Sequence analysis, which is shown in the reverse direction to transcription, demonstrates two nucleotides at the indicated position, as the inherited mutation is always heterozygous. One of the at-risk children was shown to carry the same mutation, the other was a non-gene carrier.

polypeptide. 80% of the mutations which have been identified are in exon 15. Two specific exon 15 mutations occurring at codons 1061 and 1309 account for up to 20% of all APC mutations but the remainder are spread throughout the gene with no particular ‘hotspots’. A study of 22 unrelated Japanese patients with FAP predicted that the site of mutation may determine the number of colorectal polyps. Other groups have identified families with identical APC mutations but diverse phenotype in terms of colorectal polyposis and
extracolonic disease. It may be some time before valid genotype-phenotype correlations can be proposed and it is likely that the phenotype is influenced by other genetic or environmental factors.

The lack of mutational 'hotspots' dictates that the entire gene be screened for each different family. Although laborious, once the mutation has been identified, mutation analysis can be used for presymptomatic diagnosis with complete accuracy employing user-friendly methods of mutation detection. Most mutations are small deletions or single base substitutions which result in the generation of a premature STOP codon and consequently truncation of the protein. A typical example of sequence analysis of such a substitution is given in Figure 3.

Antibodies to the APC protein have identified a 300 KDa protein expressed in the cytoplasm of colonic epithelial cells. Expression is greater in the upper portions of the colonic crypts suggesting some involvement in colonocyte maturation. Recent work suggests that APC protein, which is truncated due to a mutation, may interact with the normal APC product and the resulting heterodimer may impair the function of the normal protein. Understanding the structure, function and regulation of this important gene product will lead to new strategies for the treatment of the FAP syndrome and non-FAP colorectal cancer.

Monoclonal antibodies to APC may be used for presymptomatic diagnosis of FAP. DNA from affected and at-risk individuals can be transcribed and translated in vitro and the abnormal truncated protein be detected by western blotting. This has been shown to be a feasible alternative to direct mutation detection.

Issues in the clinical management of FAP

Despite advances in the molecular aspects of FAP, clinical recognition of those at-risk remains paramount importance. An integrated screening strategy involving clinical and molecular genetic methods should be the aim. Clinical screening should include colonoscopic examination and indirect endoscopy for CHRPE lesions. Clinical screening can be modified and avoided depending on the results of genetic studies incorporating linkage and mutational analysis data. If sufficient individuals within a family are affected, linkage studies with DNA markers mapping close to the gene can identify those inheriting the disease with a reliability of more than 99%. Studies have shown the value of such testing with the identification of affected individuals who had previously been discharged from clinical follow-up. Mutation analysis gives a 100% accurate result and for individuals at-risk who are shown to be non-gene carriers clinical screening is unnecessary and follow-up is not required. Family members who are known to carry an APC mutation will be the only at-risk individuals who will require follow-up and this will be performed as a management exercise to assess the optimum timing for surgery.

There is considerable controversy over the most appropriate form of prophylactic colectomy in patients with FAP. Proctocolectomy and ileostomy is now redundant except in those presenting with very low rectal cancers. Restorative proctocolectomy (RPC) appears an attractive option as it abolishes the risk of rectal cancer while preserving continence. It is associated with considerable postoperative morbidity, however, and there is uncertainty over long-term functional results. The neorectum has to be surveyed for the development of malignancy in the pouch and in residual rectal mucosa if the anastomosis has been stapled. Colectomy and ileorectal anastomosis (IRA) provides similar functional results to RPC but there are fewer postoperative sequelae. The persistent risk of rectal cancer is the obvious disadvantage with IRA and most cancers are asymptomatic when detected by screening. Rectal cancer occurs in around 15% of patients within 25 years following IRA with a mean onset at 13 years after surgery. Many patients are subsequently cured from rectal cancer and death occurs in only 2% of patients 15 years after IRA but the life-time risk of death from rectal cancer following IRA may be considerably higher. This may be improved by more frequent and effective surveillance measures, e.g. the use of video-endoscopy every 4 months although such a rigorous protocol is poorly tolerated by patients. Rectal polyps undergo partial regression following IRA, but the risk of rectal cancer after IRA becomes significantly greater after 50 years of age and it has been suggested that patients with a rectum in situ should be converted to RPC during the fourth decade of life after child-bearing years. The possibility of modulation of FAP adenomata by pharmacological means and gene therapy may obviate the need for further surgery following IRA. There is no definite conclusion in the choice of surgery in patients with FAP but it seems reasonable to offer IRA with pharmacological manipulation and careful surveillance to those less than 30 years who have yet to have children and RPC should be offered as the primary procedure in those with middle or upper rectal cancers or extensive rectal adenomatosis at the time of diagnosis. Even those having RPC require continued screening of the neorectum since polyps are known to develop in the pouch and the long-term risk of cancer at this site is uncertain.

Encouraging results have been reported with the use of the NSAID, sulindac, with a reduction in colorectal polyp size and number. The assumed reduction in cancer risk remains to be confirmed. The mode of action of this effect is unknown but inhibition of prostaglandin H synthetase is likely to be important. It is clear that biological modulation of FAP is feasible and so the development of pharmacological agents for use in the management of the colonic and extracolonic manifestations of this disease may be a realistic possibility.

This review has addressed some of the challenges and exciting issues in familial adenomatous polyposis. Following the recent cloning, sequencing and early functional studies of the APC gene, presymptomatic testing of patients has been already dramatically affected. It seems likely that fundamental knowledge of the APC gene and its function will result in
novel approaches to therapy for patients with familial adenomatous polyposis.

References

Molecular genetic basis of colorectal cancer susceptibility

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The past decade has seen considerable advances in understanding of the molecular processes involved in the development of colorectal cancer. With an increased awareness of genetic aspects of the disease there have already been significant changes in clinical management. This is exemplified by familial adenomatous polyposis, where identification of mutations in the adenomatous polyposis coli (APC) gene in affected individuals can be used directly to reduce the requirement for clinical screening in at-risk relatives. In other more common but less well defined heritable forms of colorectal cancer, testing to identify individuals for early diagnosis and treatment will soon become routine practice. This review does not set out to discuss all aspects of the molecular genetics of colorectal cancer but concentrates on the roles of the APC gene and the recently discovered DNA mismatch repair genes in colorectal cancer. The identification of these genes and their functional significance in the neoplastic process is discussed, and the relevance of such discoveries to future research and clinical management explored.

Colorectal cancer is the commonest cause of death due to malignancy in non-smokers in western countries and consumes considerable health care resources. There has been marginal improvement in survival from the disease in the past 50 years, mostly attributable to reduction in perioperative death resulting from advances in surgical technique and anaesthesia. This improvement has been offset by an increase in incidence of colorectal cancer and it is clear that prevailing clinical approaches often fail to influence the natural history of the disease. Presentation with late stage disease is common and the overall 5-year survival rate from population data is 37 per cent; current treatment modalities of surgery, adjuvant chemotherapy and radiotherapy frequently offer no hope of cure. There is a need to improve understanding of the fundamental mechanisms of colorectal carcinogenesis to facilitate development of new approaches aimed at arresting and even preventing the malignant process. Identification of those individuals who are at high risk of developing the disease may allow the application of prophylactic measures such as chemoprevention, endoscopic polypectomy and even colectomy.

This review discusses two syndromes responsible for the majority of known genetic susceptibility to colorectal cancer, namely familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPPC). Because of effective prophylaxis, patients with FAP should now account for fewer than 0.2 per cent of colorectal cancers. However, HNPPC constitutes a significant proportion of the disease with 2–5 per cent of patients with colorectal cancer fulfilling criteria for definition of the syndrome of HNPPC. These stringent criteria were designed to select kindreds suitable for genetic research rather than provide diagnostic criteria relevant to clinical practice. As a consequence, the prevalence of HNPPC is likely to be underestimated when such empirical criteria are employed. Indeed, there is evidence to suggest that a substantial majority of large bowel tumours arise as a result of heritable susceptibility traits\(^1\). Complete characterization of susceptibility genes will allow the identification of individuals at high risk of developing colorectal cancer, such that genetic and clinical screening may be targeted. Understanding the function of such genes also offers hope for the rational development of therapeutic agents capable of interfering with the process of malignant transformation.

The APC gene and FAP

FAP results from germline mutations in the adenomatous polyposis coli (APC) gene. APC was localized to the long arm of chromosome 5 by genetic linkage analysis\(^2\), following a clue from cytogenetic studies which noted a chromosomal deletion in a patient with FAP. A positional cloning strategy was then adopted by a number of groups and eventually the gene was cloned, sequenced and mutations identified in FAP kindreds\(^3\). Somatic mutations in the APC gene have been detected in early adenomas\(^4,5\) and even in dysplastic aberrant crypt foci\(^6\) which are believed to be precursors of adenomas\(^7\). This suggests that the initiation of adenoma formation in FAP requires inactivation of both copies of APC. This is supported by research in the multiple intestinal neoplasia (Min) mouse model of human FAP in which the remaining copy of the APC gene is also inactivated in adenomas which arise in the murine intestine\(^7\). In sporadic, non-polyposis neoplasms, inactivation of APC has also been demonstrated in the majority of carcinomas and the very smallest premalignant adenomas\(^8,10\). The combination of the causative role of APC gene mutations in the FAP syndrome and the detection of APC mutations at the very earliest stages of colorectal neoplasia indicates the pivotal role of APC mutations in the genesis of colorectal cancer.

APC gene: structure and function

APC is a large gene consisting of 15 exons encoding a protein containing 2843 amino acid residues. Exons 1–14 are small, while exon 15 is large, accounting for 77 per
cent of the coding region. Antibodies to the APC protein have identified a 300 kDa cytoplasmic protein expressed in epithelial cells. This protein has been shown to associate with alpha- and beta-catenins which are known to bind to the cell surface molecule E-cadherin and are essential for its role in cellular adhesion. It is proposed that APC protein may affect the interaction between catenins and E-cadherin, thus influencing cellular adhesion and possibly intercellular communication. Further subcellular localization has demonstrated that wild type APC binds to microtubules and may be important in their formation. However, mutant APC product (missing the carboxyl terminal part of the molecule as a result of premature truncation) appears to lack this ability. The organization and structure of microtubules are vital to cell division and there is speculation that the association of APC with the catenins, and hence cadherin, may afford a direct intracellular line of communication from cell surface to microtubule formation. In view of the central role in the development of colorectal cancer, further study to elucidate APC gene function holds great potential for understanding the fundamental basis of tumour formation. In the future, it may be possible to augment or replace the function of APC by pharmacological means in those with germ line APC mutation or even prevent neoplastic change in the general population.

