HEREDITARY COLORECTAL CANCER:

A CLINICAL AND MOLECULAR GENETIC STUDY

by

Stuart Charles Whitelaw

M.B. Ch.B. , F.R.C.S. (Edin), F.R.C.S. R.C.P.S (Glas)

St Mark's Hospital, London

and

The Imperial Cancer Research Fund, Lincoln's Inn Fields, London.

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
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Dedication

To Sarah, Ross, Kristin and Murray

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Declaration

This thesis is the result of my own work. The material contained in this thesis has not been presented nor is currently being presented either wholly or in part for any other degree or diploma. The clinical work was undertaken at St Mark's Hospital, London. The laboratory work was undertaken in the Somatic Cell Genetics Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, with the exception of the scanning densitometry analysis which was performed in the University Department of Surgery, Glasgow Royal Infirmary. Except where referred to in the text, the analysis of all data, and the writing up of this work was carried out by myself.

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Summary

The general aim of this thesis was to advance our understanding of the genetics of colorectal cancer. The specific aims were 1) to assess the psychological impact of familial adenomatous polyposis and patient attitude to predictive DNA testing, 2) to assess the prognostic value of allele loss in colorectal cancer and 3) to assess the effectiveness of a family cancer clinic for the targeted screening of colorectal cancer.

Attitudes to predictive DNA testing and the psychological impact of familial adenomatous polyposis (FAP) were documented in 62 affected adults. In the majority of cases, FAP, appeared to have a fairly minimal impact on the everyday life of the patient. However, in a significant minority (20%), a diagnosis of FAP had a devastating effect on psychological well being. Factors which might be important include a previous unpleasant experience with an ileostomy, a history of cancer death within the family, a poor understanding of the mode of transmission of FAP and a perceived delay in diagnosis. With respect to patient views on prenatal testing and termination of pregancy for FAP, fifteen (24%) of those questioned stated that they would proceed to termination of pregnancy if a prenatal test indicated that the unborn baby was affected. Six (10%) of people who had refrained from having children for fear of passing on the polyposis gene felt that the arrival of prenatal testing would enable them to consider planning a family. The majority of patients (93%) said that they would like their children tested by DNA analysis at birth or infancy, but felt that 10-12 years was the most appropriate time to discuss the diagnosis with the child.

The frequency of allele loss at the APC, P53, Nm23 and DCC gene loci was investigated in a panel of 63 colorectal cancer specimens by Southern blotting

analysis and correlation with prognosis studied. Sixty-percent of the specimens studied, exhibited allele loss for at least one genetic marker (52% at the P53 locus, 38% at the APC locus and 36% at the DCC locus). No allele loss was found at the Nm 23 locus. Univariate analysis found that allele loss on chromosome 17p was related to prognosis ($P < 0.02$), although a multivariate analysis, including other accepted prognostic indicators for colorectal cancer, failed to support this association.

The results of screening individuals referred to the Family Cancer Clinic at St Mark's Hospital, London, are described. Colonoscopy was performed in 644 asymptomatic individuals (from 436 families) with a family history of colorectal cancer over a six year period. Families were subdivided according to family history and sixty nine (15.8%) of the families fulfilled the Amsterdam criteria for the hereditary non-polyposis colorectal cancer syndromes (HNPCC). Seven cases of colorectal cancer were diagnosed at an average age of 49 years; six at Dukes' stage A and one at stage C; four in HNPCC families. One hundred and forty four (22.4%) subjects had one or more adenomas. The prevalence of adenomas in the subjects from Amsterdam criteria families was 34 of 127 (26.8%) compared with 110 of 517 (21.3%) in non - Amsterdam criteria families. Men were twice as likely to develop adenomas as women, and the prevalence of adenomas increased in both sexes with age; the odds ratio (O.R.) increasing approximately two-fold for each decade ($p < 0.0001$). A multivariate analysis showed that the number of generations of relatives ($>$ or $= 2$ versus 1) affected by colorectal cancer or adenomas was a highly significant independent variable associated with a an increased risk of adenoma development.

As part of this project, a very large kindred, St Marks' Family 96, was identified, which appears to have an autosomal dominant predisposition to an atypical polyposis syndrome and colorectal cancer. This syndrome has been called the " Hereditary Mixed Polyposis Syndrome" (HMPS) by the author. Affected individuals usually present in the fourth decade with symptomatic polyps or cancer. Although

adenomatous and hyperplastic polyps occur in affected members, the characteristic lesion is an atypical juvenile polyp. Some individuals have developed polyps of more than one type, and individual polyps may have mixed histological features. Typically, fewer than 15 polyps are found at colonoscopy, and there is no extracolonic disease associated with the development of polyps. St Mark's Family 96 consist of 10 second generation, 35 third generation, 63 fourth generation and 42 fifth generation individuals. All surviving members are derived from the third, fourth and fifth generations, and updated clinical information has been obtained in 71 patients over the age of 21 years. Thirty three members (13 females, 19 males) are known to have developed either colorectal cancer or polyps.

A genetic linkage study was performed on this family using 77 genetic markers spanning the genome. Data did not support linkage to the APC locus or any of the loci responsible for HNPCC. The most positive LOD score (0.69) was obtained with the marker D6S44 which maps to chromosome 6p21-qter.

Although the gene responsible for HMPS was not localised within the time period of this project, a subsequent linkage study found significant two-point LOD scores for linkage between HMPS and the D6S283 locus. Analysis of recombinants and multipoint linkage analysis suggests that the gene responsible for HMPS lies in a 4-cM interval containing the D8S283 locus and flanked by markers D6S468 and D6S301.

Chapter 1

The Clinical Genetics of Colorectal Cancer

" The successive steps in carcinogenesis may be analogous to hurdles in a race in which individuals who are genetically predisposed have a head start because one of the hurdles has already been jumped " (1)

Introduction

The incidence of most common cancers varies widely between different populations, often by a factor of a hundred or more (2). Evidence from studies on migrant populations and correlation of incidence with various lifestyle factors, suggest that most of this variation can be attributed to environmental factors. However, genetic predisposition is also clearly important as cancer incidence varies markedly within both resident and migrating groups. Some authors believe, that in the case of colorectal cancer, dietary carcinogens can only initiate or promote malignancy in the presence of inherited susceptibility (3).

Studies of familial colorectal cancer suggest that the same genes are involved in the pathogenesis of sporadic forms of the disease. In the genetically susceptible individual, every cell in the body will possess the critical mutation, whereas in non-susceptible individuals, only the cancer cells of the target organ will contain the same mutation. Several steps are needed to turn a normal cell into a cancer cell, and so the inheritance of one susceptible gene does not make the development of cancer a certainty. However, it does mean that the individual carrying the mutation has an increased likelihood of developing cancer at an earlier age than in the general population. Therefore, identification of the genes responsible for increased susceptibility and their aberrant sequences may define steps critical to cancer development in "sporadic" malignancy, bringing the prospect of cure and prevention one step closer.

There are at least two main categories of germ-line susceptibility to colorectal cancer; firstly, susceptibility due to the inheritance of a high penetrant mutation(s), and secondly, susceptibility due to the inheritance of a low penetrant mutation(s).

High penetrant disorders, transmitted in an autosomal dominant manner, can be sub-divided into two main groups:

1) Polyposis Syndromes, such as Familial Adenomatous Polyposis (FAP), the Peutz-Jeghers syndrome (PJS) and Juvenile Polyposis (JPS), in which the small and/or large intestine is carpeted with hundreds and sometimes thousands of polyps, and

2) Hereditary Non-Polyposis Colorectal Cancer (HNPCC) Syndromes, characterised by early onset cancer, a preponderance for right sided lesions, relatively few adenomas and extra-colonic malignancies.

Collectively, these syndromes may account for up to 10% of the total colorectal cancer burden (4, 5).

Low penetrant mutations, on the other hand, also thought to be dominantly inherited, are believed to be much more common in the general population, and may account for the vast majority of colorectal cancer after the fifth decade (3).

Familial Adenomatous Polyposis

FAP is an autosomal dominant disorder in which affected individuals develop multiple adenomatous polyps in the large bowel and elsewhere in the gastrointestinal tract. The original report of FAP is attributed to Cripps in 1882 (6). Left untreated, the vast majority of patients will develop colorectal cancer before the age of 50 years (7). The gene responsible for this disease, designated Adenomatous Polyposis Coli (APC)[1], was mapped to chromosome 5q21-22 by linkage studies in families (8) and this led to the characterisation of mutations in affected individuals (9).

Incidence

FAP is uniformly distributed world-wide and occurs in approximately 1:8000 individuals in all populations (10). This figure is based on data from Denmark where completeness of registration approaches 100%. The point prevalence rate has been calculated at 35.6 per million inhabitants, the mutation rate between 1:100,000 and 1:125,000, and the frequency of sporadic cases or new mutants between 20-25% (10,11).

Clinical Features

It is now appreciated that FAP is a multi-system disease affecting tissues derived from endodermal, mesodermal and ectodermal germ layers. Adenomas are found not only in large numbers in the large intestine, but may occur in the stomach, duodenum and small bowel, and abnormalities of the skin, connective tissue, teeth, bone, central nervous system, thyroid, eye and liver are not unusual (12)

Large Bowel Neoplasia

The number of adenomas in the large bowel is highly variable but there are usually between 100 and 5000, with an average of just over 1000 (Fig. 1.1)(13). Maeda et al categorised FAP into a *sparse* and a *profuse* form based on the density of polyps in the colon (14), and Bussey observed that polyp density tends to decrease from the

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[1] Human gene names have not been conventionally put into italics

right side of the colon to the rectum (13). Rectal sparing has been reported, but is extremely rare, and has not been documented at the St Mark's Polyposis Registry (15).

Although FAP patients may present with symptoms such as abdominal pain, altered bowel habit or rectal bleeding, the vast majority are asymptomatic and are diagnosed as a consequence of screening those at risk. In a comprehensive study of the Danish Polyposis Registry, Bullow found that most large bowel adenomas appeared at a median age of 16 years (range 5-38 years). The median age at the time of colorectal cancer diagnosis was 36 years (range 17-64, N=114) (4).

Two main treatment options are available for patients who have been diagnosed with FAP without cancer of the middle or distal rectum: total colectomy with ileorectal anastomosis (TC + IRA) (16), or restorative proctocolectomy with ileoanal pouch formation (17). Balance of opinion holds that preservation of the rectum generally affords the patient a better quality of life, and restorative proctocolectomy tends to be reserved for patients whose rectums are carpeted with large confluent adenomas, or who will not have access to regular follow-up (17). Retention of the rectum, however, still leaves the patient with a significant residual risk of malignancy. A recent review of 297 patients who had undergone TC + IRA over a 40 year period reported a cumulative risk for rectal cancer of 13.1% at 25 years (18). In the St Mark's series of 224 patients who have undergone an IRA for FAP since 1948, 10% have developed rectal cancer by the age of 50 years, and 29% by the age of 60 years (19).

Upper Gastrointestinal Tract Neoplasia

It is now recognised that duodenal adenomas are present in nearly all patients who have FAP (20-22). These lesions have considerable malignant potential and in some series, duodenal cancer is now the most common cancer-related death in patients who have undergone total colectomy (23,24).

The finding of residual adenomatous tissue in some duodenal cancers, and the co-existence of adenomas in the presence of the cancer, suggests that duodenal polyps may progress through an adenoma-carcinoma sequence analogous to that seen in the colorectum (24,25). It has been speculated that bile from FAP patients may contain carcinogens which promote adenoma growth (24).

A hamartomatous polyposis of the stomach, known as fundic gland polyposis, has also been documented in about 50% of FAP patients (26,27). Fundic gland polyposis, i.e. cystic dilated fundic glands without epithelial dysplasia, is not believed to be premalignant, although a few reports of FAP associated gastric cancer have emanated from Japan where this malignancy is extremely common (28,29).

Adenomas are occasionally seen in the mucosa of the terminal ileum at the time of colectomy and cases have been reported in which adenomas have been detected in the ileum when inspected by endoscopy (30). Such lesions have also been observed in ileo-anal pouch reservoirs but their significance at this site is not known (17). Small bowel carcinoma is a rare complication (31).

Congenital Hypertrophy of the Retinal Pigment Epithelium (CHRPE)

Pigmentation of the retinal epithelium occurs in 50-100 % of all patients with FAP (32-35). The lesions are usually discrete between 50-200 mm. in diameter (Fig. 1.2), and may have a depigmented halo around them. Smaller solitary lesions may be seen in unaffected people, but it is rare for unaffected individuals to have more than 2-5 such small lesions (35). Histological information is scanty, but the larger lesions probably consist of myelinated axons while the smaller lesions appear to be areas of enlarged retinal epithelial cells with increased pigment (36). The presence of CHRPE in the retina of an individual at a 50:50 risk of inheriting the gene has been taken as a positive test for the condition with a specificity of about 99% and a sensitivity of 65% (37). In a minority of families however, there is no expression of CHRPE in affected individuals, and two cases have been reported of parents with FAP and CHRPE in

whom an offspring had FAP but did not have CHRPE (36). Such heterogeneity limits the value of CHRPE as a reliable diagnostic marker.

Extra-intestinal Malignancy

An increased incidence of extra-intestinal tumours has also been described (12). In 1959, Turcot et al described two siblings in whom multiple colonic polyps were associated with astrocytoma and medulloblastoma (38), and since then, there have been further reports of an association between colorectal polyposis and the development of central nervous system tumours (39). Originally considered a separate clinical entity, it is now recognised that Turcot syndrome is due to APC mutations (40).

Female patients under the age of 35 years are said to have a 160 fold increased risk of developing papillary carcinoma of the thyroid relative to the general population (41), although this association has been disputed (12).

Hepatoblastoma has been documented in 35 children of FAP patients (42,43), and it seems probable that most of these tumours developed in gene carriers (44-46).

Desmoid Tumours

Desmoid tumours are benign proliferative lesions of fibroblasts, arising from fasciae, muscles and aponeuroses which show a marked tendency for local growth but never metastasise (Fig. 1.3)(47). Although extremely rare in the general population, accounting for less than 0.03% of all neoplasms (48), they occur in 4- 29% of patients with FAP (49). Desmoids may reach a very large size, causing death by ureteric obstruction or invasion of the small bowel mesentery, resulting in intra-abdominal haemorrhage or intestinal obstruction (50). Histologically, desmoids are indistinguishable from fibromatosis elsewhere, but there is a tendency for desmoids associated with FAP to be extremely vascular, and this may explain the torrential haemorrhage that sometimes accompanies their removal (51).

It is not known why desmoids should develop in some patients with FAP and not others. Desmoids tend to more common in women (3:1) , and often enlarge in pregnancy or in those taking oral contraceptives. Interestingly, guinea pigs injected with high doses of oestrogen develop lesions which are histologically indistinguishable from human desmoids (52) and occasionally desmoids regress when treated with the anti-oestrogen agents, tamoxifen and torimifene (47,49,51,53). This supports the hypothesis that desmoid growth may be hormonally dependent.