**APC mutational spectrum**

Inactivation of APC function is universal in both FAP and sporadic colorectal neoplasia. This occurs most frequently by mutations which create premature stop codons (i.e. the base triplet sequence TAG, TAA or TGA). This results in a premature signal for the end point of translation, resulting in the generation of a truncated protein product. The mutations are mainly deletions or insertions of short sequences, suggesting errors of replication rather than the action of mutagens. Single nucleotide substitutions (most commonly C to T transitions) account for around 40% of germline mutations already identified. In both FAP and sporadic colorectal neoplasms, 97% per cent of mutations occur in the 5' half of the gene and 60% per cent in a region of 600 codons within exon 15. Of the mutations responsible for FAP, 10-20% per cent are short deletions at codons 1061 and 1309. Mutation analysis of over 800 FAP kindreds has been reported in the world literature and the overall frequency of identifying the causative mutation is only 30 per cent, ranging from 21 per cent to 67% per cent. This relatively poor rate of mutation detection reflects a number of factors including the selection criteria for the diagnosis of FAP, the sensitivity and robustness of the mutation detection technique employed and the assiduousness of the search for mutations. In dedicated laboratories, employing multiple methods of APC mutation analysis, detection rates of 80 to 90% per cent may be possible.

Identification of the APC gene mutation responsible for the FAP syndrome in any particular family is time-consuming and laborious. This has led to the development of functional assays for APC mutation detection which capitalize on the core defect of causative APC mutations, namely premature truncation of the protein product. One such technique involves in vitro synthesis of APC protein from patient DNA. The presence of an APC gene mutation is indicated by the detection of truncated protein on electrophoresis. This protein truncation test is a rapid and efficient means of obtaining de facto evidence of the presence of a truncating and therefore causative mutation. The mutation can be fairly accurately localized using well characterized mutations as controls and then sequencing the relevant segment if so desired.

**Genotype-phenotype correlations in FAP**

It is becoming clear that the location of mutations within the APC gene has important clinical implications in terms of the colonic and extracolonic manifestations of FAP. Some appreciation of how mutation site could have an impact on clinical presentation can be gained from considering the molecular biology of the APC protein interaction. Normal APC protein molecules are believed to function as dimers. However, mutant APC protein has been shown to interact with normal APC, blocking normal APC function. The extent to which normal APC function is impaired may be related to the structure and residual ability of the mutant protein to form dimers. Severely truncated APC protein, due to mutations occurring in the most proximal part of the gene, may be incapable of binding with wild type APC. More distal 3' mutations allow translation of a larger protein structure containing the repeat sequences which permit heterodimer formation, resulting in impaired APC function. It may be predicted that a more aggressive phenotype will result from distal 3' mutations where overall APC function is impaired. Observational data bear this out, suggesting a functional boundary between codons 157 and 168 in exon 4 (Fig. 1). FAP families with an attenuated, usually late onset, disease have truncating mutations upstream of this boundary. In contrast, mutations in the region between codons 1250 and 1464 (exon 15) result in an extreme phenotype with profuse early onset polyps in the colon and rectum. These findings support the notion of a dominant-negative effect of truncating APC gene mutations, but other factors are certainly involved.

The phenotypic effects of mutations at different locations in APC are also demonstrated in the extent of extracolonic manifestations in FAP (Fig. 1). For some time, congenital hypertrophy of the retinal pigment epithelium (CHRPE) has been recognized as a common sign in FAP. Olschwang et al. defined a critical boundary in exon 9 with mutations 3' to this region associated with CHRPE lesions, whereas 5' mutations are associated with a normal retina. More recently, further data suggest that mutations occurring in the region between codons 1445 and 1578 in exon 15 do not induce the CHRPE phenotype (Fig. 1). Although CHRPE lesions are themselves harmless, these findings are of considerable clinical relevance. Their association with a particular spectrum of mutation will permit the presence or absence of CHRPE lesions to guide the search for causative mutations in FAP families, thereby limiting the laborious and time-consuming analyses and providing more rapid results.

An inverse relationship between the presence of CHRPE lesions and desmoid tumours was suggested by Hodgson et al., and recently Caspari et al. provided mutation data supporting this relationship. The majority of individuals with mutations between codons 1445 and 1578 showing no CHRPE lesions had desmoid tumours. Furthermore, these desmoids tended to be aggressive, often predated symptomatic colorectal disease and, in some cases, resulted directly in death. Clearly, the ability
to predict the clinical course in FAP on the basis of causative mutations will have significant effects on clinical management and patient counselling. Those with mutations predicted to result in uncontrollable rectal polyps (e.g. deletion at codon 1309) may be better served by restorative proctocolectomy with ileal pouch rather than by colectomy and ileorectal anastomosis. Antenatal diagnosis in FAP is a highly contentious area, but those individuals whose mutations are associated with severe, debilitating disease may wish to ensure that offspring are not affected. In the longer term, as the number of reported mutations increases and the quality of clinical data improves, complete elucidation of the clinical effects of different FAP gene mutations will be of great value, yielding clues to the functional significance of various domains of the APC protein and ultimately means by which therapeutic intervention may succeed.

Other genetic and environmental phenotypic modifiers are suggested by the fact that identical FAP gene mutations have been associated with diverse FAP phenotypes⁵⁴⁻⁵⁶. It is clear that environmental modulation does occur since a reduction in number and size of colorectal polyps can be achieved following treatment with the non-steroidal anti-inflammatory agent sulindac⁵⁷. In addition, alteration of the rectal milieu after colectomy and ileorectal anastomosis is associated with regression of rectal polyps⁵⁸. There is evidence from the Min mouse⁵⁹ model of FAP that phenotypic expression is modified by a locus⁶⁰ on mouse chromosome 4 corresponding to human chromosome 1p35-36. Interestingly, this chromosomal region has been noted to exhibit deletion or rearrangement in sporadic colorectal tumour tissue. Characterization of such a modifier gene may allow pharmacological modulation of the FAP phenotype.

Predictive genetic testing for FAP

As a direct consequence of identification of FAP, it became possible to perform accurate predictive testing for FAP. This can employ either genetic linkage analysis or mutation analysis. In linkage analysis, markers near the FAP gene are traced through a family to give an assessment of the risk of gene inheritance. Mutation analysis involves identification of the causative mutation within the FAP gene sequence of the affected individual. Linkage analysis has been shown to be of considerable practical value in FAP predictive testing⁶⁰ and the recent availability of highly polymorphic microsatellite markers has increased its predictive power. However, it suffers from several handicaps. The analysis requires DNA from two affected individuals in the family, which restricts its applicability. Early death of gene carriers, uncertain paternity within families and the fact that around 25% of FAP patients carry a new mutation with no family history, reduces the value of linkage studies in many clinical situations. Furthermore, by its nature, linkage analysis provides only an estimation of risk of being a gene carrier rather than a definitive diagnosis. Consequently, linkage analysis is reserved for those families in which the causative FAP mutation eludes detection.

Mutation analysis does not suffer the restrictions of linkage analysis and is undoubtedly the optimal means of predictive testing in FAP. It offers a firm diagnosis in members of a FAP family in whom the FAP mutation has been characterized. Those individuals proven to be non-gene carriers need never be enrolled in clinical screening programmes, resulting in reduction in patient distress and health care expenditure. Individuals found to carry an FAP mutation are provided with appropriate counselling. Prophylactic surgery can then be planned, taking account of the predicted phenotype of the mutation along with the patient's aspirations in education, partnerships and reproduction.

In the longer term, understanding the molecular mechanisms involved in FAP will lead to novel therapeutic approaches manipulating the disease process. While the benefits of prophylactic colectomy are unlikely to be supplanted, we may hope that the devastating effects of extracolonic disease such as desmoid tumours and upper gastrointestinal malignancy will be controlled. It is also reasonable to speculate that treatment of sporadic, non-FAP colorectal cancer will also be enhanced by such developments.
DNA mismatch repair genes in hereditary non-polyposis colorectal cancer

Phenotype and penetrance of HNPCC

Elucidation of the genetic basis of FAP was aided by the clear association of myriads of adenomas, subsequent cancer risk and the presence of a highly penetrant single gene disorder. In contrast, there are no clinical features which are pathognomonic of HNPCC other than early onset cancer with a familial aggregation. Four different genes have been implicated in the aetiology of HNPCC and penetrance is likely to be only 70–80 per cent (i.e. 20–30 per cent of individuals with a predisposing mutation may never develop cancer). Therefore, HNPCC presents a more elusive problem for the researcher and clinician. Cancers occurring as part of the HNPCC syndrome tend to arise in the proximal colon. In some families, colorectal cancer may be the only malignancy, while in others there is also segregation of uterine, ovarian, gastric, upper urinary tract, pancreatic, small bowel and skin cancers.

Fig. 2 shows an HNPCC kindred in which all six cases of colorectal cancer were of early onset, before 55 years of age. There are at least two unaffected presumptive obligate gene carriers in the second and third generations (II:4 and III:5), one of whom was elderly when she died from an unrelated condition and another who is as yet unaffected. This pedigree provides strong evidence that the penetrance of HNPCC gene mutations is lower than was widely believed. In addition, female gene carriers seem more protected from colorectal cancer than male gene carriers, suggesting the intriguing possibility of a sex-linked modifier gene. There is also evidence of cosegregation of early onset colorectal polyps and cases of colorectal, uterine and pancreatic cancers.

The prevalence of HNPCC is around 5 per cent; however, this is likely to be an underestimate since it is derived from the application of stringent criteria proposed by the International Collaborative Group on HNPCC. These require: three or more relatives with histologically proven colorectal cancer, one being a first degree relative of the other two; two or more generations affected; and at least one family member affected before age 50 years. These criteria will identify only large families where the gene defect is highly penetrant; therefore many small families will be excluded inappropriately. When these criteria are loosened, the prevalence of HNPCC will increase with a corresponding decrease in the apparent penetrance of the gene defect(s). The true penetrance of HNPCC genes can be determined only through an extensive search for mutations in colorectal cancer families ascertained on a population basis.

HNPCC results from mutations in mismatch repair genes

Four human genes have recently been identified which, when mutated, result in predisposition to colorectal cancer which is hereditary. These genes are human homologues of yeast and bacterial DNA repair genes and are known as hMSH2 on chromosome 2p16.3, hMLH1 on chromosome 3p10.5-11.3, hPMS1 on chromosome 2q31.1 and hPMS2 on chromosome 7q31. HMLH2 was originally localized to chromosome 2p by genetic linkage studies in two large unrelated HNPCC pedigrees. A subsequent study reported linkage to a marker on chromosome 3p in another family, providing evidence for genetic heterogeneity in HNPCC. Several apparently diverse areas of research subsequently converged to elucidate the underlying functional defect in HNPCC and provide clues to the genes responsible.