Intra-abdominal desmoids are notoriously difficult to treat and do not respond to radiotherapy or most forms of chemotherapy (54).

Polyposis Registries

The first polyposis registry was established at St Mark's Hospital, London, in 1925 (55), and since then, numerous national and regional registries have been established throughout the world. The benefits are shown by the dramatic decrease in the aggregated incidence of colorectal cancer in call-up cases (56,57).

The Peutz-Jeghers Syndrome

The Peutz-Jeghers syndrome (PJS) is an autosomal dominant disorder characterised by the presence of hamartomatous polyps of the gastro-intestinal tract and mucocutaneous melanin pigmentation. First reported by Hutchinson in 1896 (58), it was more fully described by Peutz in 1921 (59) and Jeghers in 1949 (60). The patient with the PJS typically presents in childhood or adolescence with intussusception, iron deficiency anaemia or with a characteristic facial pigmentation. Polyps are most commonly found in the jejunum or ileum, but they may be found throughout the gastrointestinal tract (61,62).

Incidence

The exact incidence of the Peutz-Jeghers syndrome is not known. In Scotland, fewer than 50 people were discharged from hospital with a diagnosis of Peutz-Jeghers

syndrome between 1980 and 1994 (written communication with Dr J Clarke, Community Medicine Specialist, Scottish Health Services Agency, Trinity House, Edinburgh). A family history of this disorder is said to be present in about 55% of cases (63).

Histology

Peutz-Jeghers polyps have a typical histological appearance. The core of the polyp is composed of tree-like smooth muscle covered by normal mucosa, and it may be the predominant smooth muscle component of these polyps that predisposes the Peutz-Jeghers patient to intususseption (64).

Risk of Malignancy

Although, Peutz-Jeghers polyps are classified as hamartomas, the Peutz-Jeghers syndrome is associated with a significantly increased risk of malignancy, particularly of the pancreas, breast and gastrointestinal tract (65). When breast cancer occurs, it may be bilateral (66,67). The patient with the PJS also has a predilection for some rather unusual tumours such as ovarian sex cord tumour with annular tubules (SCTAT) (68-70), feminising Sertoli cell tumour (71) and adenoma malignum of the cervix, a lesion which appears benign histologically, but behaves in a malignant fashion (70).

Screening of the index patient and at-risk family members involves colonoscopy, radiological studies of the large bowel, regular breast examination, pelvic ultrasonography in girls and physical examination of the gonads on boys.

The gene responsible for the Peutz-Jeghers syndrome has not yet been mapped.

Juvenile Polyposis

The solitary juvenile polyp of childhood is generally considered to be a benign inflammatory lesion with a self-limiting course due to spontaneous auto-amputation. By comparison, Juvenile Polyposis, first described in 1964 (72), appears to be a heterogeneous group of disorders, some of which have considerable malignant potential. There are believed to be at least three forms:

1) Juvenile Polyposis of Infancy, which is associated with malnutrition, diarrhoea, haemorrhage and intussusception, and death before 2 years (73).

2) Juvenile Polyposis coli (the most common form) where polyps are confined to the large bowel.

3) Generalised Juvenile Gastrointestinal Polyposis, characterised by the development of numerous juvenile polyps in the stomach, small intestine, colon and rectum in various combinations. This type may be associated with congenital defects which include abnormalities of the heart, cleft palate, cranium, polydactyly, malrotations of the gut, and pulmonary arteriovenous malformations (74).

Although non familial cases have been reported (75,76) most forms appear to be transmitted in an autosomal dominant manner (77).

Histology

A typical juvenile polyp is a smooth surfaced lesion with a rounded contour (Fig. 1.5). Macroscopically, it often has an ulcerated outer surface with numerous mucin filled cysts present on cut section. Although pedunculated, the pedicle is thin and devoid of muscle, making it particularly susceptible to volvulus, venous congestion, haemorrhage and auto-amputation. Microscopically, the polyp consists of epithelial tubules embedded in a mass of oedematous lamina propria with prominent inflammatory cell infiltrates (78).

Risk of Malignancy

It is now recognised that patients with JP coli have a significantly increased risk of developing colorectal cancer. One author has estimated that the cumulative life-time risk may exceed 50% (79). Juvenile polyposis has also been reported in association with adenocarcinoma of the stomach (80) and pancreas (76).

Juvenile polyposis remains a comparatively poorly defined syndrome and no clear guidelines have been established for screening and management of affected families. However, the premalignant nature of the disorder suggests that the families should be kept under close medical surveillance, and colonoscopy performed on a regular basis.

The Muir-Torre Syndrome

In 1967, Muir described a patient with six primary carcinomas, four in the colon, one in the duodenum, and one in the larynx in addition to multiple karato-acanthomas and a sebaceous adenoma of the skin (81). The following year, Torre reported the cases of a 57 year old man who had over 100 sebaceous adenomas and 2 adenocarcinomas of the gastrointestinal tract (82). A hereditary basis for this syndrome was proposed in 1971(83).

The Muir-Torre Syndrome (MTS) is an autosomal dominant disorder characterised by: 1) at least a single sebaceous gland tumour (either an adenoma, an epithelioma, or a carcinoma) (Figs. 1.6,1.7) and 2) a minimum of one visceral malignancy . To date, 120 patients with MTS have been documented world-wide (84). The majority of internal malignancies in this disorder arise in the colorectum (51%) or the genitourinary tract (25%), but tumours may arise at any site, and an association with lymphoma and haematological malignancies has also been described (85-87). Unlike "sporadic" colorectal cancer, the majority of colorectal cancers reported in this syndrome have occurred proximal to the splenic flexure (58%), and nearly half the patients have more than one malignancy. The sebaceous gland tumours may precede, appear concurrently or follow the diagnosis of the patient's cancer and more than 20%

of the patients also have a kerato-acanthoma. (85). A characteristic feature of the MTS, is the prolonged survival often oberved following the diagnosis of the visceral malignancy (88).

Sebaceous adenomas are rare skin tumours, and their occurrence as multiple neoplasms is even rarer. In a retrospective review of histo-pathological specimens stored within the department of dermatology at the Mayo Clinic over a 60 year period between 1923-83, only 59 patients with one or more of these lesions were found; 42% of these patients had one or more visceral malignancies (90).

The Muir-Torre syndrome may be a variant of the Hereditary Non Polyposis Syndrome (90).

Cowden's Syndrome

In 1963, Lloyd and Dennis reported the case of a 20 year old woman who had multiple hyperkeratotic papules of the lips and palate, gross deformity of the breast with nodularity, discolouration and ulceration, and a multinodular goitre. Biopsy of the breast and thyroid showed epithelial overgrowth "indistinguishable from carcinoma", and ten years later, the patient died from metastatic breast cancer (91). It was proposed at the time that this constellation of findings might represent a new symptom complex, and it was named Cowden's Syndrome after the surname of the propositus. Cowden's syndrome has since been referred to as the " multiple hamartoma syndrome" to emphasise the multiple hamartomatous anomalies of various organs (92).

There are now 76 reports of Cowden's syndrome in the literature, and of these 15 (20%) have developed breast cancer (5 bilateral), 5 (7%) thyroid cancer and 14 (18%) malignancy elsewhere (93).

Mucocutaneous lesions are prominent and distinctive, and include facial hair follicle tumours, (Fig.1.8), keratoses and oral papillomas (94). Polyps of varying histology

may occur anywhere in the gastro-intestinal tract but the malignant potential of these polyps is believed to be low (95).

The characteristic facial appearance of a patient with Cowden's syndrome is shown in Fig. 1.9

Hereditary Mixed Polyposis Syndrome

An atypical polyposis syndrome characterised by histologically unusual polyps and early onset colorectal cancer will be described in Chapter 3.

Hereditary Non-Polyposis Colorectal Cancer

In 1913, Aldred Warthin, a pathologist at Ann Arbor University, Michigan, USA, reported a large kindred, "Family G" which appeared to have a dominantly inherited susceptibility to colorectal and endometrial cancer (96). Clinical information on 650 descendants of the proband of this family has been updated over a 75 year period, during which 95 persons have developed malignant neoplasms (19 before the age of 40 years). Thirteen have developed multiple primary tumours (97-99).

Many similar families have since been reported, in other parts of North America, the Netherlands, Finland, United Kingdom, Japan and South Africa, suggesting a worldwide distribution for this syndrome (100-104).

The hereditary non- polyposis colorectal cancer (HNPCC) syndromes are now regarded as a group of dominantly inherited disorders, characterised by 1) an early age of onset of colorectal cancer (40-45 years), 2) a preponderance of tumour excess in the right side of the colon , 3) a high risk of metachronous disease and 4) sometimes an excess of other adenocarcinomas in the kinship (Fig. 1.10)(5). Extra-colonic malignancies most commonly include those affecting the stomach, endometrium and urinary tract and typically, these cancers present 20-30 years earlier than would be expected in the general population (105-108).

Incidence

Before 1993, there were no specific genetic markers for the HNPCC syndrome, and the lack of a verifiable biomarker and an easily recognisable phenotype greatly hindered documentation of HNPCC in both family studies and population surveys.

It is now known that the majority of cases of HNPCC result from mutations in at least four DNA mismatch-repair genes: the hMSH2 gene on chromosome 2p, the hMLH1 gene on chromosome 3p, the PMS1 gene on chromosome 2q and the PMS 2 gene on chromosome 7 (108-11).

The exact frequency and penetrance of the genes responsible for HNPCC has yet to be determined, but most studies estimate that HNPCC may account for between 1-5% of all colorectal cancer cases (112-116).

Histology

Despite claims of improved survival for HNPCC compared to a general series of patients with colorectal cancer (117), the pathology of adenocarcinomas appears to be very similar to that seen in "sporadic" cancers, and there are no reliable histological feature which can identify a cancer as hereditary in nature (5). However, the relative rarity of adenomas in HNPCC, the earlier age at which they occur, the higher incidence of villous growth pattern and associated dysplasia, suggests that the polyps associated with HNPCC may have a greater potential for malignant conversion (118).

Anticipation

In a follow-up to his original report, Warthin noted that " in Family "G", there was shown a decided tendency for the neoplasm to develop at an earlier age in successive generations " (97). Other authors have also commented that in some families, HNPCC appears to skip a generation (119). At least two possibilities exist to explain this observation; either the mutation may exhibit non-penetrance, exerting no effect

on phenotype, or the phenomenon of anticipation may be present, where age of disease onset becomes earlier in successive generations.

In some other hereditary disorders such as myotonic dystrophy, anticipation appears to result from the expansion of a trinucleotide DNA repeat sequence within the gene as the gene defect is passed from parent to child (120,121). It is too early to comment on whether such a mechanism also operates in HNPCC.

Familial Clustering of Colorectal Cancer

Although the vast majority of colorectal cancer occurs in the absence of any obvious pattern of Mendelian inheritance, it has been noted for many years, that this malignancy tends to occur more often in some families than in others. This was previously attributed either to a chance clustering of cases or to shared exposure to environmental carcinogens. However, several different modes of investigation, involving mortality, family history and endoscopy studies, have consistently demonstrated a 2-4 fold increase in colorectal neoplasia in first degree relatives of cases, making a hereditary explanation more likely (Tables 1.2-1.3). (112,122-129).

Pedigree Analysis Studies

Pedigree analysis is a useful technique which utilises hypothetical mathematical models to assess patterns of disease within families for different models of inheritance.

In 1985, Burt et al conducted a pedigree analysis study of a large kindred in Utah, which appeared to have an increased susceptibility to colorectal cancer. This kindred included more than 5000 subjects spanning 6 generations, and 4 branches of this family were studied. Although there were several cases of colorectal cancer among kindred members, initial pedigree analysis failed to elucidate a recognised pattern of inheritance, with tumour distribution within the family being similar to that seen in the Utah Cancer Registry. However, when kindred members were selected and

examined for adenomatous polyps using the 60 cm fibre-optic sigmoidoscope (F.O.S.), a statistically significant difference was observed between the family members and spouse controls. A repeat analysis of the data, then provided strongest evidence for an autosomal dominant mode of inheritance, with little evidence for autosomal recessive inheritance or chance occurrence (3).

Burt's original study was then extended to estimate the frequency of inherited susceptibility to adenomas in the general population . This study also supported the theory that susceptibility was probably inherited in an autosomal dominant manner. Gene frequency in the Utah population was estimated to be 19% with a life-time penetrance of 0.4 for adenomas or colorectal cancer in susceptible individuals (Fig.1.11)(130). In an Italian population, however, the penetrance of the gene was estimated to be only 0.1, and the true penetrance therefore may lie somewhere between these values (131).

Genetic predisposition to adenoma formation may yet turn out to be the most important factor in the pathogenesis of colorectal cancer, with dietery carcinogens acting as promoters in the adenoma-carcinoma sequence. Once the susceptibility locus has been identified, gene carriers who develop colorectal cancer can be compared with carriers who develop adenomas and those carriers who remain adenoma free. This may shed more light on the mechanisms underlying gene-diet interaction, and provide a more rational basis for developing cancer prevention strategies.

Fig 1.1 A colectomy specimen from a patient with Familial Adenomatous Polyposis.