Studies on repeat sequences in genomic DNA from cancers in HNPCC identified widespread instability in short repetitive tracts, suggestive of defective DNA mismatch repair42-45. A similar pattern of DNA instability was described in Escherichia coli50 and yeast51-53 when mutations were induced experimentally in mismatch repair genes. This group of genes became a strong candidate for the human condition of HNPCC with the characteristic change in repeat DNA. Efforts to isolate the genes responsible for HNPCC focused on identifying human homologues of the yeast and bacterial DNA mismatch repair genes.

The first to be isolated was hMSH2 on chromosome

Fig. 2. A four generation family with hereditary non-polyposis colorectal cancer. Women are represented by circles and men by squares. Diagonal line denotes dead. Further definitions are given in the key. All six cases of colorectal cancer were early onset and four arose proximal to the splenic flexure. In this family, colorectal cancer segregates with colorectal polyps and malignant disease of the uterus and pancreas. This pedigree displays incomplete penetrance, where females II:4 and III:5 were asymptomatic gene carriers, passing the causative mutation to their children.

Fichel et al. exploited the fact that genes with such fundamental function frequently display homology between species. Using DNA sequence from yeast, the human homologue *hMSH2* was localized to chromosome 2p. Vogelstein's group (Leach et al.) used detailed linkage studies to localize the gene, then screened candidate genes in that region for the presence of germline mutations in HNPCC kindreds. A 2802 nucleotide complementary DNA from *hMSH2* was identified and mutations were shown to co-segregate with HNPCC in families linked to chromosome 2p.

One now become clear that defects in mismatch repair were responsible for HNPCC, other genes with similar sequence and known to be involved in mismatch repair were identified and mutations demonstrated to be responsible for the HNPCC in other families. *hMLH1* mutations were shown to be responsible for disease segregating with chromosome 3p markers, whereas, *hPMS1* and *hPMS2*, on chromosomes 2q and 7q respectively, account for a proportion of remaining HNPCC families. At this stage there are only limited mutation data on *hMLH1*, *hMLH2* and *hPMS* genes. These include nucleotide substitutions, short deletions and deletions of several hundred base pairs often with the creation of premature stop codons and generation of truncated protein.

More recently a further gene, GTBP, has been identified which is a member of the same family of genes as *hMSH2*. GTBP-protein forms a complex with *hMSH2* and this heterodimer, known as *hMutsX*, is essential for correcting subtle mismatch errors (Inguaggiato et al.). Mutations in GTBP are associated with defective mismatch repair in colorectal cancer cell lines; however, as yet, no germline mutations have been demonstrated in HNPCC. At present, the GTBP gene is the focus of considerable research and its contribution to colorectal carcinogenesis is likely to be elucidated in the near future.

There are at least four genes responsible for HNPCC and this has important implications for HNPCC population genetics and screening. The true penetrance of HNPCC gene mutations is unknown and HNPCC gene frequency may be substantially lower than the prevalence suggested by families selected through the International Collaborative Group criteria. Lynch et al. have calculated that the population gene frequency may be around 1 in 200. It is possible that some individuals may carry mutations in two or more predisposition genes. Thus, in a family with a characterized mutation in *hMSH2*, some individuals may be reassured as being non-carriers and discharged from follow-up when, in fact, they have a coincidental mutation in *hMLH1*. Such a possibility demands extreme caution before widespread introduction of genetic screening for HNPCC. At present, it seems wise to recommend continued colonoscopic screening, even in those at-risk individuals who are shown to be non-gene carriers, until further information is produced regarding penetrance and potential effects of abnormalities in more than one mismatch repair gene.

**DNA instability in HNPCC and 'sporadic' colorectal cancers**

DNA from cancers associated with HNPCC displays a characteristic instability in repetitive DNA sequence as a result of replication errors. Fig. 3 compares three cancers, one of which displays instability at a microsatellite repeat locus. Each allelic band in normal DNA contains the repeat sequence, and in patients B and C these are identical in both normal and tumour DNA. However, in patient A there is a shift in the allele pattern in tumour DNA, consistent with microsatellite instability and indicating a replication error in this tumour. Such changes are also detectable in around 1 in 6 apparently sporadic colorectal cancers. Such 'sporadic' tumours with DNA microsatellite instability tend to be right-sided, diploid, and have an inverse relationship with *p53* mutation features which are shared with cancers arising in HNPCC. Surprisingly, there is no significant age difference between patients with tumours exhibiting microsatellite instability and those whose tumours do not. However, 60 per cent of very young patients have been shown to have tumour DNA instability. The inverse relationship between microsatellite instability and aneuploidy/p53 mutation suggests that one of two mechanisms is required in colorectal cancer development. One involves defects in mismatch repair in diploid cells, while the other involves *p53* mutation and the development of aneuploidy.

Tumours with DNA instability may represent a useful marker of a subset of patients more likely to carry germline mismatch repair gene mutations. Liu et al. found that 15 per cent of cancers displayed DNA instability but detected germline mutations in *hMSH2* and *hMLH1* in only 10 per cent of these. This low frequency suggests that defects in mismatch repair systems may be involved. At present, it is appropriate to concentrate efforts on large cancer families and individuals with early onset

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**Fig. 3** Microsatellite instability in colorectal cancer DNA. Autoradiograph demonstrating microsatellite locus on chromosome 8 which is amplified by polymerase chain reaction and separated by polyacrylamide gel electrophoresis. Each allelic band in normal (N) DNA contains the repeat sequence (patients A and C are heterozygous, and B homozygous). In patients B and C, identical alleles are present in both normal and tumour (T) DNA. However, in patient A there is a shift in the allele pattern in tumour DNA, consistent with microsatellite instability at one allele and indicating a mismatch repair error in this tumour.
of colorectal cancer and there is every reason to hope that new approaches in management will lead to a reduction in cancer incidence in at-risk populations.

Other loci involved in genetic predisposition to colorectal cancer

Infidelity in other systems of DNA replication

Evidence of microsatellite instability has been demonstrated in over 60 per cent of patients presenting with colorectal cancer before the age of 35 years. This group would be expected to have constitutional predisposition to their disease and, indeed, extensive mutation analysis has revealed mismatch repair mutations in 60 per cent of cases. However, this presents the problem that the other 40 per cent are caused by as yet undetermined factors. It seems likely that a substantial proportion of such early onset cases are indeed due to genetic predisposition, but the genes involved remain to be identified. One candidate group involves the nucleotide-specific mismatch repair system which recognizes G-T mismatches. If uncorrected, these mismatches lead to a C to T transition. As previously discussed, such transitions are frequently seen in the APC gene in both FAP and sporadic colorectal tumours. This raises the intriguing possibility that many of the somatic changes that are involved in the very genesis of colorectal cancer and indeed of FAP are caused by defects in DNA repair systems.

Adenomas in patients carrying HNPCC gene mutations display microsatellite instability, suggesting that mismatch repair defects are important early events in colorectal carcinogenesis. Tumour formation requires inactivation of both copies of a given mismatch repair gene, one copy by germline mutation and the other by somatic (acquired) mutation. Colorectal cancer cell lines exhibiting microsatellite instability have a continuing defect in mismatch repair, which persists through transformation, leading to progressive alterations in repetitive DNA sequences. It seems reasonable to speculate that in human cancers the functional defect in mismatch repair will also persist, leading to further mutations and genetic derangement.

There is substantial evidence to suggest that inactivation of both copies of a DNA repair gene by mutation, or loss of one gene copy, is required for tumour formation. However, it is also clear that a subtle phenotype is associated with heterozygous germline mutations, since abnormal colonic crypt cell production rate and DNA repair have been reported in the apparently normal colorectal mucosa of affected and at-risk HNPCC family members. In addition, it has been shown that a subset of patients with germline mismatch repair gene mutations do, in fact, have an increased mutation rate in their normal tissues. This is of considerable importance because such individuals may develop mutations in many tissues, but develop relatively few tumours. It may be that mutations must occur in important genes, such as APC, before a cancer can develop and it seems likely that environmental influences must have a major involvement in this process.

Research into the molecular basis of HNPCC has proceeded at a staggering pace and it will take some time for these developments to be appreciated and incorporated into routine clinical practice. However, HNPCC accounts for a significant proportion of colorectal cancer and there is every reason to hope that new approaches in management will lead to a reduction in cancer incidence in at-risk populations.

The ras genes

Activated proto-oncogenes have been shown to play a major role in many human cancers. The ras gene family are cytoplasmic proto-oncogenes with signal transduction functions. Activating mutations in codons 12 and 13 of Kirsten ras occur in 50 per cent of colorectal cancers and adenomas greater than 1 cm in diameter. The frequency of K-ras mutations is much lower in adenomas less than 1 cm, which suggests that this oncogene is involved in the progression of adenoma to carcinoma. K-ras expression, although high in primary carcinomas, tends to be lower in clinically advanced cases, indicating that when the tumour progresses to a certain stage ras activation is no longer required.

There is some evidence to suggest that ras genes may be involved in colorectal cancer susceptibility. A meta-analysis of all published studies identified rare Harvey ras alleles as likely predisposition factors in cancer and from their findings the authors propose that 1 in 11 colorectal cancers occurs as a result of inheritance of a predisposing H-ras allele. It is possible that inheritance of a rare H-ras allele may indicate an increased risk of colorectal cancer which may even interact with predisposition due to inheritance of a hMSH2 or other DNA repair gene mutation.

Perspective

This review has summarized the more clinically relevant aspects of current research in molecular genetics of colorectal cancer, with particular regard to HNPCC and FAP. The contributions of other important genes involved in colorectal cancer, including p53 and DCC, have not
been considered but the events described above must be taken in the context of alterations in these genes\textsuperscript{24}. The central roles of APC and the mutator genes in colorectal cancer are clear. It is even possible that changes in other cancer genes may be induced by defects in mismatch repair.

The clinical relevance of genetic studies of the APC gene in FAP is now beyond doubt. However, unlike FAP, the morbidity could be considerable and may last for years,\textsuperscript{1,12} and involves the targeting of long-term surveillance measures. Thus, microsatellite instability could be used as a biomarker indicating an increased likelihood that the cancer host carries an inherited HNPCC gene mutation. Those shown to have germline mutations could be counselled with a view to offering clinical and genetic screening to their relatives. This would allow efficient targeting of long-term colonoscopic screening to those who most need it. At present, this screening is recommended every 3 years, but recent investigations in high-risk individuals support a more intensive approach with colonoscopy every 1–2 years.\textsuperscript{7,8} As such screening may last up to 50 years, the burden in terms of cost and patient morbidity could be considerable and these difficulties may be addressed only by prophylactic measures. However, unlike FAP, the absence of a definite premalignant phenotype in HNPCC and the present uncertainty over gene penetrance make prophylactic colectomy unappealing. Fortunately, it is likely that these problems will be resolved in the near future, leading to novel approaches in the management of HNPCC and indeed other cancers.

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Detailed physical and deletion mapping of 8p with isolation of YAC clones from tumour suppressor loci involved in colorectal cancer

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Loss of heterozygosity (LOH) of markers at chromosome 8p is frequently noted in many different tumour types, including colorectal cancer. Numerous investigations indicate the presence of more than one tumour suppressor gene (TSG) located on 8p. In this study, we describe a detailed LOH map in colorectal cancer and relate this to physical mapping data from reduced radiation 8p hybrids, yeast artificial chromosome (YAC) co-localisation of markers and fluorescence in situ hybridisation data. These data indicate the presence of two regions harbouring putative TSG's between the polymorphic markers for the LPL gene-D8S298 (~4 Mb) and the markers D8S136-D8S137 (~8 Mb). Yeast Artificial Chromosomes (YAC) have been isolated from these regions of interest to aid the localisation of the putative TSG's.

Keywords: chromosome 8p; colorectal cancer; mapping; YAC isolation; TSG

Introduction

The development of colorectal cancer is associated with the accumulation of abnormalities in specific genes. These include the inactivation of APC gene, and mutational activation of the Ki-ras proto-oncogene as early events, along with p53 and DCC tumour suppressor gene defects later in the progression to malignancy. Defective mismatch repair has been described in a proportion of cancers, resulting in widespread genomic instability and has been attributed to underlying mutations in mismatch repair genes (Aaltonen et al., 1993; Ionov et al., 1993; Sen Gupta et al., 1993). Non-random genetic events have been described at other chromosome locations, suggesting that other loci may be important in the development of this disease.

There is strong evidence to suggest a tumour suppressor gene locus on chromosome 8p. Frequent non-random loss of heterozygosity (LOH) at polymorphic markers mapping to 8p has been reported in colorectal cancer (van der Bosch et al., 1992) and several other cancers including lung, liver, bladder, prostate, head and neck, breast and kidney (Ohata et al., 1993; Emi et al., 1992, 1993; Knowles et al., 1993; Bergerheim et al., 1991; Li et al., 1994; Pykett et al., 1994; Schoenberg et al., 1995). Several lines of investigation suggest that the gene on 8p is involved in the later stages of colorectal carcinogenesis. Adenomas, the benign precursors of colorectal carcinoma, rarely show 8p loss (Cunningham et al., 1994) and LOH at 8p has been shown to be associated with micro-invasion (Keleman et al., 1994). Also suppression of metastasis in rat prostate cancer has been induced by introduction of human chromosome 8 (Ichikawa et al., 1994). Together, these data strongly suggest that 8p harbours a tumour suppressor gene or genes involved in many epithelial malignancies. Localisation of the putative loci on 8p has proved difficult for several reasons. Many investigators have employed different, often poorly localised polymorphic markers in their studies. Also, a number of different tumour types have been analysed for the involvement of 8p and the validity of extrapolating between cancers is uncertain. Finally, there are now substantial data supporting the presence of two TSG loci (Fujiwara et al., 1993; Yaremko et al., 1994), which may account for the discrepancies in earlier reports. Studies in colorectal cancer have suggested a region within 8p22-p23.1 and a more centromeric region of 8p21.3-p11.2. These localisations are supported by findings in other cancers. More recently Fujiwara et al. (1994) indicate a common region of deletion in colon, lung and liver cancers within the 8p22-p21.3 area and the Japanese group (Fujiwara et al., 1995), have now proposed a candidate gene, PRLTS, isolated from this region, although the evidence of mutation in the residual allele of cancers is slender. The data in prostate cancer also indicate two regions of 8p loss, in 8p22 (Bova et al., 1994; Macoska et al., 1994; Cher et al., 1994; Matsysma et al., 1994) and a more proximal region between the markers D8S87 and D8S133 (Trapman et al., 1994). Bladder cancer and breast cancer cell lines show a more proximal area of deletion, 8p21-q11.2 (Knowles et al., 1993) and 8p21-p12 (Pykett et al., 1994) respectively.

In this paper we report detailed localisation data on the region of smallest deletional overlap in 8p22-8p21, derived from LOH studies in a series of 119 sporadic colorectal cancers. We have isolated yeast artificial chromosome (YAC) clones from this region, allowing construction of a physical map from radiation hybrid mapping and FISH analysis. These mapping data and YAC clones will aid in the final localisation of the target tumour suppressor genes.
Results

Initial 8p LOH studies in colorectal cancer

As a first screen to delineate a region of interest, 14 loci were assessed in LOH studies on chromosome 8, 13 on 8p and 1 on the 8q arm. Five markers were restriction fragment length polymorphisms (RFLP) and the other nine dinucleotide repeat polymorphisms. Details of these markers can be found in the Methods section. Of 119 cancers, 99 were found to be informative at one or more loci with 59 (59.6%) of these demonstrating LOH. Figure 1 shows the percentage of informative cases that demonstrated LOH for each marker. Peak losses occur at the LPL gene and the D8S136 marker. The most telomeric marker also showed loss but this is likely to be due to telomeric shortening (Hastie et al., 1993). Due to the increased frequency of LOH at D8S136 and LPL, our efforts were concentrated on physical mapping of this interval.

Figure 1 Percentage loss of heterozygosity for each of the 14 polymorphic markers employed in this series. pMS502 was assigned to chromosome 8q but the rest are all on 8p

Physical mapping of (CA)n markers on a radiation hybrid panel

Several (CA)n repeat polymorphic markers were mapped on to a panel of somatic cell hybrids and reduced chromosome 8 radiation hybrids encompassing this region (Wagner et al., 1991; Sapru et al., 1994). Two human/hamster somatic cell hybrids were employed. 8HL12 containing the whole of chromosome 8p and including intervals A–C as described by Wagner et al. (1991) and also 20xP0455-2 containing intervals B and C. Radiation hybrids 2A6, 2B1, 2N2, 2Q1, 2R1 and 2T1 were obtained from Michael Wagner (described in Sapru et al., 1994) and used for mapping of markers.

Physical mapping data are shown in Table 1 and incorporate published data (Wood et al., 1993; Sapru et al., 1994) where our own marker order was ambiguous. Genetic linkage has shown that D8S283, D8S278, D8S259 and D8S87 are all very close with zero recombination (Weissenbach et al., 1992; Wood et al., 1993) and so the order of these markers is not completely established. The NEFL gene marker is present in the hybrid 2B1 but not 2A6 and therefore would be placed telomeric to D8S124, a marker used by Sapru et al. (1994).

Primers were designed from the newly isolated candidate gene, PRLTS, (Fujimura et al., 1995) and used for investigation with the hybrids described above. The gene was present in both human/hamster hybrids 20xP0455-2 and 8HL12 and also in the radiation hybrids 2A6 and 2B1 but not in the hybrids ID1, IE1, 2F4, 2N2, 2R1, 2Q1 and 2T1.

Localisation of the target gene

Having delineated the region likely to harbour the tumour suppressor gene, we concentrated on this area using the physical mapping data from the radiation hybrid panel in further detailed LOH studies. A consensus map from our own studies and other linkage data is shown in Figure 2a.

<table>
<thead>
<tr>
<th>Locus name</th>
<th>Interval*</th>
<th>8HL12</th>
<th>20xP</th>
<th>2A6</th>
<th>2B1</th>
<th>2N2</th>
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<th>2R1</th>
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Key: + = presence of loci, -- = absence of loci. Neither indicates not tested. *Refers to intervals defined by Wagner et al. (1991)
Loss at all informative markers was noted for many cases (40 out of 99), however 19 tumours had LOH patterns which allowed us to delineate small regions of interest on 8p. Tumours showing microsatellite instability were excluded from critical localisation, to remove the possibility of mis-interpretation of data. Two areas of interstitial loss were clearly identified, one between the LPL gene and D8S298 markers and the other between the D8S136 and D8S137 markers. Figure 2b shows the critical cases delineating the common regions of deletion and photographs of example autorads are given in Figure 3. Tumour 115 shows two areas of loss including loss of an LPL gene marker. Tumour 181 has only one deletion – that of the D8S298 marker – while tumour 117 has only one allele of the NEFL gene marker. These data indicate two areas of deletion, a distal area from the LPL gene to the D8S298 marker and a proximal region between the D8S136 and D8S137 markers. To delineate the regions more closely, we isolated YAC clones using (CA)n repeat markers as sequence tagged sites (STS's) from the LPL gene to D8S137.

Isolation of yeast artificial chromosomes (YAC) and ordering by FISH analysis

A YAC library (HGMP/IIC, UK) was screened by PCR from pooled colonies, using the 8p markers from the regions of interest. The following markers were used as sequence tagged sites (STS) – LPL-BstNI/MnlI polymorphism, D8S133, D8S280, D8S258, D8S282, D8S298, D8S136, NEFL gene polymorphism, D8S137 and D8S278. On average, three out of 40 primary pools were positive for each marker, three out of nine secondary pools and two of the 20 tertiary pools, therefore generally two clones were produced for each marker. Each individual YAC clone was isolated and mapped by FISH. Ten YAC’s were mapped to chromosome 8p, with six mapping to chromosome 8p alone, LPL, D8S282, D8S258, D8S298, D8S278 and D8S137. These six YAC clones (which were not chimaeric for other chromosomes) were ordered with respect to a centromeric probe. The final order by FISH analysis was centomere-D8S278-D8S137-D8S282-D8S258-D8S298-LPL. YAC size and respective STS’s are given in Table 2.
that described in tumours was (8p21.3-8p12). During LOH delineated is may distance of genetic gene. The telomeric et (Bova et al., 1993; Sen Gupta et al., 1993); such tumours were excluded from LOH analysis to avoid ambiguities in defining the deleted regions.