Fig 1.2 Congenital Hypertrophy of the Retinal Pigment Epithelium

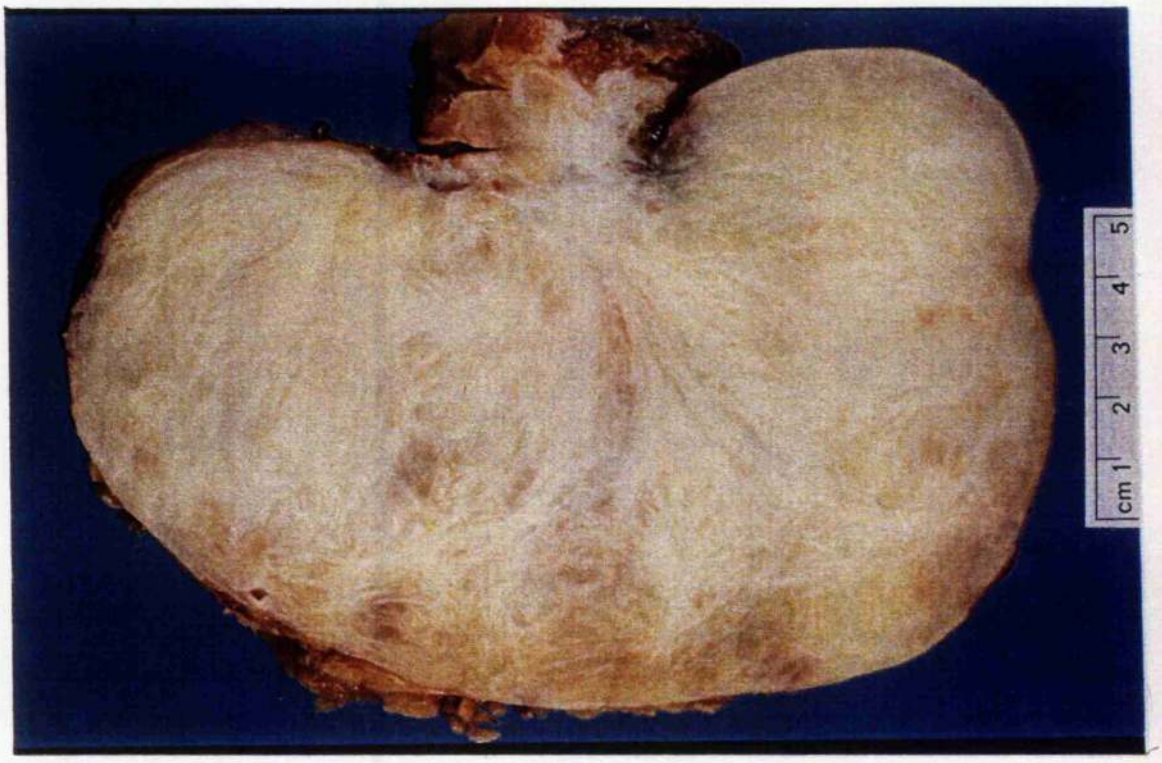


Fig 1.3 A desmoid tumour from a patient with FAP. Note the homogene - ous appearance of the cut surface resulting from high collagen content of the stroma.

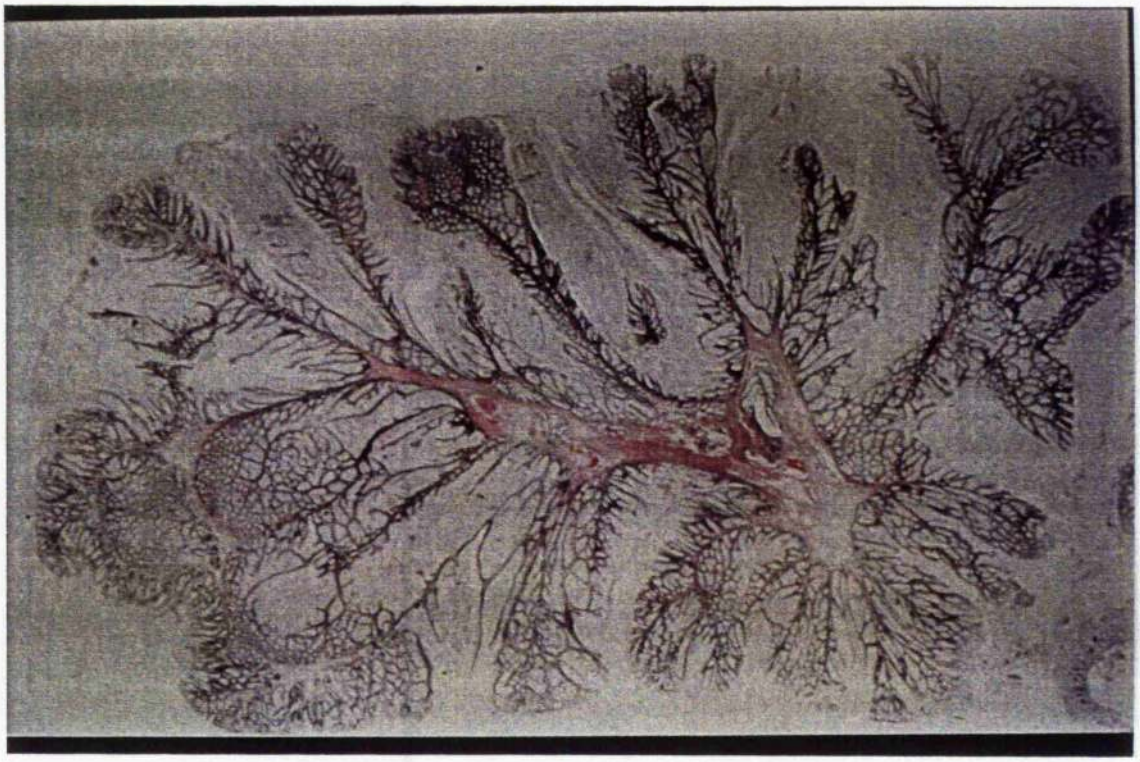


Fig 1.4 A typical Peutz-Jeghers polyp

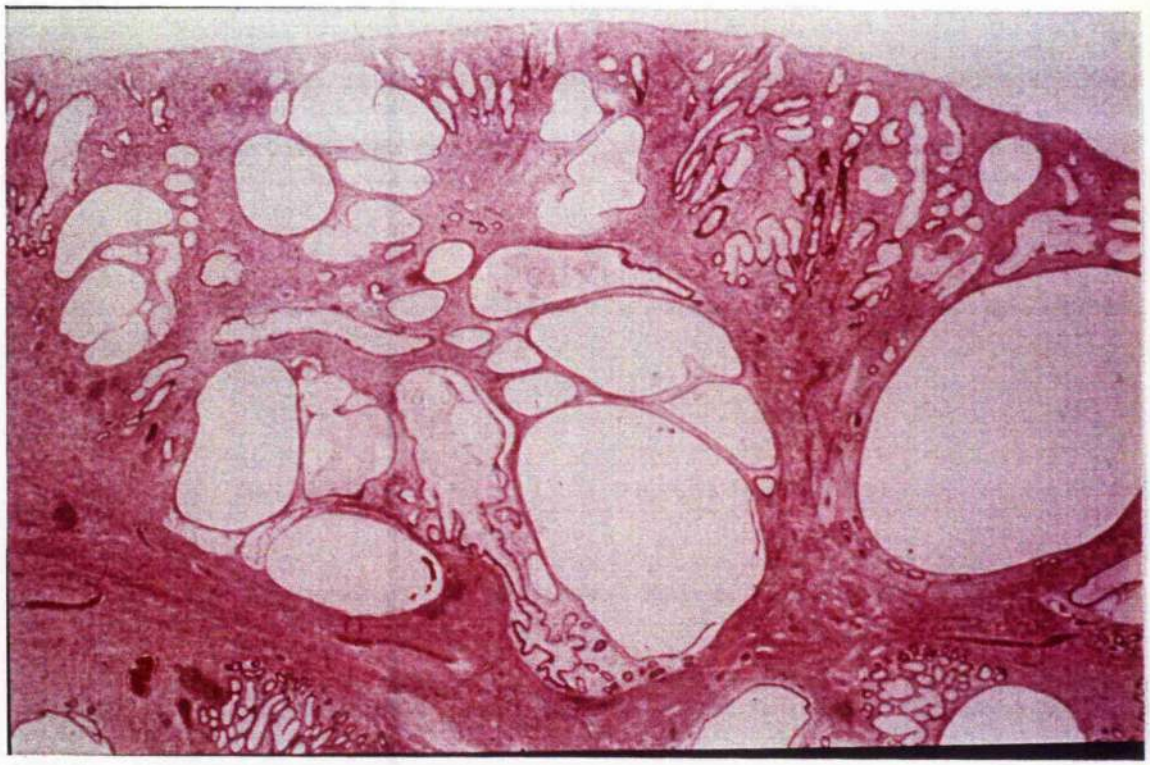


Fig 1.5 A typical juvenile polyp

Fig 1.6 Macroscopic appearance of a sebaceous adenoma

Fig 1.7 Microscopic appearance of a sebaceous adenoma

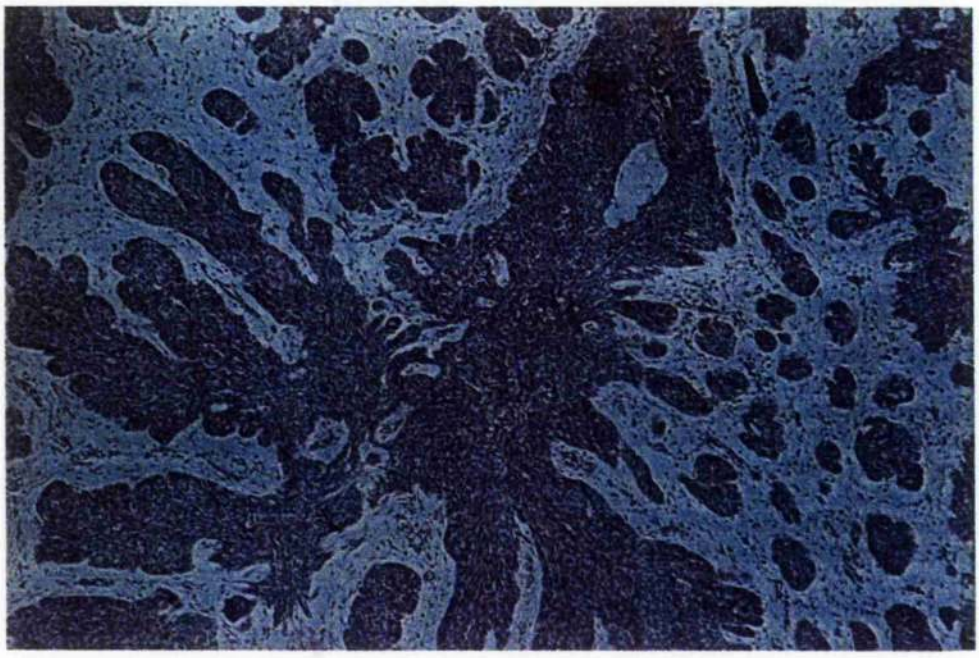


Fig 1.8 Histological appearance of a hair follicle tumour in a patient with Cowden's syndrome.

Fig 1.9 Facial appearance of a patient with Cowden's syndrome.

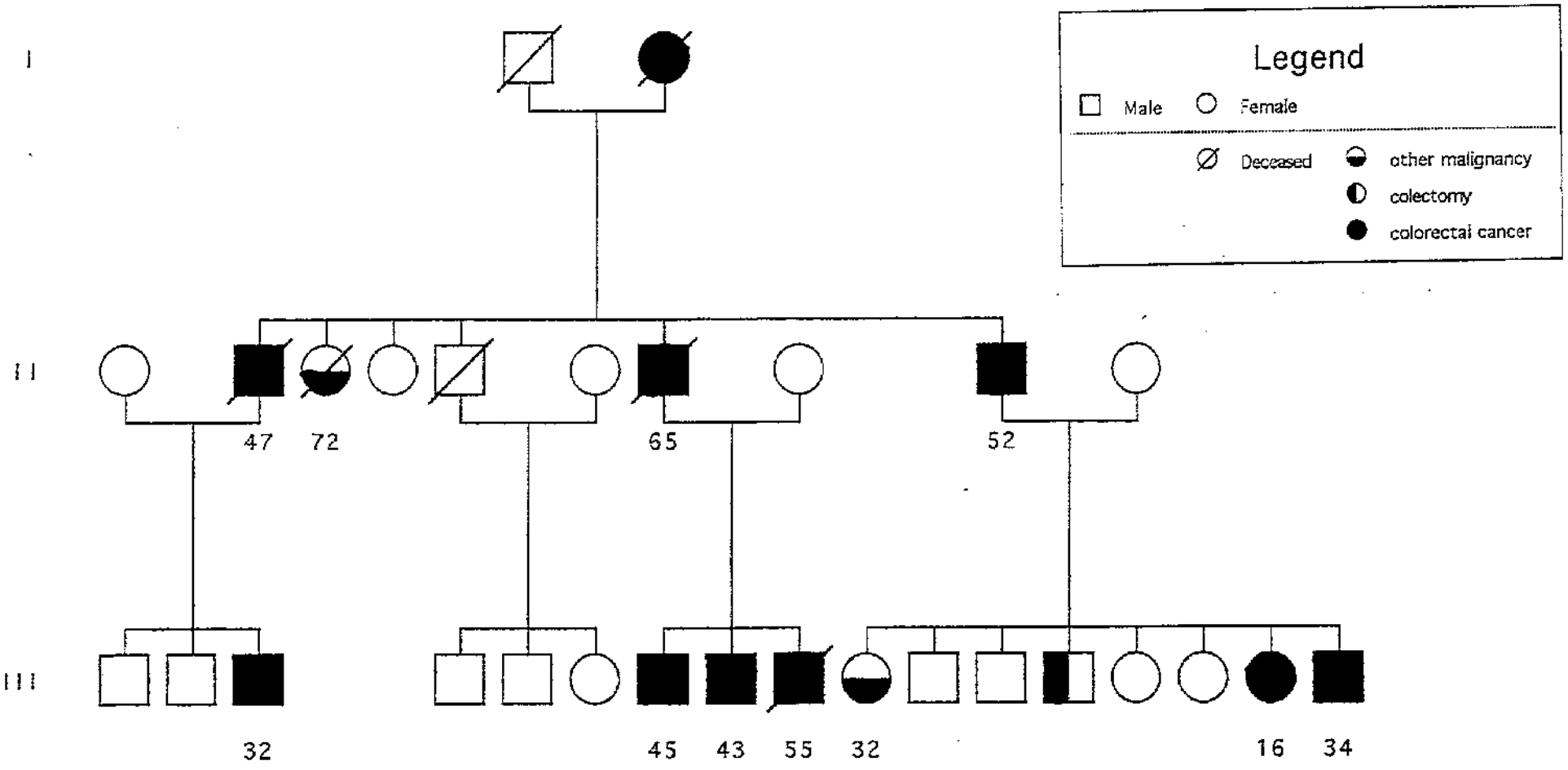


Fig.1.10 Pedigree of a family with HNPCC syndrome registered at St Mark's Hospital

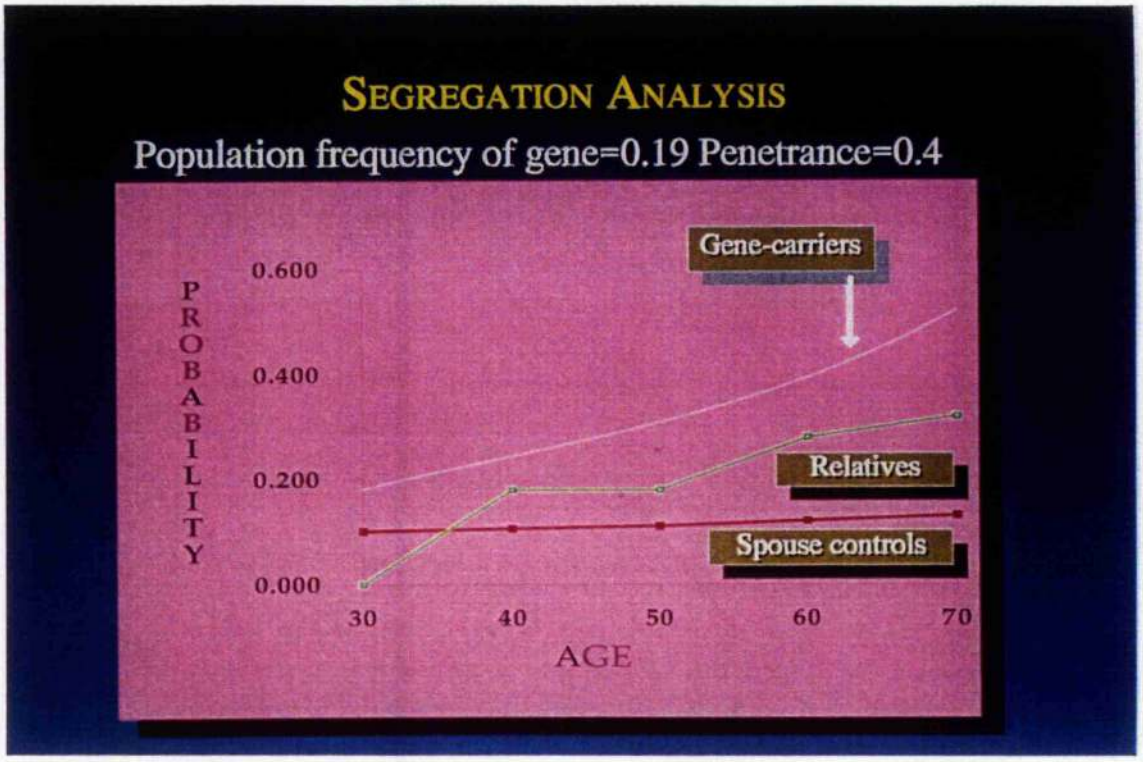


Fig 1.11 The probability of detecting adenomas in endoscopically screened relatives and spouses.

Reference	Relatives of cases who developed CRC	Relatives of controls who developed CRC	Relative Risk
(132)	18%	2%	9
(133)	19%	3.5%	5.3
(114)	20.9%	5.4%	3.9
(134)	11.3%	5.1%	2.4
(112)	23%	5%	4.6

Table 1.1 A Summary of Family History Studies of Colorectal Cancer.

Reference	Mean Age	Sample Size	Method	Neoplasia	Trial
(135)	N.S.	125	Colonoscopy	12%	P
(136)	51	114	Colonoscopy	20%	P
(137)	54	154	Colonoscopy	18%	P
(124)	55	201	Colonoscopy	27%	R
(138)	54	49	Colonoscopy	63%	R
(139)	N.S.	48	Colonoscopy	25%	R
(140)	41	644	Colonoscopy	22.4%	P

Table 1.2 Uncontrolled endoscopy studies in first degree relatives of colorectal cancer cases. N.S. = not stated. P= prospective. R = retrospective. F.O.B.T. = faecal occult blood testing. F.O.S. = fibre-optic sigmoidoscopy.

Reference	Group	Mean Age	Sample Size	Method	Neoplasia
(130)	Relatives	51	407	F.O.S.	19%
	Controls	52	265	F.O.S.	12%
(125)	Relatives	50	128	Colonoscopy	12%
	Controls	54	49	Colonoscopy	8%
(123)	Relatives	46	471	F.O.B.T./F.O.S	8%
	Controls	57	457	F.O.B.T./F.O.S	4%
(363)	Relatives	58	92	F.O.S.	15%
	Controls	63	30	F.O.S	10%

Table 1.3 Case-controlled studies of adenoma incidence in first degree relative of colorectal cancer cases. F.O.B.T.= Faecal Occult Blood Testing. F.O.S. = Flexible Sigmoidoscopy.

Chapter 2

The Molecular Genetics of Colorectal Cancer

" Eventually, the techniques of nucleic acid chemistry should allow us to itemise all the differences in nucleotide sequence and gene expression that distinguishes a cancer cell from its normal counterpart, and perhaps at that point the steps in carcinogenesis will cease to be in doubt " (141)

Introduction

Although Theodore Boveri suggested at the beginning of this century that chromosomal abnormalities might be the basis for cancer (142), it was not until the src gene of the Rous sarcoma virus was characterised, that a conclusive link between genes and cancer was demonstrated (143). Since then, there has been a virtual explosion in our knowledge of cancer genetics, offering real hope for earlier diagnosis, more effective therapy and accurate assessment of prognosis.

Cancer is now considered to be a genetic disease which develops as a result of a stepwise accumulation of errors in the genome. Such errors may be due to the integration of viruses, radiation damage, exposure to chemical carcinogens or may be inherited in the germ-line. Regardless of the mechanism involved, the end result is usually the same; alterations in certain DNA sequences lead to unregulated cell growth, proliferation and differentiation, transforming a normal cell to a malignant one.

Two features of colorectal cancer make it a particularly good model for studying neoplasia. Firstly, abundant clinical and histo-pathological data support a theory that the majority of cancers arise from pre-existing adenomatous polyps, making it possible to study genetic changes at different stages of carcinogenesis. Secondly, there are well defined hereditary bowel syndromes which predispose to colorectal cancer, and which are particularly suitable for gene mapping using linkage analysis.

In 1987 it was demonstrated that the APC gene was mutated in a significant percentage of "sporadic " colorectal cancer specimens (144-146). This landmark observation formed the basis for a genetic model for colorectal cancer, and established a common link between the hereditary and sporadic forms of disease.

It is now hypothesised that colorectal cancer results from a net accumulation of genetic errors involving the activation of growth promoting oncogenes, and/or inactivation of growth restraining tumour suppressor genes (147). The tendency for such mutations to occur may be accentuated by mutations in mismatch-repair genes conferring a mutator phenotype (148).

Clonality of Colorectal Cancer

There are at least two scenarios by which a cancer might develop from normal tissue. In the first scenario, a large cohort of normal cells may be recruited en masse by some unknown agent into becoming cancer cells. In the second scenario, the cells in the tumour may descend from a single cell that has gained a selective growth advantage and undergone clonal expansion. In the latter situation, cells making up the tumour mass will be members of a single lineage forming a monoclonal population. Present evidence suggests that the monoclonal theory is the more likely. Monoclonality has been demonstrated for lymphomas, uterine leiomyomas and bladder cancer (149-151), and there is some evidence to suggest that colorectal cancer might also be monoclonal (152).

Multistep Nature of Cancer

Measurements of age dependent cancer incidence predict that at least 4-6 rate limiting independent steps are necessary for most common cancers to develop (153). However, it has been difficult until recently to reconcile this observation with current cancer models. Each postulated genetic change is a low probability event occurring perhaps once in a thousand to more typically once in a million times per cell generation (154). The likelihood of all events occurring together therefore (the product of the probabilities) would seem extremely small and the 10 to 30 years that it actually takes for most tumours to develop, would seem too short a time period for such mutations to occur at random.

It is now appreciated that the cancer genome is extremely unstable, with dramatically increased mutation rates per cell generation (155). While normal cells maintain a fixed stable complement of chromosomes over dozens of cell generations, cancer cell seem intrinsically erratic, with a tendency to rearrangement, duplication, and deletion during cell division. As a consequence they often display novel, bizarre traits in their progeny cells (156). This suggests that the cellular machinery required to maintain the number and configuration of genes often breaks down in cancer cells.

Current thinking is that certain genes, when altered, may have the ability to confer a mutator phenotype. Lane has proposed that the p53 gene may function as a "molecular policeman", by regulating the onset of DNA replication at the G1-S interphase of the cell cycle. When the p53 gene is functioning normally, cells with potentially damaging mutations are prevented from entering the cell cycle, and the damaged cell can either have the mutation corrected or be directed into programmed cell death or apoptosis . However, in cells with deficient p53 function, the narrow limits under which plasticity is controlled, breakdown, and the genome becomes hypermutable (157).

The mismatch- repair genes, hMSH2, hMLH1, PMS1 and 2 may serve a similar genome surveillance function by correcting DNA replication errors (108-111)

Gain of Function Mutations

In 1910, Peyton Rous, working at the Rockefeller Institute for Medical Research, demonstrated that a cell free filtrate from a chicken sarcoma could induce the same tumour in other chickens, raising the possibility of a transmissible agent with the potential to induce malignant change (159). However, it was not until Bishop and Varmus described the first human oncogene over 70 years later that the full significance of Rous's observation was appreciated (160,161).

Oncogenes are a family of unique sequences of DNA whose abnormal expression is associated with uncontrolled cell proliferation and differentiation arrest. These

sequences have been shown to transform normal cells in- vitro and to cause tumours after a short latency period at the site of inoculation in-vivo (162,163). In contrast to tumour suppressor genes, oncogene mutations are capable of inducing tumours with single hit kinetics in susceptible hosts (164). Although first demonstrated in rapidly transforming RNA viruses (v-onc), their importance lies in the discovery that their DNA sequences are derived from normal cellular DNA (c-onc). Each viral oncogene has a region of highly conserved sequence homology to normal vertebrate DNA, and it is believed that they arose through illegitimate transduction of the normal cellular proto-oncogene into the viral genome. Over three dozen oncogenes have now been described, several of which are implicated in colorectal cancer (165).

It is still not known exactly how all the known oncogenes exert their effect, but some interesting clues are appearing (165,166). Normal cells produce growth factors, which promote cell division, differentiation and development. These proteins act through a chain of command on their target cells. After binding to surface receptors, their activity is modified by kinases which are part of the cytoplasmic domain of the receptor. They then interact with intracellular second messenger systems, which in turn can influence the activity of other proteins, bind directly with nuclear DNA, and regulate gene expression. However, this intricate means of communication often breaks down in malignancy, and growth factors can form self-perpetuating autocrine feedback loops, leading to unregulated cell growth (166).

Proto-oncogenes may be activated by point mutation (167), translocation (168), or gene amplification (169).

Ras gene mutations

The ras family of oncogenes was originally discovered in two murine retroviruses known as the Harvey and Kirsten murine sarcoma viruses. To date, three members, Ki-ras, Ha-ras and N-ras, have been identified and the molecular weight of their encoded proteins is 21 kiloDaltons (170,171). Present evidence suggests that the principal role of ras genes is to regulate signal transduction across the cell membrane . A cytoplasmic protein called GAP (GTPase activating protein) has been identified which can interact with the p21 ras protein to amplify its hydrolytic GTP activity by as much as 50 fold (172). Diminished GTPase activity as a result of ras mutation therefore could interfere with signal transduction from surface cell receptors, leading to enhanced ATPase activity and increased cell metabolism (173).

In 1982, it was established that the transforming sequence from a human bladder cancer was a modified form of H-ras, with a point mutation at codon 12 which resulted in the substitution of a glycine for a valine residue, altering the conformation of the oncogene encoded protein (167). Other transfection assays have demonstrated that ras point mutations invariably cluster around codons 12,13 and 61 (174). Ras gene DNA extracted from bladder cancer on its own does not have the capacity to transform normal cells, and in animal model systems, ras gene point mutations require combined co-transfection with c-myc or P53 for malignant conversion to occur (175).

Ras genes are the most commonly mutated oncogenes in colorectal neoplasia, occurring in between 40-60% of all carcinomas, and a similar number of adenomas greater than 1cm (170,176). Although these mutations appear to be an early event in the adenoma-carcinoma sequence (147), they may be absent from the most poorly differentiated cancers suggesting that ras gene activation may be switched off in the more advanced stages of malignancy (177). No difference in ras mutation frequency has been observed between proximal and distal tumours (178), and there appears to be

no correlation between the presence of ras oncogene expression and prognosis (179,180).

The polymerase chain reaction (PCR) can detect neoplastic ras point mutations in DNA from shed epithelial cells in patients' stool samples, but the usefulness of this test for diagnosis and screening remains uncertain (181).

C-Myc Oncogene

C-myc is the oncogene of the avian leukosis virus, which in chickens leads to the development of myelocytomas, lymphomas, sarcomas and carcinomas (182). The protein product of the c-myc oncogene is a 62kD nuclear phosphoprotein which functions as a transmembrane regulator of transcription (183,184). Abnormal levels of c-myc mRNA levels and the c-myc protein in colorectal cancer have been reported in between 6-98% of cases (185,186), but deregulation of c-myc gene expression does not appear to be associated with amplification or rearrangement of the gene (187). Some correlation has been claimed between c-myc oncogene expression and tumour differentiation, but the correlation between c-myc expression and prognosis is poor (188).

Other oncogene products less frequently altered in colorectal tumours include c-src, overexpressed in 62% of cancers: c-myb deleted in 9% and c-erb2 amplified in 3-4%. (189-191)

Loss of Function Mutations

The concept that tumours may arise as a result of a *loss* of genetic material was first proposed by Boveri in 1914, who concluded, after studying abnormal mitoses during the development of sea urchin embryos, that malignant cells were mutant clones that had acquired an unbalanced chromosome complement:

" Another possibility is that in every normal cell there is a specific arrangement for inhibiting, which allows the process of division only when the inhibition has been

overcome by a specific stimulus. To assume the presence of definite chromosomes which inhibit division would harmonise best with my fundamental idea " (142).

Nonetheless, it was not until the somatic cell hybridisation experiments of the late 1960s that strong evidence emerged to support Boveri's hypothesis (192). Somatic cell hybrids have inherently unstable karyotypes and frequently shed chromosomes arising from one or other parent cell. The expulsion of mouse chromosome 4 in the studies of Harris and Klein, led to reversion to the malignant phenotype, strongly suggesting that a gene (or genes) from a normal cell might replace a defective function in the cancer cell (193).

In the 1970s, methods were developed which permitted the transfer of a single specific chromosome which would be retained as a complete structural unit in succeeding generations of recipient tumour cell lines (194). In most cases it was found that transfer of the missing normal chromosome region in the tumour was able to temporarily suppress the immortalised phenotype, whereas the transfer of irrelevant chromosomes had no such effect (195). In colorectal cancer, the transfer of chromosomes 5,17 or 18 into tumour cell lines, can significantly inhibit growth in-vitro, abolish anchorage independent growth, and completely suppress tumour development (196,197).

In the 1980s, Cavanee, demonstrated a molecular basis for tumour suppressor gene inactivation using restriction fragment length polymorphism analysis (198). The steps that lead to homozygosity in a cancer cell usually involve flanking chromosomal regions as well as the genetic region of interest, and accordingly, anonymous DNA probes mapping to nearby chromosomal sites, which show heterozygosity in normal cells, may suffer a parallel reduction to homozygosity (Fig 2.1). A low level of non-specific allele loss occurs throughout the genome in a wide variety of tumours, but regions which consistently demonstrate a high frequency of fractional allelic deletion (>10%) frequently harbour tumour suppressor gene loci (199,200). Such mutations may either be inherited in the germ-line, or occur in

somatic tumour cells. Loss of heterozygosity (LOH) studies using matched tumour/normal DNA samples have proven to be an extremely useful tool for identifying genes involved in inherited cancer syndromes (201).