To improve mapping data in the regions of interest, 19 mini-satellite loci from chromosome 8p have been mapped on to radiation hybrids and 17 YACs have been isolated. The results concur broadly with published reports except for two discrepancies. Data from radiation hybrids suggest the order centromere-D8S87-D8S278-D8S283 which is in contrast to linkage studies. We do not know how to interpret this discrepancy, as all these markers are in very close proximity and radiation hybrids are subject to rearrangement. The isolation of a YAC containing the D8S278 marker may confirm its position on the 8p arm. Another anomaly was the position of the YAC isolated using the D8S298 marker, which we have placed telomeric to D8S258 by FISH. Bookstein et al. (1994) have isolated YACs from 8p22 and obtained one which contains both the LPL gene and D8S258 marker, suggesting that D8S258 is telomeric to D8S298. However, our FISH data indicate that all three YACs, D8S298, D8S282 and D8S258 are actually very close in proximity. The physical distance between LPL and D8S258 was found to be much smaller than would be predicted by its genetic distance in cM and so the distance between D8S258 and D8S298 may also be smaller than the maximum of 3 cM obtained from recombination data.

Our data indicate the presence of more than one tumour suppressor gene on the short arm of chromosome 8, which would be consistent with the high frequency of loss in many different cancers. Several of our tumours exhibited two regions of LOH while composite data also suggest two target regions. One area lies between the LPL gene and the D8S298 marker whilst a second, more proximal location is near the NEFL gene (8p21). The more distal, 8p22, location has also been shown to be of importance in prostate cancer (Bova et al., 1993; Cher et al., 1994; Macoska et al., 1994; Matsyama et al., 1994) and a YAC contig has now been constructed for this band (Bookstein et al., 1994). The second important region around the NEFL gene, agrees with
a recent report by Trapman et al. (1994), who identified an area between D8S87 and D8S133, markers also used in this study.

Some candidate genes critical to these deletions have been suggested. Wang et al. (1992) reported the polymerase β gene as being a site for frequent mutations in six colorectal cancer cell lines. However the same 8p deletion has been described in patients with Werners’ syndrome (Sadakane et al., 1994) and the specific role of pol β in colorectal carcinogenesis is uncertain. LOH data presented here strongly suggest that pol β is not the target tumour suppressor gene, as it lies outwith the maximum region of deletion. Fujiwara et al. (1995) have recently published evidence of the isolation of a candidate tumour suppressor gene (PRLTS) from the region 8p22-21.3. However, only one colorectal carcinoma and two hepatocellular carcinomas contained any somatic mutation in this gene, which throws open the possibility that PRLTS is not the target gene. Our radiation hybrid data indicate that the PRLTS gene is in the vicinity of the D8S124 marker, but this is more proximal than one would expect, as the markers used by Fujiwara et al. are thought to be distal to LPL which is positioned at the 8p22/2p21.3 border (data not shown). Another possible area of overlap of these hybrids is between the markers D8S265 and D8S206, although this would appear distal to the area indicated by the group.

These studies have contributed to physical mapping data from the 8p22-8p12 region and we have further refined two regions of LOH on 8p, strongly suggestive of TSGs important in the later stages of colorectal carcinogenesis. We have isolated YACs from 8p which will allow construction of a contig of the region and due to their close proximity to the area of maximal LOH, may even harbour the gene of interest. Hopefully this will allow definitive isolation of the target tumour suppressor gene which is likely to be involved in tumorigenesis in breast, prostate, kidney, lung, liver, head and neck, as well as colorectal cancer.

Materials and methods

Analysis of DNA for LOH

DNA was extracted from 119 colorectal cancers and matched normal tissues as described (see Cunningham et al., 1993). Details of the RFLP markers for D8S165, LPL, POLβ, D8S162 and the (CA), repeat markers for D8S201, LPL, ANKI1 have already been described (see Cunningham et al., 1993). The (CA), repeat markers D8S265, D8S261, D8S258, D8S280, D8S282, D8S298, D8S259, D8S283, D8S278, D8S255 and D8S268 were described by Weisenbach et al. (1992); D8S133, D8S136, D8S137 have been described by Steinbrueck et al. (1992) and the NEFL (CA), repeat marker was from Rogaev et al. (1992).

Tumours which displayed microsatellite instability were not included in localising the critical areas of deletion, due to the difficulty in interpreting the data and the potential for mis-interpreting data.

Mapping of CAₙ markers onto hybrid panel

Somatic cell hybrids and radiation hybrids were obtained from Michael Wagner (Wagner et al., 1991, Supru et al., 1994). The hybrids were screened with the various CAₙ repeat markers using PCR, the production of a PCR product indicating its presence of that marker in the hybrid.

Screening of hybrids for the presence of the PRLTS gene was performed using primers from exons 2 and 7, the sequences of which were obtained from Fujinawa et al. (1995). Control DNA was consistently used to indicate the correct size PCR product.

YAC library screening

A YAC library (ICI, UK) was obtained from the Human Genome Mapping Project Resource Centre. This consisted of 40 pools of YAC clones which could be screened by PCR. Positive pools underwent further screening of the associated 2° pools and then 3° pools, until a single yeast clone was finally obtained for that marker. PCR reactions were performed using 200 mM of each nucleotide, 0.625 units of Taq polymerase (Cetus), 250 mM MgCl₂, 2.5 µl 10× buffer (Cetus), 50 ng of each primer and 0.2 mM β-mercaptoethanol. The reactions were carried out in a Hybaid Omnimere (Hybaid) and consisted of one cycle of 95°C for 1 min, 35 cycles of 95°C for 30 s, 55°C for 45 s, 72°C for 1 min and one cycle of 72°C for 5 min. Products were separated by electrophoresis on 3% agarose, stained with ethidium bromide and visualised using u.v. light.

YAC isolation

The YAC was isolated by preparing yeast chromosomes. The yeast strain was grown to late log phase at 30°C, in 100 ml of selective AMC media with 2% glucose added. The pelleted cells were washed twice in 0.05 M EDTA pH 7.5 and then resuspended in 3 ml. To this was added 1 ml of SCE (1 m sorbitol, 0.1 M Na citrate, 0.06 M EDTA pH 7.0), 50 µl β-mercaptoethanol, 1 mg zymolase and 5 ml of 1% low melting point agarose (prepared in 0.125 M EDTA pH 7.5). This was aliquoted into plug molds, allowed to set and incubated overnight at 37°C in 0.45 M EDTA pH 9.0, 10 mM Tris- HCl pH 8.0, 7.5% β-mercaptoethanol, to allow spheroplasts to form. The plugs were then transferred to NDS (0.5 M EDTA pH 9.5, 10 mM Tris-HCl pH 9.5, 1% lauryl sarcosine), proteinase K added to 1 mg/ml and incubated overnight at 50°C. plugs were then stored in fresh NDS.

YAC characterisation

The YAC DNA was separated from the other yeast chromosomes by Pulsed Field Gel Electrophoresis (PFGE). The plugs were loaded onto a 1% 0.5×TAE gel and subjected to 60–120s switch times over 40 h at 150 V on a CHEF gel apparatus (BioRad). The gel was stained with ethidium bromide and visualised by u.v. light, the YAC can be seen as an extra chromosome when compared to yeast standards. For any YAC which was not clearly visible, the gel was treated with 1/40th dilution HCl and a Southern blot analysis performed (Maniatis et al., 1982). Probes of the (CA), repeat marker PCR products were labelled by the random primer method, with [-32P]dCTP using the Random Prime Labelling Kit (Boehringer Mannheim). Hybridisations were performed at 65°C in 6× SSC with 10% Dextran Sulphate. Filters were washed to a stringency of 0.1× SSC at 65°C and exposed to Kodak film for 2–5 days at –70°C.

Once the size and position of the YAC was known, a preparative gel was performed and the chromosome cut out of the gel. YAC DNA was prepared as described in Fantes et al. (1995) and labelled by Nick translation with Bio-16-dUTP (Boehringer) and digoxigenin-11-dUTP (Boehringer), and hybridised to human diploid male fibroblasts as described.
in Fantes et al. (1992). Hybridisation signals were detected with successive layers of FITC-conjugated anti-digoxigenin, FITC-conjugated anti-sheep plus avidin-Texas Red, biotinylated anti-avidin and avidin-Texas Red. Chimerism of the individual YAC clones was excluded by detailed assessment of all the chromosomes during FISH.

References


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Microsatellite instability and the role of hMSH2 in sporadic colorectal cancer

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Microsatellite instability (MSI) occurs in most tumours from patients with hereditary non-polyposis colorectal cancer (HNPCC) and in around 17% of sporadic colorectal cancers. Germline defects in mismatch repair (MMR) genes are responsible for the majority of large HNPCC families, with hMSH2 accounting for at least 50%. MMR gene defects also occur in a small proportion of sporadic colorectal tumours with MSI. Here we report a systematic analysis of mismatch repair deficiency in 215 Scottish patients with sporadic colorectal tumours. We found that 16.4% of tumours exhibited MSI; survival analysis by Cox proportional hazards method showed a substantial survival advantage for patients with tumours showing MSI, independent of other prognostic factors. Tumours with MSI were screened for hMSH2 mutations and although 61% were found to have alterations, of these only 1/24 was exonic. The majority of these changes were reductions in length at intronic mononucleotide tracts and we postulate that these alterations are the result of a genetic defect elsewhere, although they may compromise hMSH2 function as a second step in tumourigenesis. Our findings indicate that instability confers an improved prognosis in colorectal cancer and, despite the fact that these two groups of tumours share similar biological characteristics, the genetic basis of HNPCC and sporadic colorectal cancer with MSI is different.

Keywords: replication errors; sporadic colorectal cancer; hMSH2; prognosis

Introduction

Tumours from hereditary non-polyposis colorectal cancer (HNPCC) patients are characterised by the presence of multiple replication errors (Ionov et al., 1993; Aaltonen et al., 1993). As a consequence of defects in the mismatch repair pathway in such tumours, mutations are unpaired. Within microsatellite sequences such replication slippage is manifest as an alteration in length, termed microsatellite instability (MSI). MSI has also been demonstrated in sporadic colorectal cancers (Thibodeau et al., 1993) and subsequently in many other cancers, including pancreatic (Han et al., 1993), gastric (Han et al., 1993; Peltomaki et al., 1993; Mironov et al., 1994; Rhyu et al., 1994; Chong et al., 1994), prostatic (Gao et al., 1994), endometrial (Risinger et al., 1993; Peltomaki et al., 1993), breast (Patel et al., 1994), non-small cell (Shridhar et al., 1994; Fong et al., 1995) and small cell lung cancers (Merlo et al., 1994) and in Muir-Torre syndrome (Honchel et al., 1994).