The Retinoblastoma Paradigm

The childhood tumour retinoblastoma serves as a paradigm for understanding the recessive nature of tumour suppressor gene inactivation. This malignancy has been observed in two distinct forms (202). In 25-30% of retinoblastoma cases, tumours appear bilaterally, may be multifocal in each eye and appear at a very young age. In the remaining 70-75% of cases, tumours are unilateral, unifocal and tend to develop later. In a statistical study, Alfred Knudson proposed a two hit model to explain the differing age of onset of this malignancy between children with and without a positive family history (203). The essential feature of Knudson's hypothesis is that in the familial form of retinoblastoma, the affected individual inherits a mutant loss of function allele from the affected parent, and then a somatic event inactivates the normal allele from the other parent; the implication being that any somatic cell of the gene carrier can be examined for this first mutation.

Several mechanisms have been proposed to explain the way in which the surviving wild-type allele may be inactivated after the first copy has been inactivated. These include, complete loss of the normal chromosome, loss and reduplication of the abnormal chromosome, chromosomal non-disjunction, mitotic recombination and reduplication of the mutant allele, gene deletion or point mutation (Fig 2.2).

Isolation of the Retinoblastoma (Rb) gene was made through a series of family, cytogenetic and molecular studies (198,204-207). A germ-line mutation in the retinoblastoma gene has been found to confer a 90% chance of malignancy, as the background somatic mutation rate is sufficiently high for the normal allele to be inactivated (202).

The Rb gene encodes a 110 kDa protein product which is believed to be principally involved in cell-cycle regulation (208). It has the ability also to bind DNA and form specific complexes with the transforming proteins of several DNA tumour viruses, including SV40 large T, adenovirus E1A, and human papilloma virus (209-211).

Rb gene inactivation does not appear to be a frequent event in colorectal cancer. Indeed, cytogenetic studies have found 48-55% of colorectal tumours exhibit non random *gains* of chromosome 13, (212,213), suggesting that an increase in Rb gene copy number, may be one explanation for increased Rb gene expression observed in some colorectal cancers (214).

The APC Gene

The key clue to the localisation of the gene responsible for FAP appeared in 1986 when Herrera and colleagues reported the case of a mentally handicapped patient with FAP who had a cytogenetically visible interstitial deletion on the long arm of chromosome 5 (215) . (Fig 2.3). This observation was quickly followed up by Bodmer et al in London and Leppert et al in Salt Lake City, who regionally localised the APC gene by linkage analysis to chromosome 5q21-22 (8,216). It was demonstrated subsequently that LOH was present in this region of the genome in 40-80% of "sporadic" colorectal tumours suggesting a tumour suppressor function for the APC gene in both hereditary and sporadic colorectal cancer (144,145).

Using a combination of yeast artificial chromosome (YAC) vectors and chromosome walking techniques, a large region of 5q21 was then cloned (217). Three candidate cDNAs were identified which mapped within a 100kb constitutional microdeletion in one FAP patient, and point mutations were detected using a polymerase chain reaction strategy in the germ-line DNAs of one of these genes. Transmission of a mutation was found to produce the FAP phenotype in the offspring of one of the patients thus confirming that APC was the gene responsible for this disorder (9).

The APC gene consists of 15 exons and has an 8.5 kb coding sequence, encoding a predicted protein product of 2843 amino-acids. The coding region can be examined by PCR using 31 pairs of primers. Exons 1-14 are small, but exon 15 is large, accounting for 77% of the coding region (9) (Fig 2.4).

To date, examination of more than 400 unrelated individuals with FAP has revealed 126 germ-line mutations, 61 of which are unique (218). More than two-thirds of the mutations are clustered in the 5' half of the last exon, with the most common mutation being a 5 bp. deletion at codon 1309 (219,220). Thirty- four percent of the mutations are point mutations which generate stop codons, 59% are insertions or deletions resulting in frame-shift mutations , 5-7% occur at splice junctions, and fewer than 2% are large deletions which remove part or all of the APC gene from the chromosome. More than 90% of the mutations detected can be considered "knockout" mutations resulting in premature termination of transcription and a truncated protein product.

Function of the APC Gene and its Role in Carcinogenesis.

The protein product of APC contains a potential coiled structure in the NH2 -terminal region, a repeated 20 amino acid sequence in the central region, and a stretch of basic amino acids in the COOH- terminal region (9). A search of the protein database predicted weak sequence homology to the G-proteins, and originally it was thought that the APC protein might be involved in signal transduction across the cell membrane, given the function of G proteins as coupling factors (221).

APC protein is localised in the cytoskeleton (222) and the presence of short coiled regions within the APC protein suggests that the molecule may have the ability to form dimers with other molecules as well as APC protein itself (223). This means that the site of the mutation, and hence the length of the truncated peptide may determine the ability of mutant APC protein to form dimers, modifying function and influencing phenotypic expression.

It is now known that APC protein associates with alpha and beta catenin- components of the adhesional cell molecule E-cadherin complex (223,224). Cadherins are cell surface molecules that mediate calcium-dependent intercellular interactions, and are important for morphogenesis (225). Loss of E-cadherin function or alpha catenin has been shown to be associated with invasion and metastasis in colorectal cancer models (226). Binding to catenins is essential for E-cadherin function and it has been suggested that catenins bind the cadherins to the cytoskeleton (227). It is postulated that the APC protein may modulate interactions between the catenins and the cadherin molecules, and control pathways critical for epithelial growth (228).

Mutations of the APC gene have been identified in 60% of sporadic colorectal cancers and 63% of sporadic adenomas, with nearly 2/3rd of the mutations occurring in exon 15 (146). This frequency may be an underestimate, as the promoter region and introns of the genes have not been studied, and present methods of analysis may miss mutations.

APC mutations have also been identified in breast, pancreatic, gastric and oesophageal cancer , suggesting a more general role for the APC gene in malignant disease (229-231).

Animal Models

A murine equivalent of FAP has been described, which has been named " multiple intestinal neoplasia" or M.I.N.. Mice expressing this trait develop large number of adenomas in the small and large bowel and are susceptible to gastro-intestinal cancer (232). Like FAP, this trait is transmitted as an autosomal dominant, and linkage analysis has shown cosegregation with a nonsense mutation in the murine homologue of the APC gene (233). Furthermore, inactivation of the remaining allele of the murine APC has been demonstrated in 100% of tumours from Min mice supporting Knudson's hypothesis (234,235).

Recently, a frame-shift mutation was introduced at codon 1638 of the mouse APC gene by homologous recombination in embryonic stem (ES) cells. Both the corresponding chimeric and heterozygous animals developed epithelial hyperplasia, adenomas and adenocarcinomas of the small intestine within 20 weeks of birth (236).

Predictive DNA Testing

Linkage analysis is a powerful diagnostic tool which permits presymptomatic diagnosis in 95% of at risk individuals with a predicted accuracy of 98% ((Fig. 2.5)(237). A disadvantage of this technique is that a DNA sample has to be available from at least two affected family members to establish linkage relationships. When linkage analysis is not possible, other methods of presymptomatic diagnosis are required to identify the mutations within each family. These methods include single strand conformational analysis (SSCP) (238) denaturing gradient gel electrophoresis (DGGE) (239), in-vitro reverse transcription assays (240), functional assays (241) and heteroduplex analysis (242). Each of these methods has advantages and disadvantages, and it is difficult to apply specific tests to an extended group of unrelated individuals as the spectrum of mutations identified is very large. Once a nucleotide alteration has been detected however, the region of interest can be sequenced, the exact site and character of the mutation identified, and a more user friendly direct method developed for screening family members.

Overall, the causative APC mutation has been identified in only 30% of the patients with FAP reported in the literature (243).

MCC gene

While searching for the APC gene, another gene termed MCC (Mutated in Colorectal Cancer), centromeric to APC was cloned from the region of chromosome 5q21 (244). MCC has a 2.5 kb coding sequence, 17 exons and encodes an 829 amino acid protein with a very short region of homology to the G protein m3 muscarinic acteylcholine receptor. A rearrangement disrupting the coding region was found in one colorectal tumour, and two additional tumours were found to contain somatically acquired mutations which resulted in amino-acid substitutions (244). Up to 55% of large bowel cancers contain deletions involving the MCC locus (245,246), and it has been postulated that MCC may represent another tumour suppressor gene. In common with the APC gene, the MCC gene also contains many coiled regions, and it has been proposed that the two gene products may have the capacity to dimerise with one another to form a biologically active complex (247). If this is the case, a mutation in either gene might have the potential to inactivate the entire complex.

The P53 Gene

The P53 gene is the most ubiquitously mutated gene in human malignancy, and increased expression of this gene has been identified at both mRNA and protein level in a wide variety of tumours (248). The product of p53, a 393 amino-acid nuclear phosphoprotein, was originally identified in1979 by Lane and Crawford through its ability to form a tight complex with the large T antigen of SV40 DNA virus (249). The p53 oncoprotein was found to be present in large quantities (5-100 fold) in transformed cells in culture, but in very low quantities in normal tissues. This was principally because mutant p53 oncoprotein is stable, whereas wild-type p53 is unstable with a very short half life (250).

The p53 gene maps to chromosome 17p13.1, encompasses 16-20 kb of DNA, and is composed of 11 exons, the first of which is non-coding and is localised 8-10 kb from exons 2-11 (251). There are five highly conserved regions among the amino-acid

residues, 13-19, 117-142,171-181, 234-258, and 270-286 (252) . All point mutations affect the sequence specific binding domains of the p53 gene and result in amino acid substitutions (251). The spectrum of mutations appear to be tissue specific and in colorectal cancer, the majority of mutations occur in codons 175, 248, 278 and 282 (252,253).

Evidence for a role for p53 in malignancy is derived from at least 6 lines of experiment:

1) Loss of heterozygosity in the region of the p53 locus can be demonstrated in over 70 % of all sporadic and familial colorectal cancers , and sequence analysis reveals point mutations in nearly 80% of all tumours (177,254-255)

2) Wild type 53 transfected into colorectal cancer cell lines is capable of reverting the transformed phenotype (197)

3) Germ-line mutations in the p53 gene are associated with the rare autosomal dominant Li-Fraumeni syndrome, in which affected individuals have an increased risk of developing breast cancer, soft tissue sarcomas, brain tumours and leukaemia (256,257). However, for reasons not yet clear, patients with the Li-Fraumeni syndrome do not appear to be at increased risk of developing colorectal cancer (258).

4) Total loss of normal p53 function in transgenic mice significantly increases the susceptibility of the mice to a broad spectrum of tumours including sarcoma and lymphoma, although the mice are born normal (259). This contrasts with total loss of Rb gene function which has been shown to be essential for normal mouse development (207,260).

5) Loss of function due to the formation of stable complexes between wild type p53 and viral oncoproteins is linked to virus mediated tumourogenesis (261,262).

6) Loss of function due to the formation of stable complexes between wild type p53 and specific amplified cellular oncoproteins, such as the MDM2 oncogene product, is associated with p53 over-expression, and the development of malignancy (263-265).

Function of the P53 Gene

Although initially thought to be an oncogene, it is now recognised that p53 invariably acts in a dominant negative manner, and can perhaps more accurately therefore be classified as a tumour suppressor gene (157). The p53 protein normally exists as a tetramer or a higher order aggregate, meaning that 4 or more identical copies of the p53 protein assemble to form a single functional unit. This molecular architecture renders the p53 protein particularly susceptible to mutational inactivation, for heterodimer formation may compromise the function of the entire molecule, explaining the spectrum of inactivation caused by over a hundred mutations, characterised in this gene (252).

Current thinking is that p53 plays a vital role in cell cycle regulation (266), DNA transcription (267), genome surveillance (157), and programmed cell death or apoptosis (268). Such pleiotropy may be due to the fact that the p53 protein induces the transcription of several different genes containing a specific DNA sequence in the upstream regulatory region (269,270). Recently it has been demonstrated that p53 promotes the expression of a gene called WAF1/Cip1, whose protein product p21 can bind to cyclin dependent kinase and halt cell division in mid-cycle (271). This gives the cell an opportunity to either to correct DNA errors or trigger programmed cell death. A point mutation can eliminate this surveillance capability of the p53 gene leading to a pool of proliferating cells, increased mutation and neoplasia.
P53 mutations may be caused by ultraviolet light (272,273), radiation (274) or fungal toxins (275). The cancer specific spectrum of these mutations makes it possible to generate hypothesis regarding the nature of the carcinogen in a series of tumours (251).

The DCC Gene

Chromosome 18 is a common site of cytogenetic abnormalities in colorectal cancer (276), and non random allelic deletion on chromosome 18q is observed in over 70% of large "sporadic" adenomas and carcinomas (277). This suggests that this region may be mutated at an intermediate stage of the adenoma-carcinoma sequence. A mapping study identified that most allele loss was centred at the18q21 locus , and a contiguous stretch of DNA comprising 370 kilobases was cloned in this region. One probe known as p15-65 pinpointed a homozygous deletion in one of the cancers examined, and a large gene, called DCC (**D**eleted in **C**olorectal **C**ancer) was subsequently identified (277).

The DCC gene is approximately 1.2 megabases in length, and encodes a transcript of 12.5 kb . The predicted amino acid of the specified cDNA was highly similar to the neural adhesion molecules and related surface glycoproteins, containing 4 immunoglobulin like domains, multiple repeats of fibronectin type III domain, and a cytoplasmic domain not related to any previously identified gene product (277). Neural adhesion molecules may be important in the regulation of cell to cell interaction, alterations of which have been noted in several studies of neoplasia (278-280)

Three lines of evidence suggest that DCC has a tumour suppressor function. Firstly this gene is expressed in most tissues, including normal colonic mucosa, but has reduced expression in colorectal cancer (277). Secondly, there is a high frequency of allele loss in LOH studies (177). Finally, the transfer of chromosome 18 into a colorectal cancer cell line has been found to suppress tumour growth on soft agar, and tumour formation in athymic nude mice (196).

There are no reports of germ-line mutations in the DCC gene in HNPCC kindreds, but the DCC gene is very large and mutations may be difficult to identify (281).

Mismatch-Repair Genes

In an exhaustive linkage analysis study involving over 350 genetic markers in two large HNPCC kindreds from USA and New Zealand, it was demonstrated that predisposition to early onset colon cancer was unequivocally linked to a marker on chromosome 2p (282). This locus was subsequently designated COCA1 in the Genome Database (283).