Up to 86% of tumours from HNPCC patients exhibit instability at multiple microsatellite sites (Aaltonen et al., 1993, 1994; Wu et al., 1994). A significant, although much lower, proportion (in most studies around 17%) of sporadic colorectal carcinomas show shifts in allele size in at least one microsatellite locus relative to their corresponding normal tissue (Aaltonen et al., 1993, 1994; Ionov et al., 1993; Lothe et al., 1993; Young et al., 1993; Kim et al., 1994), although others have found the frequency to be as high as 28% in the population studied (Thibodeau et al., 1993). The prevalence of tumour MSI is greater when cases with some family history of cancer are included (Lothe et al., 1993). Sporadic tumours with microsatellite instability have characteristics in common with HNPCC tumours. They are usually located in the proximal colon (Ionov et al., 1993; Thibodeau et al., 1993; Lothe et al., 1993; Aaltonen et al., 1993; Kim et al., 1994), associated with extracellular mucin production (Kim et al., 1994), poor differentiation (Ionov et al., 1993; Lothe et al., 1993; Kim et al., 1994) and diploidy (Aaltonen et al., 1993; Lothe et al., 1993). They also show a negative correlation with mutation (Ionov et al., 1993) or immunocytochemical stabilisation of p53 (Kim et al., 1994) and Ki-ras mutation (Ionov et al., 1993). In addition, loss of heterozygosity at known tumour suppressor gene loci on chromosomes 5q, 17p and 18q is relatively less frequent in tumours with MSI compared to those without (Thibodeau et al., 1993). Finally, these tumours show a tendency towards increased patient survival (Thibodeau et al., 1993; Lothe et al., 1993).

Recently, a number of genes that participate in human mismatch repair have been identified, namely hMSH2 (Fishel et al., 1993; Leach et al., 1993), hMLH1 (Bronner et al., 1994; Papadopoulos et al., 1994), hPMS1 and hPMS2 (Nicolaides et al., 1994) and p16/GTBP (Palombo et al., 1995; Drummond et al., 1995; Papadopoulos et al., 1995). The roles of these genes were inferred from their homology with bacterial and yeast mismatch repair genes (Prolla et al., 1994), and germ-line mutations of all except p16/GTBP have been found in HNPCC families. Defects in the mismatch repair pathway account for the vast majority of HNPCC families; mutations in hMSH2 are thought to account for at least 50% of kindreds.

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(Liu et al., 1994) and mutations in hMLH1 for up to 33% (Han et al., 1995; Liu et al., 1996). Mutations in these genes may also play a role in the development of sporadic colorectal cancer. However, mutations in mismatch repair genes have so far been demonstrated in only a small percentage of sporadic cancers or cell lines derived from colorectal carcinomas (Liu et al., 1995; Borresen et al., 1995).

We sought to clarify further the prevalence of mismatch repair deficiency in a large unselected series of 215 Scottish sporadic colorectal cancer cases. We confirm the previously reported characteristics of such tumours: their predilection for origin in the proximal colon, tendency for mucinous histology, negative correlation with abnormalities in p53 expression and substantially better prognosis than tumours in patients without MSI. To delineate the role of a specific mismatch repair gene known to be important in hereditary cancers sharing these characteristics, we carried out an extensive mutation analysis of hMSH2 on selected cases. Our results revealed a substantial difference in the nature and incidence of mutations in this gene between sporadic and hereditary (HNPPC) colorectal cancers, emphasising that the mechanism underlying tumourigenesis is different in these two groups.

Results

Microsatellite instability analysis

We analysed 215 patients diagnosed as having primary sporadic colorectal cancer. This group included patients with more than one tumour and those with cancer at other sites (either synchronous or asynchronous) who did not fit the criteria for HNPPC (Percesepe et al., 1994). When patients had more than one carcinoma, each lesion was analysed separately if DNA was available. Thus, a total of 219 carcinomas were analysed, four patients each having two synchronous cancers.

These 219 cancers were staged as follows: 25 Duke's Stage A, 100 Duke's Stage B and 94 Duke's Stage CD (for these purposes stages C and D were amalgamated as information regarding distant metastasis was not always available). The average age was 70 years, range 28–95 years, with one patient of unknown age. The group comprised 46.5% (100/215) males and 53.5% (115/215) females.

All samples were analysed for genetic instability at a minimum of four microsatellite loci. 16.4% (36/219) exhibited instability at one or more loci. The average age of this group was 68.75 years, range 37–93 years. This is not significantly different from the age of the group without evidence of MSI (70.02 years, range 28–95). During the course of this study we identified a polyadenine tract located in the 5' region of hMSH2 intron 5 which exhibited replication errors in 58% (19/33) of samples showing microsatellite instability at another loci. When instability at this locus was included, we identified three additional patients with evidence of MSI, one of which appeared to show instability at another locus but despite repetition had not given an unequivocal result. Thus, in total, 17.8% (39/219) of sporadic colorectal cancers in this series exhibited MSI.

When we applied the more stringent requirement of instability at two or more loci (including intron 5 of hMSH2), a criterion often used to define a replication error positive (RER+) phenotype (Alstonen et al., 1993; Parsons et al., 1993), the incidence of MSI was 10.5% (23/219).

The relationship between microsatellite instability and site of tumour, p53 stabilisation and Duke's stage was examined (Table 3). We found 59% (23/39) of lesions with instability at one or more loci were in the proximal or right side of the bowel (ie proximal to and including the splenic flexure). This was significantly different ($\chi^2=7.73$, $P<0.01$) from the distribution of those without MSI (non-shifters), where 35% (63/180) were right-sided, similar to the general distribution of all colorectal cancers in the UK of which 28% are right-sided (Cancer Research Campaign, 1993). Of lesions with at least two unstable sites, 74% were proximal ($\chi^2=12.93$, $P<0.001$). When proximal tumours alone were considered, 27% (23/86) demonstrated microsatellite instability at one or more loci, as compared with only 12% (16/133) of distal cancers.

When we looked for evidence of p53 stabilisation in our series (scored positive if any tumour nucleus in the section stained (Purdie et al., 1991)) 56.4% (22/39) of tumours with MSI at one or more loci were positive for p53 protein, which was not significantly different from those without MSI, in which 60% (108/180) had evidence of stabilised p53 ($\chi^2=0.43$, $P>0.5$). However, a significant inverse relationship was seen between MSI status and p53 staining in tumours with instability at more than one loci ($\chi^2=4.43$, $P<0.05$). Fifty-nine percent of our total population had positively stained nuclei. When the site of lesion was considered 54% of proximal tumours and 64.5% of distal tumours had evidence of stabilised p53. We compared these results to data generated from the analysis of a subset of these cases for loss of heterozygosity (LOH) at 17p using three restriction fragment length polymorphic (RFLP) markers intragenic to p53 (Purdie et al., in preparation). 125 lesions were analysed for intragenic loss of p53, of which 49 were informative at one or more loci. Of these informative cases, 19 had allele loss involving p53 and in three (16%) of these we demonstrated MSI, all located on the left side of the bowel. Of the 30 lesions where heterozygosity in p53 appeared to be retained, nine (30%) exhibited MSI; of these, six were located on the right side of the colon. Thus there is an apparent trend for tumours that exhibit MSI to retain heterozygosity in p53, but this difference was not statistically significant. There was no significant difference between Duke's Stage and MSI status in tumours with MSI at one or more loci ($\chi^2=2.37$, $P>0.3$) or at more than one loci ($\chi^2=2.028$, $P>0.3$) (Table 3), although lesions with MSI appeared to be less common within the Duke's A group.

Histological examination of the 39 tumours with MSI revealed that 41% (16/39) of these tumours were mucin-secreting. This is a much higher proportion than in the unselected Scottish population of sporadic colorectal cancers; in such a series overlapping with the present study we observed only 17% (59/346) mucinous carcinomas.
Survival analysis

Survival analysis was carried out on 169 patients for whom information was available on all factors (46 excluded) using the Cox proportional hazards method. The most significant predictor of survival was Duke’s stage ($\chi^2=38.4$, $P<0.0001$) followed by age ($\chi^2=9.11$, $P=0.0025$), MSI ($\chi^2=7.83$, $P=0.0051$) and side ($\chi^2=6.35$, $P=0.012$). The hazard ratio of patients with tumours showing MSI to those without was estimated to be 0.39 (95% C.I. 0.19–0.82). Thus, at any point in time after diagnosis, a patient whose tumour showed MSI had a risk of dying which was estimated to be about 39% of that of a patient with a tumour without MSI, even after allowing for the influence of other predictive factors. The results are presented as an estimated survival curve (Figure 3). The effects of p53 immunohistochemical status and sex were unrelated to prognosis.

In order to eliminate any possibility that tumour side is a consequence of MSI status the test was repeated excluding ‘side’. The result showed negligible influence on the predicted survival of patients whose tumours had MSI to those without [hazard ratio of 0.37 (95% C.I. 0.18–0.77)]. No evidence was found of interactions between Duke’s stage, age, MSI status, side and sex.

Mutation analysis of hMSH2

We analysed 36 tumours with microsatellite instability at one or more loci (including hMSH2 intron 5) for mutations in the hMSH2 gene (Table 4). In addition, 32 tumours without MSI were analysed as controls. We found two tumours which each had a single exonic germ-line mutation. Both of these were missense mutations and both occurred in the control group. Only one tumour with MSI was found to carry any exonic mutation, a frameshift creating a new stop codon. This tumour also showed a reduction in length of a polyadenine tract located three nucleotides downstream of the 3’ end of exon 5, a change which was found in an additional 21 tumours. Of these 21 tumours the length change was somatic in 20 cases. These alterations were initially detected by abnormal banding on SSCP gels, but were later found to be easily detectable on denaturing polyacrylamide gels. Abnormal allele lengths ranged from 10 to 23 bases compared with matching normal tissue which had 25 adenine nucleotides in this tract, which differs from the previously published sequence of 26 residues (Liu et al., 1994).

A further tumour with MSI had an intronic transition, an alteration of unknown function, as well as the length reduction in intron 5 and a second reduction in the length of intron 1. Seven other tumours had this somatic reduction in the length of the polythymine tract located 3’ in intron 1, predicted to be part of the splice site. Whereas in normal tissue this tract extends for 12 or 13 nucleotides, this was reduced to as few as nine base pairs in some tumours. Analysis of cancer-free control samples demonstrated that 12 or 13 bases was a normal polymorphic variation. Removal of this polyadenylate tract by PvuI digestion and repetition of SSCP on the residual 176 nucleotide fragment (containing 90% of the exonic sequence) revealed no further mutations.

When these results are combined, 15 tumours had a single change in the hMSH2 gene, eight tumours had two alterations and one had three. Thus, a total of 61% (22/36) of tumours with MSI had an alteration in hMSH2 compared to 6% (2/32) of non-shifters analysed. Forty-six percent (11/24) of tumours with hMSH2 mutations showed mucinous histology.

Polymorphisms within the hMSH2 gene

Two alleles were detected by heteroduplex analysis in intron 1, which sequencing revealed to be a C-G substitution at the +9 position. This destroyed a BsrI cleavage site, permitting use of a PCR-RFLP assay to calculate allele frequency in 106 unselected healthy blood donors. The allele frequencies were A1 = 0.38 and A2 = 0.62 in this local population. The same frequency for each allele was found for the cases analysed for hMSH2 mutation in our study population. Thus this polymorphism does not appear to influence susceptibility to sporadic colorectal cancer.

Twenty-seven percent of patients with MSI were heterozygous for a previously described polymorphism in intron 12 (Fishel et al., 1993; Hall et al., 1994). Two patients carried both the polymorphism described at +12 position of intron 10 (Wijnen et al., 1994) and the polymorphism described at -9 position of intron 9, GTCGTT-GTCA TT (Børresen et al., 1995).

Discussion

This study has identified a group comprising a sixth of a population of Scottish sporadic colorectal cancer patients that exhibit microsatellite instability. This proportion is in agreement with the incidence of MSI identified by others in sporadic CRC from other geographic locations, including Lothe et al. (1993) (16.5%); Aaltonen et al. (1993) (13%) and Kim et al. (1994) (13%). Our data include a number of cases where only one locus is altered, a phenomenon which has been previously noted in colorectal cancer (Peltomäki et al., 1993; Lothe et al., 1993).
We examined other clinico-pathological factors that may distinguish this group from the general population of colorectal cancer patients. Although we found no significant difference between microsatellite unstable and stable tumours for age of onset, Duke's Stage, or gender distribution, the data did confirm the proximal location of colonic lesions with MSI which has been noted by others (Lothe et al., 1993; Kim et al., 1994; Thibodeau et al., 1993). Additionally, we found that there was a significant inverse relationship between p53 stabilisation and MSI status in tumours with instability at more than one loci. This relationship has also been reported by others (Jonov et al., 1993), although without indication whether the tumours analysed were sporadic or hereditary in nature. Moreover, we find that MSI tumours are about twice as common within the group of tumours which have retained both alleles of p53.

The results of the Cox analysis indicate a highly significant favourable effect of MSI status on post-operative survival, even after allowing for the effects of age, Duke's stage, sex and tumour side. Paradoxically, histological features generally associated with aggressive tumour behaviour are common in MSI tumours. In our series 41% are mucin-secreting. The frequency is 46% in tumours with a hMSH2 mutation. In addition, a higher proportion of poorly differentiated tumours associated with MSI has been reported by others (Jonov et al., 1993; Lothe et al., 1993; Kim et al., 1994). It has been suggested that this survival advantage stems from an enhanced host immune response to cancer cells arising as a result of a high mutation rate of tumour-associated antigens (Kim et al., 1994). Alternatively perhaps the situation is reached where the tumour can no longer survive with such a mutation burden if, for example, it compromises the function of essential housekeeping genes. Recent insights into the mechanism of development of tumours with MSI suggest that many of these carcinomas have inactivation of the TGFβ receptor, rendering cells insensitive to TGFβ-mediated growth inhibition (Markowitz et al., 1995). Thus, determination of the MSI status of a colorectal lesion could be important not only as a predictor of prognosis but also in terms of potential cancer therapy.

Although sporadic colorectal cancers with MSI demonstrate replication slippage in a similar manner to HNPCC lesions and share similar biological characteristics, there are clear differences. Unlike the situation seen in sporadic colorectal cancer, microsatellite instability occurs as an early event in HNPCC adenomas (Young et al., 1993; Aaltonen et al., 1993), as it does in Barrett's oesophagus, oesophageal adenocarcinoma (Meltzer et al., 1994) and ulcerative colitis (Suzuki et al., 1994). The role of hMSH2 is clearly different in HNPCC and sporadic colorectal cancer. Only 3% (1/36) of unstable tumours and 6% (2/32) of stable tumours had one or more mutation in the exonic portions, splice acceptor or donor regions of hMSH2. This concurs with the results of the analysis of a similar series of sporadic tumours (Borresen et al., 1995) and is akin to the incidence of hMSH2 mutation in 20 cell lines derived from sporadic colorectal cancers with MSI (Liu et al., 1995). However, it is in direct contrast to the situation found in HNPCC where analysis of these regions identifies a mutation incidence of up to 40% (Liu et al., 1994; Wijnen et al., 1995).

We have, however, shown many intronic defects within the hMSH2 gene, regions which to our knowledge have not been examined extensively for mutations in HNPCC tumours. Introns are likely potential targets for mutation because they contain repetitive sequences and the observed alterations in length of mononucleotide repeated units would be consistent with a primary defect in another mismatch repair gene. Instability of polyadenin tracts in colorectal cancer, this time in random stretches of DNA, has been demonstrated by others (Chen et al., 1995) and it is interesting to note that the mutation spectrum associated with a recently discovered hMSH2 binding protein, GTBP or p160, tends towards reductions in length of mononucleotide repeated units such as this (Papadopoulos et al., 1995). The effect on gene function of these intronic alterations within the hMSH2 gene is as yet unknown. We postulate that they may compromise transcriptional efficiency of hMSH2, but at this time we cannot predict whether this would act globally or have a local effect by a mechanism such as exon skipping. Unfortunately RNA is not available from these lesions to fully address this point. There is evidence that mutations of this type within or outwith splice sites can cause inefficient or aberrant splicing (Wierenga et al., 1984; Chu et al., 1993). Thus, these hMSH2 defects might contribute to the development of a proportion of sporadic colorectal cancers through further crippling mismatch repair.

The possible impact of double and triple mutations in hMSH2 is unclear. It is thought that both alleles of hMSH2 are inactivated, like a classical tumour suppressor gene, during the development of colorectal cancer. However others have shown a dose-response effect of this gene during in vitro studies (Parsons et al., 1993). It is also not yet known if all missense mutations described inactivate the gene to the same degree, which could account for phenotypic variation. Unlike the situation seen with other tumour suppressor genes, loss of heterozygosity is not commonly observed at the hMSH2 locus (Aaltonen et al., 1993).

The overall incidence of hMSH2 mutation in our population of sporadic colorectal cancers is at least 11%, with only 1% having exonic mutations. Amongst our population of patients with apparently sporadic colorectal cancer we found three who carried germ-line mutations in hMSH2. This small subset of patients may be thought to represent hereditary, rather than sporadic, colorectal cancers which do not fit the criteria for HNPCC. Only one of these three patients had any family history of cancer. Interestingly, this patient was identified from the group of cancers without MSI. He was younger (49 years) than our average age-group, had a Duke's C carcinoma in the ascending colon, and had a family history of pancreatic cancer. This individual may belong to the class of familial RER+ tumours including lung, breast and pancreas, with less prevalent microsatellite alterations (Liu et al., 1995) although we are unaware that this has previously been associated with hMSH2 mutation. A second patient with no family history or evidence of microsatellite instability, aged 74 and with a cancer of the caecum, was also found to have a germ-line hMSH2 mutation. Both of these mutations were missense and therefore predicted to compromise protein function. The third germline mutation was the shortened polyadenine tract.
in intron 5 of unknown function; this was found in a tumour demonstrating MSI. These observations support the view that a wide range of phenotypes are associated with germ-line hMSH2 mutation (Kolodner et al., 1994; Mary et al., 1994).

Two major conclusions can be drawn from this study. Firstly, although MSI is clearly a significant factor in the clonal expansion of one in six of all sporadic colorectal cancers, exonic hMSH2 defects are associated with this instability in a considerably lower proportion of tumours than in HNPCC. Indeed, when mutations in the intronic sequences of hMSH2 are excluded from consideration, hMSH2 mutation in sporadic tumours with MSI is no more frequent than in other tumours. Our study therefore suggests that the genetic basis of microsatellite instability in sporadic and inherited colorectal cancer is different. This is supported by evidence of a dramatic difference between the incidence of MSI in adenomas from HNPCC and sporadic colorectal cancer patients (Young et al., 1993; Aaltonen et al., 1994). Secondly, our data (from a large series of patients with extensive follow-up) clearly demonstrate a survival advantage for patients who have a tumour exhibiting MSI. It will be of great interest to determine the mechanism by which such genetic instability imparts improved prognosis since this may have substantial implications for novel therapeutic approaches.

Materials and methods

Tissue samples

Tissues were harvested fresh from consecutive colorectal carcinomas removed at operation between 1988–1993, and frozen at −70°C. DNA was extracted from paired normal (uninvolved mucosa or blood) and tumour tissue by the method of Goelz et al. (1985). Representative portions of tumour were paraffin-processed following standard methodology.

Polymerase chain reaction (PCR) of microsatellite sequences

All tumours were analysed for evidence of genetic instability at four microsatellite loci, D2S119, D3S1293, D8S282 and D13S160 (Gyapay et al., 1994). In addition, some were examined at loci D15S132 and D17S849 by Automated Laser Fluorography (ALF, Pharmacia Biodevices Ltd.). Amplification conditions were 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 μM each dNTP, 0.5 μM each primer (Table 1), 2.5 units Taq DNA polymerase (Life Technologies) in 100 μl reaction and 100 ng genomic DNA template. Magnesium concentrations for each set are indicated in Table 1. The upstream primer of each primer pair was synthesised with a fluorescein molecule at the 5’ position to allow analysis by ALF.

Analysis of microsatellite marker sequences

For silver staining, products were heat denatured in 0.5 volume STR loading buffer (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol FF) and electrophoresed on 6% 19:1 acrylamide:bis acrylamide denaturing gels containing 7 M urea. Electrophoresis buffer was 44.5 mM Tris, 44.5 mM Boric acid, 1 mM EDTA, pH 8.3 and gels were electrophoresed at constant power (70 W). To facilitate silver staining the gel was bound to the glass plate with γ-methacyloxypropyltrimethoxysilane (Kobayashi, 1988; Storts et al., 1993) and stained in situ according to the method of Bassam et al. (1991).

PCR samples analysed by ALF were prepared by adding an equal volume of denaturing solution [95% formamide, 0.88% dextran blue, and 25 mM EDTA (pH 9)] and heat denatured at 95°C for 5 min, then rapidly cooled on ice. Internal standards between 65 and 313 nucleotides long, were prepared by amplification from single stranded M13mp18 vector using a variety of 20-24mer primers. Five microlitres of PCR product in denaturing solution were loaded onto a 6% MDE gel (AT Biochem, PA, USA) containing 7 M urea and electrophoresed at constant power (55 W) and temperature (50°C). Evidence of microsatellite instability was determined by a shift of allele peak to a new position.