Loss of heterozygosity for 2p markers in HNPCC tumours was investigated because a tumour suppressor mechanism was presumed to be responsible for increased susceptibility to cancer in these families. Surprisingly, LOH was not observed in any of the 14 HNPCC tumours studied. Instead, for microsatellite markers, tumour DNA exhibited alleles that were not present in normal tissue DNA, suggesting that replication errors (RER) had occurred during tumour development (284).

Complex repair mechanisms exist within all cells to correct errors of replication during mitosis. One such system, Long patch mismatch repair, has been well characterised in bacteria, and the family of proteins that comprise this repair system are highly conserved through evolution with homologous systems in the yeast Saccharomyces Cerevisiae and in humans (285). Studies in yeast and bacteria had shown that mutations affecting these systems could lead to microsatellite instability (286), and this suggested that similar mutations might be responsible for the microsatellite instability observed in a significant percentage of HNPCC tumours.

By exploiting homology to the yeast proteins, the human homologue of MSH2 was finally mapped to the COCA1 region on chromosome 2p. Candidate genes within this region were identified and sequenced, and a search was made for germ-line mutations in HNPCC kindreds. In one of these genes, hMSH2, mutations were identified in a highly conserved region between codons 615-788, which cosegregated with colorectal cancer in the HNPCC kindreds, thus proving that hMSH2 was one of the HNPCC genes (110).

It has been shown since that purified hMSH2 protein efficiently and specifically binds DNA containing Insertion-Deletion-Loop (IDL) mismatches of up to 14 nucleotides, suggesting a direct role for hMSH2 in mutation avoidance in human cells (287).

Replication errors have not only been identified in colorectal cancers derived from chromosome 2p linked families (288), but have also been found in endometrial tumours from HNPCC patients (289). Furthermore, adenomas from HNPCC families often exhibit RER, whereas in sporadic adenomas, RER is rare (290). Thus, the presence of RER in colorectal tumours may prove valuable for identifying those individuals likely to carry a mismatch-repair mutation.

It has been shown also that RER phenotype in colorectal cancers may reflect a more general defect resulting in hypermutability of expressed genes. Mutations at the hypoxanthine phosphoribosyl transferase (hprt) gene were studied in RER and non RER colorectal cancer cell lines. Increased mutation rates of greater than 100 fold were found in RER compared to non RER cell lines (291).

A second locus that segregates with some cases of HNPCC was mapped using linkage analysis to chromosome 3p21-23, and RER was shown to occur in a tumour from a family that was linked to this locus (111). The gene responsible, hMLH1 was subsequently cloned. It has been shown to cover approximately 58 kilobases of genomic DNA and contain 19 exons, and has homology to the bacterial mutL homologue (109).

Two other human mismatch-repair genes, PMS1 and PMS2 have since been identified (108).

Clinical Application

In large HNPCC kindreds, linkage analysis with chromosome 2p and 3p markers may be sufficient to provide adequate data for clinical use. The nature of the disease, however, means that such families are relatively rare, and a direct search for germ-line mutations in affected individuals using mutation analysis will probably be the most common method of investigation (110).

It is too early to speculate whether the identification of hMSH2, hMLH1, PMS 1 and 2 genes will have any implications for population screening, as relatively little is presently known about the frequency and penetrance of these genes.

Putative Colorectal Cancer Genes

Chromosome 8p is the third most common site of LOH in colorectal cancer studies (177), occurring in about 10% of adenomas (292), 50% of cancers (293) and 90% of colorectal cancer cell lines (294). The lower frequency of LOH detected in adenomas suggests that putative genes(s) may be involved in progression to a more malignant phenotype. Abnormalities on chromosome 8p have also been reported in hepatocellular and lung cancer (295), prostate cancer (296), bladder cancer (297) and glioblastoma (298). In the case of colorectal cancer, at least two tumour suppressor loci may be implicated (299).

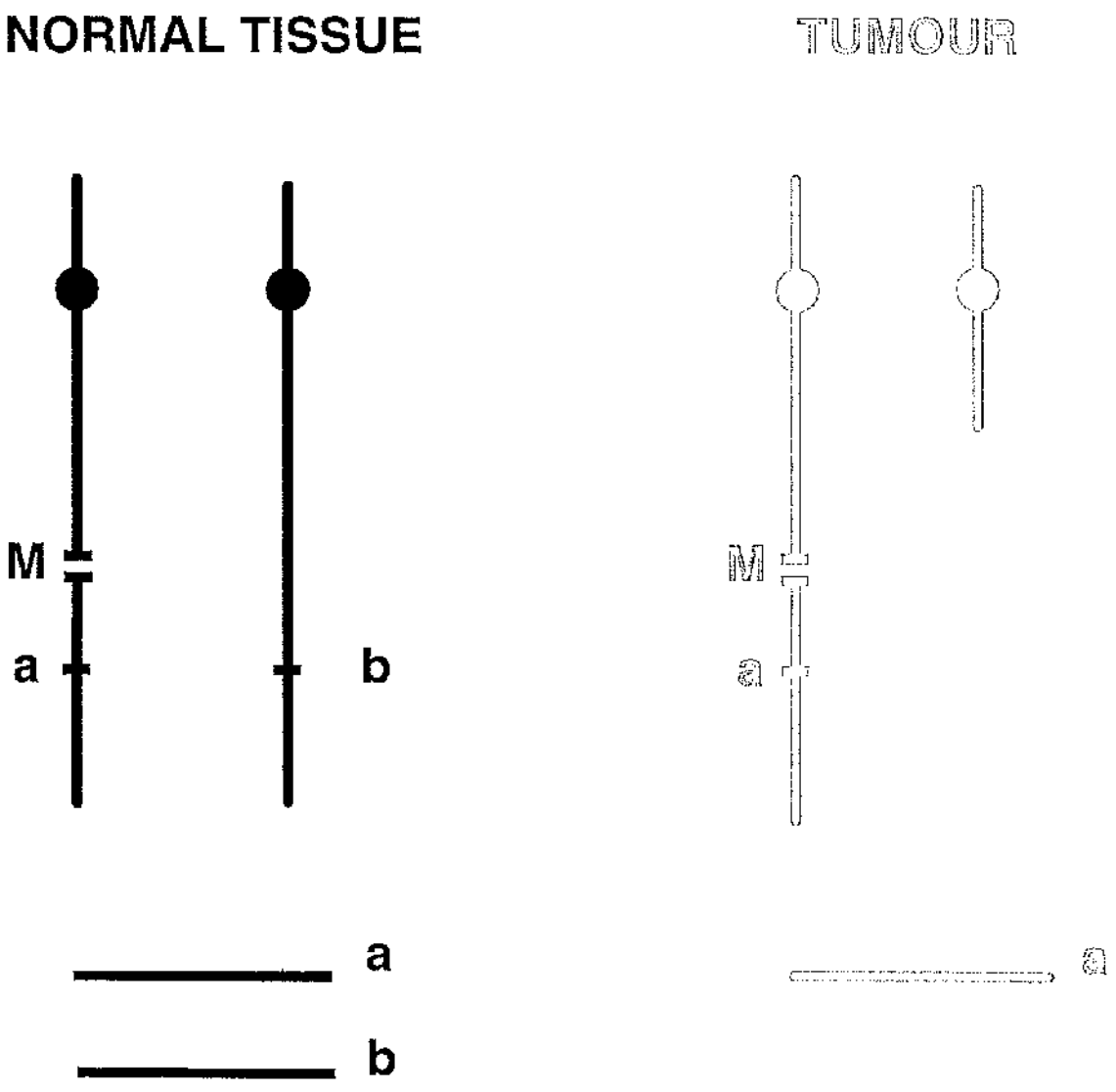


Figure 2.1 Diagram illustrating Loss of Heterozygosity in DNA from matched Tumour/Blood Pairs

M = Marker

a and b = alleles

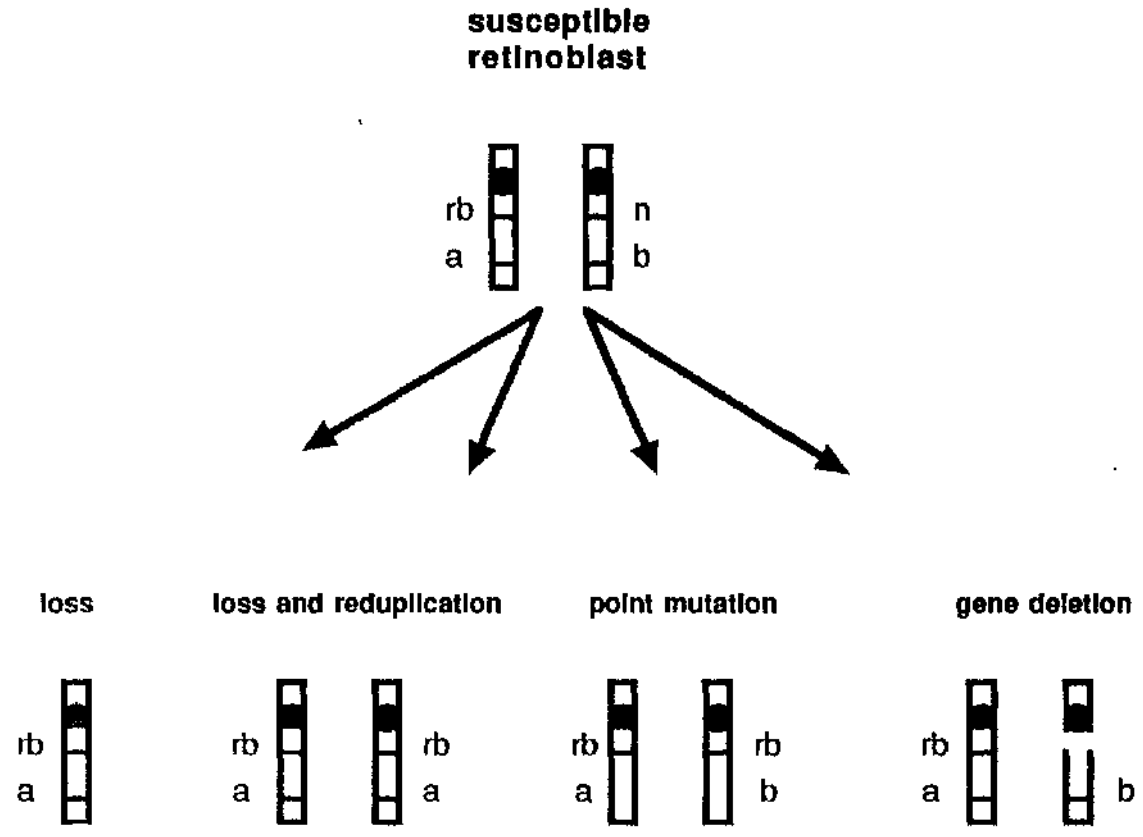


Fig 2.2 Mechanisms of Loss of Heterozygosity

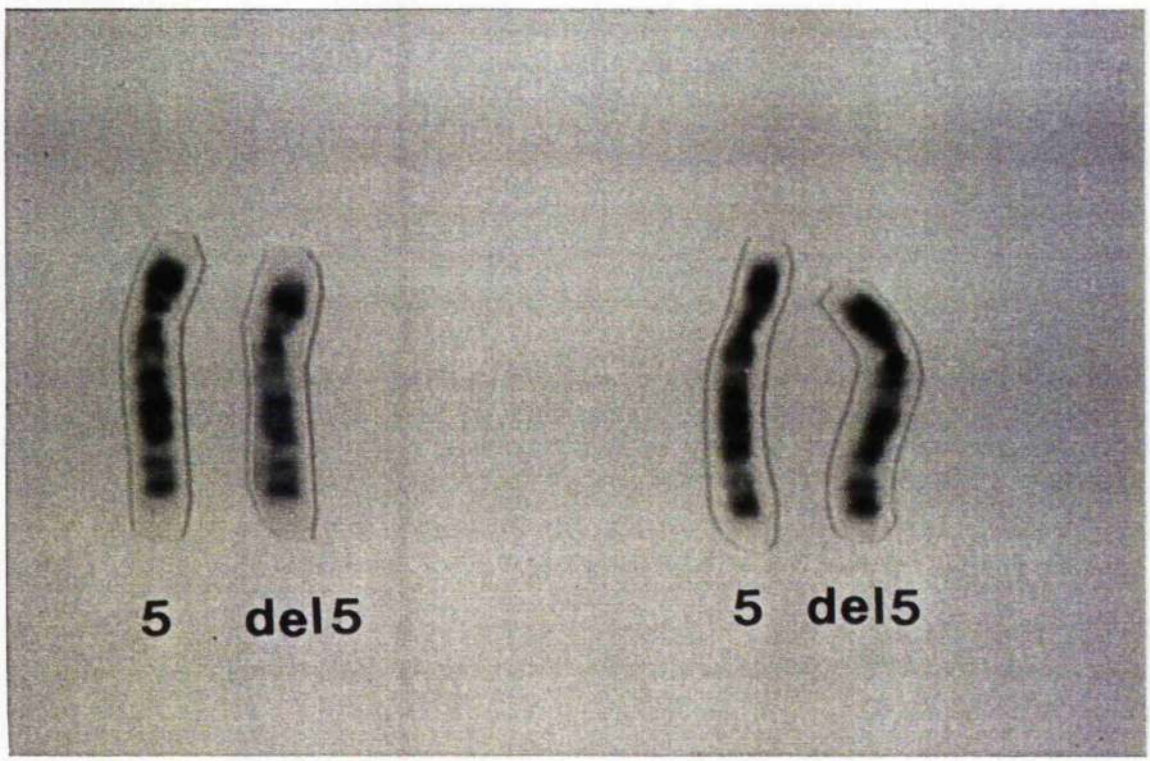


Fig 2.3 Cytogenetic deletion on chromosome 5q in two patients with FAP.

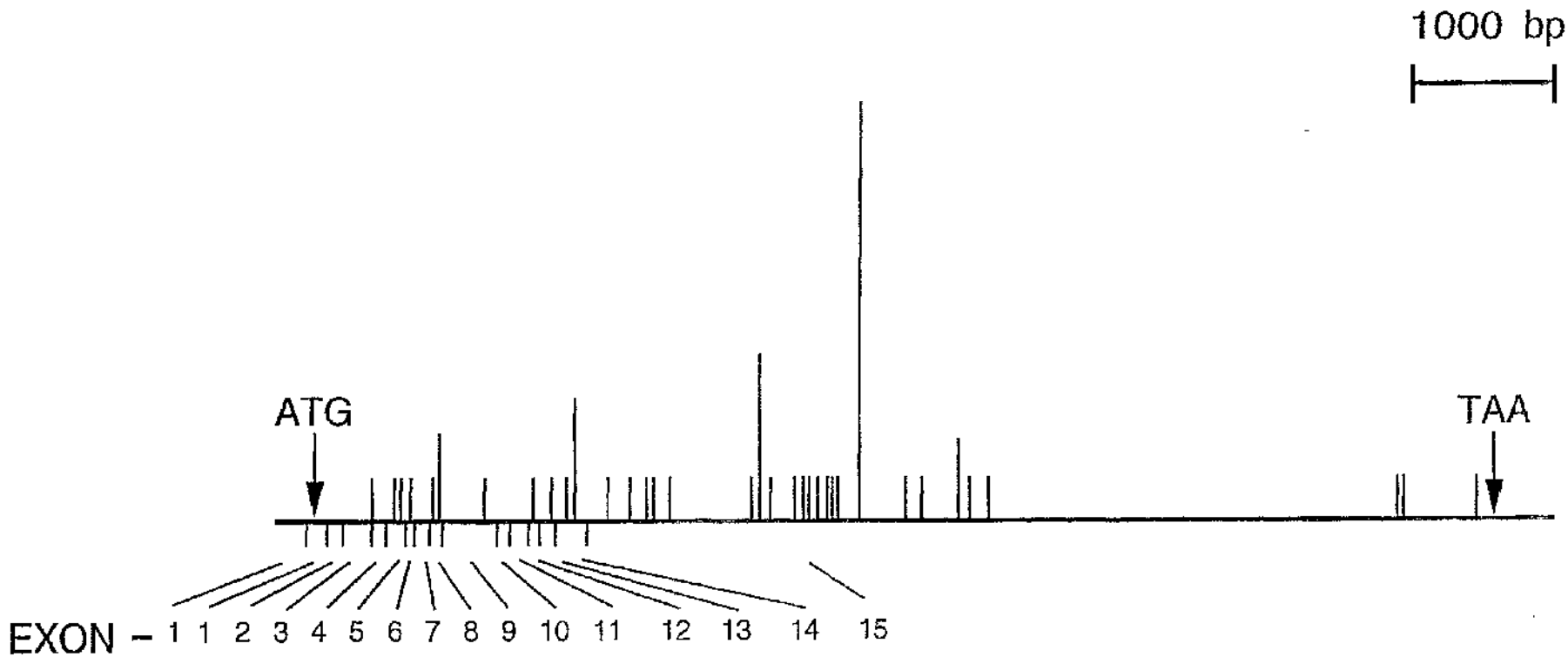


Fig. 2.4 Spectrum of germ-line mutations identified within the *APC* gene. The length of the bar indicates the number of mutations at the indicated position. Positions of translational initiation (ATG) and termination (TAA) codons are marked with arrows. (219)

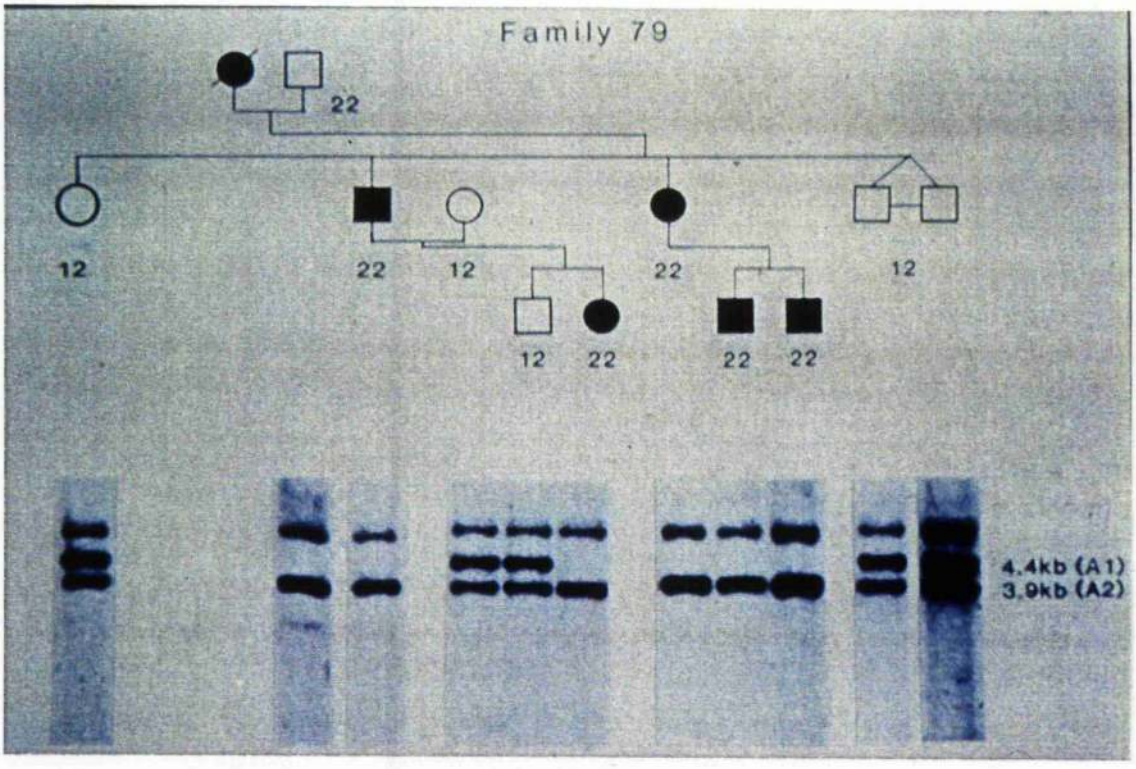


Fig 2.5 Linkage analysis in a family affected by FAP.

Chapter 3

Strategies for Prevention

" The best single measure of progress against cancer is a change in the age-adjusted mortality rate associated with all cancers combined in the total population. According to this measure, we are losing the war against cancer..... A shift in research emphasis, from research on treatment to research on prevention, seems necessary if substantial progress against cancer is to be forthcoming" (300)

Introduction

In 1992, there were approximately 20,000 deaths from colorectal cancer in the United Kingdom, and 30,000 new registered cases (301). Scotland has one of the highest incidence of colorectal cancer in the world with one in twenty- three men and one in thirty-three women expected to develop colorectal cancer during their lifetime (302). Within Scotland, colorectal cancer is three times more common in the north than in the south of the country (303). Approximately three-quarters of all patients are beyond hope of a cure at presentation, giving a poor overall five year survival rate (304,305).

Primary Prevention

Epidemiological and migration studies suggest that nutritional factors account for the vast majority of "sporadic "colorectal cancer cases, although, as discussed previously, there may be wide genetic variation on the effects of dietary carcinogens within different populations. Puerto-Rican born residents in New York, for example, are twice as likely to develop colorectal cancer as those living in Puerto-Rico, and similar changes in incidence and mortality have been observed for Hispanic migrants to New Mexico and Los Angeles (306).

Pin-pointing the specific carcinogens responsible has proven to be an elusive goal. Epidemiological studies of colorectal cancer have well recognised limitations (307), and there is some evidence that different carcinogens may be involved at different stages of carcinogenesis. An autopsy study in Northern Norway, found no difference in the prevalence of adenomas compared to the population of Oslo, yet the incidence of colorectal cancer was 70% higher in Oslo (308,309)

In 1971, Denis Burkitt put forward his now famous hypothesis of a fibre depleted aetiology for colorectal cancer. This was based on his careful clinical observations of

the difference of patterns of disease between Western and traditional African societies. Burkitt noted the rarity of colorectal cancer in Africa compared to the Western countries and observed that African diets were generally higher in fibre and lower in refined carbohydrates than in Western equivalents (310).

Kune et al compared quantitative dietary history in 715 cases and 727 age and sex matched community controls, and found that a diet containing a high intake of fibre, vitamin C, fish and pork exerted a protective effect, whereas the converse was true when a large amount of beef was eaten (310).

Since these pioneering observations, there have been many other similar fibre related epidemiological studies, although many have failed to support Burkitt's attractive hypothesis (311,312).

A meta- analysis of 12 methodologically sound and descriptively complete case controlled studies demonstrated only a slight protective effect for a high fibre diet with an odds ratio of 0.57 (313).

Willett et al reported important information on the 10 year follow up of 98,464 nurses who completed a dietary questionnaire in 1980. Of the 88,751 responses, 150 came from nurses who had developed colorectal cancer. After adjusting for energy intake, the trend for risk for total fat intake ($p< 0.05$) and animal fat intake ($p< 0.01$) was significant. Relative risks were highest using a ratio of total intake of red meats to total intake of fish, the highest versus the lowest quintile for this ratio gave a relative risk of 2.49 (314)

Winawer conducted a time trend case controlled prospective study in which serum cholesterol studies were available for up to a 10 year period prior to a diagnosis of colorectal cancer. Although the cholesterol level tended to increase slightly over time in the general population, the level of cholesterol in patients destined to develop colorectal cancer fell on average by 13%, a difference which reached statistical significance at the time of diagnosis (315).

Eskimo populations whose main source of fat is fish oil (omega 3 fatty acids) have a low incidence of colorectal cancer, and this has been attributed to the anti-prostaglandin effect of these oils (316).

Attempts to modify experimental carcinogenesis in animals have demonstrated a large arsenal of pharmacological agents which can inhibit or block the various steps of carcinogenesis (317-322). In humans, several retrospective studies have suggested a possible protective effect for aspirin therapy (323,324).

Demonstrating benefit prospectively for a chemopreventive agent is riddled with complexity There may be little information regarding appropriate dose and schedule of the chemotherapeutic agent to be studied, and end points other than mortality may be difficult to measure and interpret. The value of histological biomarkers as end-points has been questioned (325).

The greatest barrier to effective primary chemoprevention measures, however, are probably both educational and psychological in nature. A recent Scottish Opinion Survey, for example, showed that 69% of Scottish men claim to be extremely healthy eaters, and would not consider changing their eating habits, yet up to a fifth of men and an eighth of women never eat fresh fruit or green vegetables. Even more alarming was the finding that a high proportion of Scottish children eat neither green vegetables or fruit (326).

Thus, primary prevention is unlikely to be successful until a) specific dietary carcinogens have been identified, b) there is a willingness to modify diet at population level and c) the general public perceive colorectal cancer as a major health problem (327).

Secondary Prevention

Secondary prevention is the detection of *early*, usually asymptomatic disease using a test which allows earlier treatment and results in improved outcome. The main technique used is *screening*, where an attempt is made to divide the population into those who test positive, and therefore likely to have or develop the disease in question, and those who are test negative, and probably do not have the disease. Test-positive cases require further investigations to establish whether they actually do have the disease, while test negative cases should require no further investigation. The ability of any screening test to detect those with the disease is expressed by its sensitivity; exclusion of those without the disease is defined as its specificity.

Colorectal cancer fulfils only some of the criteria considered important by the World Health Organisation for screening (328). It is undoubtedly a major health problem in Western Europe and North America, and technology exists to identify and treat early disease with minimum morbidity. It does not meet other criteria, in that presently, there are not adequate resources in the community for treating those who give a positive test, and the cost effectiveness of colorectal cancer screening has not been determined.

Adenoma-Carcinoma Sequence- The Basis for Screening

Colorectal Cancer is an unusual malignancy in that it has a clearly identifiable precursor lesion, the adenoma, which has a relatively long premalignant phase (Fig 3.1). The ease with which this lesion can be detected and removed using fibre-optic technology means that colorectal cancer can truly be considered a preventable disease (Fig 3.2).

Histo-pathological and epidemiological evidence for an *adenoma-carcinoma sequence* is considerable:

• The development of CRC in patients with FAP is almost inevitable if colectomy is not performed and the risk of carcinoma increases with increasing interval from the time of FAP diagnosis (329).

• When the incidence of CRC and adenomas in a population are plotted against age, the curves for adenomas and carcinomas are very similar, with the peak in incidence for adenomas preceding that for carcinoma by 4 years (330).

• Patients with CRC frequently have benign adenomas elsewhere in the bowel at the time of diagnosis of the index cancer (331,332).

• At 25 year follow up, metachronous primary cancers are more common in those who had pre-existing adenomas at the time of the index primary than those who did not (333), and patients with a history of colonoscopic polypectomy have a six-fold increased risk of subsequently developing colorectal carcinoma (334).

• Elements of benign adenomatous tissue are found at the periphery of about 14% of carcinomas (Fig. 3.3). In a series of 1,961 malignant tumours examined at St Mark's Hospital between 1957 and 1968, there were 278 (14.2%) in which there was evidence of contiguous tumour with either a tubular or predominantly villous component. In all cases the benign component had identical histological features to adenomas which were wholly benign (332).

• Populations with little or no risk of developing carcinoma (for example some areas of Japan and the South African Bantu) have a very low incidence of large bowel adenomas. In one study involving 14,000 autopsies of South African Bantus, not one single adenomatous polyp was identified, and during a 12 year period at the Baragwanath Hospital (2,000 beds), only 6 adenomas were submitted to the hospital laboratory for pathological examination (335).

• Areas of epithelial dysplasia are only seen in adenomas or in long-standing ulcerative colitis (332).

Post-mortem and colonoscopy studies suggest that 20-30% of asymptomatic persons in the fifth decade and 40-60% thereafter develop colorectal adenomas (336-338). Clearly the vast majority of adenomas do not turn malignant, but certain pathological features such as size (>1 cm.), villous architecture and severe epithelial dysplasia are associated with increased risk of malignant conversion (332). Morson established that malignant conversion in polyps under 1 cm. in diameter is extremely rare, occurring in about 1 in 100 polyps; between 1 and 2 cm., the risk increases to about 1 in 10, and over 2 cm there is over a 50% malignancy rate (332).

Evidence documenting adenoma growth rate is scanty, as polyps diagnosed during life are usually removed at colonoscopy. In a retrospective radiological study of 226 symptomatic patients with untreated large adenomas (>1 cm.), a cumulative risk of a diagnosis of cancer at the site of the index polyp as 2.5% at five years, 8% at 10 years, and 24% at 20 years has been calculated (339). Winawer has recently shown that an interval of three years between colonoscopy examinations in those found to have adenomas can be considered safe in the vast majority of cases (340).

Polypectomy and Colorectal Cancer Reduction

There have been no reported randomised colonoscopy studies demonstrating that polypectomy reduces population mortality from colorectal cancer. The ease with which polyps can be removed at colonoscopy would make such a study in many people's minds unethical. Nonetheless, there is strong circumstantial evidence to suggest that this hypothesis is true.

Murakami et al compared relative risks for cancer over a mean period of 6 years in patients who had total colonoscopy in Osaka, Japan. With age and sex matched standardised population indices: relative risk in 760 patients in whom no polyps were found at initial colonoscopy was 1, while the risk in whom polyps were found was 5.1. The latter group was subdivided into those having a polypectomy (relative risk 2.3) and those who had only biopsies (relative risk 8). Although those treated by

biopsy only had small adenomas, they still had an excessive risk for cancer, with the polypectomy group having incomplete but some protection (341).