Polymerase chain reaction of exons from hMSH2

Each of the 16 exons of hMSH2 was amplified using the primers described in Table 2. PCR was carried out for 30 cycles at annealing temperatures calculated according to primer composition. Reactions consisted of 50 μl volume containing 100 ng genomic DNA, 12.5–25 pmoles of each primer, 200 μM of each dNTP (Pharmacia Biodevices Ltd), 2.5 mM MgCl₂ (except exons 15 and 1, which contained 1.5 mM), 1.25 U of thermostable DNA polymerase and buffer consisting of 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl and 0.1% non-ionic detergent.

Mutation analysis of hMSH2 gene

Single stranded conformational polymorphism (SSCP) analysis was carried out as previously described (Curtis et al., 1994). Heteroduplex analysis was also performed on those samples in which the banding patterns proved difficult to resolve by SSCP. PCR products for hetero-

<table>
<thead>
<tr>
<th>Marker name</th>
<th>Locus</th>
<th>[Mg²⁺]</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFM077yb7</td>
<td>D2S119</td>
<td>2.0 mM</td>
<td>CTTGGGAGGACTGTCATT</td>
</tr>
<tr>
<td>AFM2000a1</td>
<td>D3S1293</td>
<td>2.0 mM</td>
<td>GAGAACCTCCTCAATTATGGG</td>
</tr>
<tr>
<td>AFM234v64</td>
<td>D8S282</td>
<td>1.5 mM</td>
<td>AAGCTACACAGGTTCAAC</td>
</tr>
<tr>
<td>AFM135xu11</td>
<td>D13S160</td>
<td>1.5 mM</td>
<td>CTGAGAATTCAACACAGG</td>
</tr>
<tr>
<td>AFM265x9</td>
<td>D15S132</td>
<td>1 mM</td>
<td>TTATGGGTCTGAGGGTAC</td>
</tr>
<tr>
<td>AFM234xg3</td>
<td>D17S849</td>
<td>1 mM</td>
<td>CATCTCTGTTCTCAAGGATTTTGG</td>
</tr>
</tbody>
</table>

Table 1 Microsatellite loci primers
duplex analysis were heated to 95°C and cooled slowly to 7°C over 30 min. 5 µl of sample in 1 µl loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF) was electrophoresed on a MDE gel containing 3.12 M urea according to the manufacturer’s protocols (AT Biochem, PA, USA) and run at 0.71–0.8 kV for 16 h. Both glass plates were pre-treated, one with Gel Slick (AT Biochem, PA, USA) and the other with 20 µl γ-methacryloxyproptrimethoxysilane in 5 ml distilled water (pH 3.5 with acetic acid). Silver staining was performed according to the following protocol: The gel was soaked for 10 min in 10% ethanol then 10 min in 1% nitric acid, rinsed quickly with water and washed for 5 min in water. It was then soaked for 20 min in 12 mM silver nitrate, rinsed twice for 20 s, first with water then with gold developer (0.28 M sodium carbonate, 0.0185% formaldehyde) until a precipitate formed. The developer was replaced and the gel soaked until an image appeared. The reaction was stopped with 0.1 M citric acid and the gel rinsed in water. The whole procedure was carried out on a rocking platform.

Table 2 hMHS2 primers

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primers (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GGCACATTCTCTCAACCCAGG</td>
</tr>
<tr>
<td></td>
<td>AAAGGAGCCGCCCACAA</td>
</tr>
<tr>
<td>2</td>
<td>ATATCTTATTGCTTG</td>
</tr>
<tr>
<td></td>
<td>GTATGCTGGAGAGAAAC</td>
</tr>
<tr>
<td>3</td>
<td>AATAATTTTTCAATATTTGCA</td>
</tr>
<tr>
<td>4</td>
<td>TAATGAGTTGAGTTG</td>
</tr>
<tr>
<td>5</td>
<td>TAGGCAAGTTACATCC</td>
</tr>
<tr>
<td>6</td>
<td>GGTGAGTTGAGTTG</td>
</tr>
<tr>
<td>7</td>
<td>TACTTCTTCTTCAATC</td>
</tr>
<tr>
<td>8</td>
<td>TGATCATCAGTTG</td>
</tr>
<tr>
<td>9</td>
<td>ACTATTTGAGTAAAGAG</td>
</tr>
<tr>
<td>10</td>
<td>TGATATGAGTTATATTAG</td>
</tr>
<tr>
<td>11</td>
<td>CCAGGATCACTGCCAG</td>
</tr>
<tr>
<td>12</td>
<td>TTTATATTTACATCATTC</td>
</tr>
<tr>
<td>13</td>
<td>GGTGATCATCAGTTG</td>
</tr>
<tr>
<td>14</td>
<td>TACTTCTTCTTCAATC</td>
</tr>
<tr>
<td>15</td>
<td>CGATCAATTTTATAAG</td>
</tr>
<tr>
<td>16</td>
<td>AGAATTTTTTCAATATTG</td>
</tr>
</tbody>
</table>

Twenty-one products generated from exon 5 were abnormal by SSCP and sequencing revealed changes in the length of a polyadenine tract within the intron. In order to eliminate the possibility of other mutations in this exon, this portion was cleaved away with 7rI11 (MBI Fermentas, Lithuania) and the remaining exonic fragment reassayed by SSCP. Similarly, eight products generated from exon 2 which were abnormal by SSCP but revealed only a length change in an intronic polyadenine tract by sequencing, were cleaved with PstI11 to allow reassessment of the exonic portion by SSCP.

Samples which demonstrated an abnormal banding pattern by heteroduplex or SSCP analysis were reamplified with Taq polymerase and directly sequenced (Sequenase PCR Products Sequencing Kit, United States Biochemicals) or cloned into pGEM7zf+ (Promega Ltd., UK) and sequenced as pooled clones (20–100 colonies) using a modified dyeoxy

Figure 2 Exon 1 polymorphism, C to G change at the +9 position of intron 1, in normal DNA from three patients of different genotype by (a) heteroduplex, where H indicates heteroduplex formation (b) SSCP and (c) BstI restriction digest analysis. Allele sizes for BstI digestion products are 81 bp (A1) and 57 bp (A2). C indicates the constant bands of 152 bp and 48 bp (21 bp band not shown); M, DNA molecular weight marker 1 kb ladder (Life Technologies, UK).

Table 3 Characteristics of tumours with MSI

<table>
<thead>
<tr>
<th></th>
<th>Unstable &gt; 1 locus</th>
<th>MSI status Unstable &gt; 1 locus</th>
<th>Stable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Side</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left</td>
<td>16 (41%)</td>
<td>6 (26%)</td>
<td>117 (65%)</td>
</tr>
<tr>
<td>Right</td>
<td>23 (59%)</td>
<td>17 (74%)</td>
<td>63 (35%)</td>
</tr>
<tr>
<td>Dukes stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2 (5%)</td>
<td>1 (4.5%)</td>
<td>23 (13%)</td>
</tr>
<tr>
<td>B</td>
<td>21 (54%)</td>
<td>13 (56.5%)</td>
<td>79 (44%)</td>
</tr>
<tr>
<td>CD</td>
<td>16 (41%)</td>
<td>9 (39%)</td>
<td>78 (43%)</td>
</tr>
<tr>
<td>p53 Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICC Positive</td>
<td>22 (56%)</td>
<td>9 (39%)</td>
<td>108 (60%)</td>
</tr>
<tr>
<td>ICC Negative</td>
<td>17 (44%)</td>
<td>14 (61%)</td>
<td>66 (37%)</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>23</td>
<td>180</td>
</tr>
</tbody>
</table>

*a no alteration in length seen at loci tested. b immunocytochemistry.

PLP1 fixed tissue unavailable. c Three samples included with strong cytoplasmic staining, nuclei were not stained.
chain termination method (Sequenase version 2.0, United States Biochemicals) with the appropriate PCR primer. Where PCR fragments did not produce high quality sequence by this method, single colonies were sequenced using the T7 promoter primer (Promega Ltd., UK). Due to the poor fidelity of Taq enzyme, sequence data from single colonies generated by Taq polymerase were confirmed using an alternative polymerase, rTth DNA polymerase (Perkin Elmer, UK), which has proofreading activity and a significantly lower error rate. These samples were cloned into plasmid pCRII using the TA cloning kit (Invitrogen BV, NV Leek, Holland) and single colonies sequenced.

A frequent abnormality in the exon 1 PCR fragment was detected by the presence of an extra band by heteroduplex analysis and a consistent SSCP banding pattern (Figure 2). An additional allele was identified by sequence analysis and allelic status confirmed by restriction digestion (BclI) followed by electrophoresis on a 20% polyacrylamide gel.

In addition, the incidence of a previously reported polymorphism in intron 12 (Fishel et al., 1993; Hall et al., 1994) was investigated. A restriction digestion assay was developed which included digestion by SphI followed by electrophoresis on a 3% agarose gel.

**Immunocytochemical staining of p53 protein**

Representative portions of tumour were fixed in periodate-lysine-paraformaldehyde-dichromate (PLPD) and embedded in paraffin wax. Staining was performed with the monoclonal anti-p53 antibody, Pab1801 (Oncogene Science, UK) as previously described (Purdie et al., 1991). Samples were scored as positive if any positive nuclei were found in the section and were analysed by at least two observers.

**Statistical analysis**

The effect on post-operative survival of all relevant factors was investigated by analysis with the Cox proportional hazards method, using the Cox Regression routines of SPSS for Windows 6.0. Post-operative survival was established by perusal of death certificates held by the Registrar General for Scotland. Those patients who did not appear in the registry on the census date were traced through hospital records to confirm survival by continuing out-patient attendance or by letter to their family practitioner. The censoring date for survival was taken as 31.12.93; thus, all who survived later than this date were treated as `lost known alive' at this time. Factors investigated as possible predictors of survival were age, tumour side, ploidy, p53 status, gender, shift status and Duke's stage. Deaths from all causes were considered.

**Acknowledgements**

We wish to thank Dr Herb Poff for helpful advice on the method of silver staining MDE gels, Mr S Morris for preparation of molecular weight standards for ALF analysis, Ms Zsuzsana Egelstaff for database management, Mrs Norma Brown for collection of patient details and Ms Joan Flannigan for expert technical assistance. This study was funded by the Cancer Research Campaign, Scottish Hospitals Endowment Research Trust (LJC) and the Melville Trust (CC).