In a further study, a cohort of 1418 patients who had adenomas removed at colonoscopy were followed up for a mean period of 5.9 years, during which the patients underwent further colonoscopy. The incidence rate of colorectal cancer was compared in this cohort with that in three reference groups; two cohorts in which polyps were not removed, and one general population registry after adjustment for age and sex. Five asymptomatic and no symptomatic cancers were diagnosed in the follow-up cohort. In contrast, number of cancers expected on the basis of the rates in the three reference groups were 48.3, 43.4 and 20.7, for reduction in the incidence of colorectal cancer of 90, 88, and 76% respectively ($P < 0.001$) (340).

Mass Screening

Most attempts at mass population screening for adenomas and curable Dukes A carcinomas have depended on faecal occult blood testing (FOBT), the detection of occult blood in the faeces using a guaiac based test that detects the peroxidase like activity of haematin (342,343). The advantages of FOBT as a test are that it is cheap, safe and relatively simple to use and interpret. Its main disadvantages are its low sensitivity, low specificity, and poor compliance (344-47).

Mortality is the only truly reliable indicator of a successful cancer screening programme, because the stage of the cancer detected and the length of survival are affected by:

1) lead time bias, in which screening brings forward the date at which the diagnosis of the cancer is made, and this will appear to prolong survival even if the date of death is the same and,

2) length bias, in which screening detects a disproportionate number of slow growing tumours, automatically reducing the distribution of stage and increasing survival times.

There are presently five on-going prospective randomised clinical trials to determine whether mass screening with FOBT will reduce population mortality from colorectal cancer (348-352). Collectively, these trials involve over 250,000 subjects. No survival benefit has yet been announced from the Nottingham study which is the largest of these trials.

Targeted Screening

Using mathematical models to determine the best approach towards decision making and screening, Eddy has proposed that detection rate for early asymptomatic disease could be improved several fold if the screening programme concentrated on patients with known high risk parameters, such as strong family history, a history of adenomas or cancer, or long-standing ulcerative colitis (353).

Individuals with inherited susceptibility undoubtedly constitute the largest high risk group, and it is now possible to identify some gene carriers using genetic markers, making family history even more relevant. The Kings Fund Forum has recommended that a comprehensive family history should be taken as part of the assessment of all individuals with colorectal cancer (354).

The experience of the United Kingdom's first clinic specifically established to counsel and screen individuals with a strong family history of colorectal cancer will be discussed in the next chapter.

Fig 3.1 Benign tubular adenomas

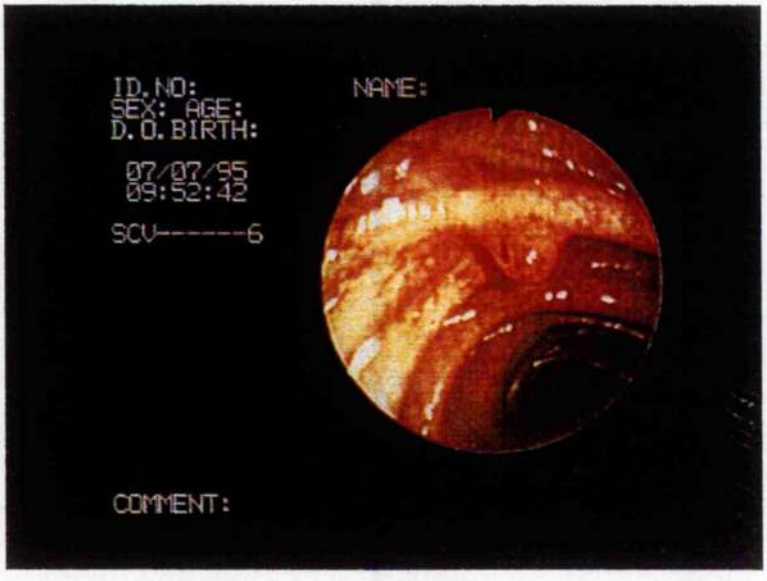


Fig 3.2 Appearance of adenoma at colonoscopy

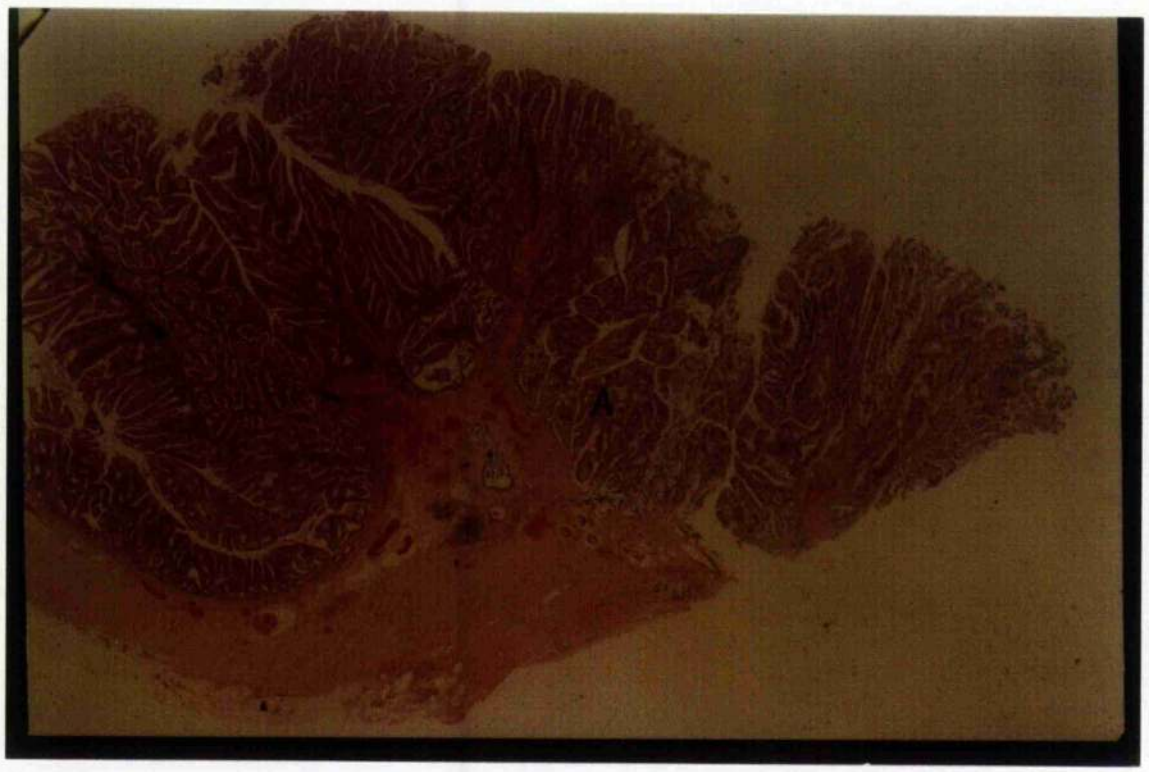


Fig 3.3 *A* lies to carcinoma side of adenoma/carcinoma junction.

Chapter 4

The St Mark's Family Cancer Clinic

Introduction

In 1986, a clinic was established at St Mark's Hospital, London, to provide genetic counselling and screening for relatives of patients who had a strong family history of non-polyposis colorectal cancer (103). Families with familial adenomatous polyposis, the Peutz-Jeghers and juvenile polyposis syndromes were followed up at a separate clinic in the same hospital. Although, the primary interest of the clinic was colorectal cancer, advice and counselling were also provided for persons with a strong family history of breast, gastric, ovarian cancer and melanoma.

Patients and Methods

The St Mark's Family Cancer Clinic was financially supported by the Imperial Cancer Research Fund and the National Health Service and individuals could either refer themselves directly or be referred by a hospital consultant or general practitioner. The clinic took place twice a month, and was staffed by a consultant in clinical genetics, a genetic nurse counsellor, a clinical research fellow, a secretary and a laboratory assistant, who received,processed and stored blood samples for DNA extraction. Information about the existence of the clinic was publicised both in the national press and in women's magazines. The endoscopic service was provided by Dr C.B. Williams, Consultant Gastroenterologist, and surgical expertise was provided by Mr J.M. Northover, Consultant Surgeon, St Mark's Hospital, London.

At the time the clinic was initiated, there was no universally accepted protocol for screening first degree relatives with a strong family history of colorectal and other cancers. The observed two to four-fold relative risk of developing cancer in first degree relatives of cases was not considered accurate enough for screening purposes, and risk estimates were calculated and stratified using pedigree analysis (Table 4.1)(103).

At interview, information was obtained about the age, number and relationship of relatives affected by colorectal cancer or adenomas. Family history was ascertained for at least three generations and verified where possible from death certificates, pathology and medical reports. For the purposes of classification and research, the "Amsterdam criteria" were adopted for the diagnosis of the HNPCC syndrome, although the limitations of these criteria are well recognised (355).

Using a protocol drawn up by Slack and colleagues(103), individuals with two or more first degree relatives (FDRs) with CRC, or one FDR affected under the age of 45 years, or a family history of more than two generations affected by CRC, were offered surveillance by colonoscopy on a five yearly basis from the age of 25 years, increasing to 3 yearly if colorectal adenomas were detected at any stage. In numerical terms, this was estimated to represent an increased life-time risk of one in ten or greater, a threshold chosen for pragmatic as well as economic considerations. Some individuals who were excessively anxious were offered screening by colonoscopy, even if they did not fulfil the stated criteria. At total colonoscopy, all polyps detected were removed for pathological examination except where surgery was required. In this situation, the polyp was biopsied only, and further histology was obtained from the surgical specimen. All polyps were reported by Dr Jeremy Jass and Dr Ian Talbot, Consultant Pathologists at St Mark's Hospital, and classified according to the WHO criteria (356). Where colonoscopic surveillance was not offered, screening for faecal occult blood was arranged on an annual basis. If the F.O.B.T. test was positive on any occasion, or the patients developed symptoms suggestive of large bowel neoplasia, they were referred for urgent colonoscopy. Any patient who gave a history of rectal bleeding, altered bowel habit or other symptoms suggestive of large bowel neoplasia was referred for further investigation and treatment. On average 8-10 individuals were counselled during each clinic session, and each interview lasted 30-40 minutes.

Statistical Analysis

A multivariate analysis was performed to assess the association between family history variables and the frequency of adenomas or cancer detected at colonoscopy using a forward stepwise logistic regression (BMDP-LR computing Facility, UCLA) (357). A significance level of $p< 0.05$ was used for the inclusion of a variable in the model. In the multivariate analysis the family history variables were coded as shown in Table 4.2. Age was entered either as a continuous or categorical variable (with categories 25-34, 35-44, 45-54,>55). Statistical Analysis was performed by Drs Wendy Atkin, and Pierro Gaglia, ICRF.

Opthalmological Examination.

Patients who had developed colorectal cancer under the age of 40 years or who had been found to have multiple adenomas at colonoscopy were referred for opthalmological evaluation to Professor Barry Jay, Moorfields Hospital, London, to determine whether retinal pigmentation diagnostic of CHRPE was present.

Skin Examination

Patients suspected of having the Muir-Torre, Cowden's , the Dysplastic naevus Syndromes or any skin lesion with features of malignant melanoma were referred to Dr Julia Newton, Consultant Dermatologist, Royal London Hospital, for further opinion .

Results

Between August 1986 and March 1993, 1283 persons attended the St Mark's Family Cancer Clinic. Twenty-seven per cent of referrals came from general practitioners, 27% were self referrals, 25% were from members of families previously ascertained, 19% were from hospital consultants and 2% were from other registries.

636 individuals from 436 families fulfilled the stated criteria for colonoscopy. Seven declined the offer of colonoscopy (compliance rate = 99%), so 629 individuals were available for study. The family history characteristics are summarised in Table 4.2.

In addition, 3 families with FAP were identified, one family with possible attenuated FAP, one with the Muir-Torre syndrome and one with possible Cowden's syndrome.

The age at colonoscopy varied from 25 years to 77 years (median = 41 years). Two hundred and sixty five (41.1%) subjects were male; the percentage of males was similar in each group category (range 39.5% - 42.8%)

Repeat colonoscopies were performed in forty-two cases, six after a 5 year interval (initial screen normal), and the others after a 3 year interval.

Incidence of Adenomas Diagnosed

One or more adenomas were found in 144 (22.9%) subjects and of these, 21 had synchronous metaplastic polyps. Twenty- nine (4.6%) patients had metaplastic polyps only. Of subjects with adenomas, 91% had a single adenoma and 7 (4.9%) had five or more adenomas (range 6-300). Such multiple adenomas were more frequent in patients with "Amsterdam criteria" families than from other families (11.8% and 2.7% respectively; p=0.03) (Table 4.3).

Distribution of Adenomas in the Large Bowel

Forty-six of the subjects with adenomas (31.9%) had lesions proximal to the splenic flexure only. There was a significant difference in the proportion of right sided adenomas between patients from the families with "Amsterdam criteria" and from other families (47.1% and 27.3% respectively; p=0.03), whereas no significant difference was found between males and females (30.4% and 33.8% respectively) (Table 4.3)

One 50 year old woman with a pedigree consistent with HNPCC was found on colonoscopy to have about 300 adenomas. There were no adenomas present in the rectum and she did not have the retinal pigmentation characteristic of FAP. Her affected relatives had less than 10 adenomas present on examination of the colon.

Size and Histology of Adenomas Detected

Fifty nine (41.0%) of subjects with adenomas had only a single diminutive adenoma (< 5 mm.) (32.9% males and 50.8% females, p=0.03). Thirty nine (27.1%) of adenoma patients had an adenoma which was large (> 10 mm..) , tubulovillous, villous , moderately severely dysplastic or malignant. The frequency of these unfavourable pathological features was higher in men than in women (31.6% and 21.5% respectively) and in the "Amsterdam criteria" versus other families (35.4% vs. 24.5%), but the numbers were too small too reach statistical significance (Table 4.3).

Age and Sex Distribution of Adenomas Diagnosed

The prevalence of adenomas increased with age, being less than 10% under the age of 35 years and rising to over 40% from 55 years, with an increasing odds ratio(OR) of approximately two fold for each decade (p<0.0001). The prevalence of adenomas was about two-fold greater in males than in females (OR=2.05, p=0.0002) (Table 4.4)

Cancers Detected

Seven cancers (six Dukes' stage A and one Dukes' C) were diagnosed. Five of the cancers appeared to arise within adenomas and two developed in the presence of synchronous adenomas). The median age at diagnosis was 49 years (range 34-63 years); three patients were male. Four of the patients belonged to an "Amsterdam criteria family", three did not. All cancers,but one (in a 63 year old woman), occurred proximal to the splenic flexure, and none of these cases were associated with the presence of distal adenomas (Table 4.5.).

Family Variables Associated with Increased Risk of Adenomas

Eight family history variables were chosen and the age and sex adjusted odds ratio of having adenomas or cancer for each was examined (Table 4.6) After adjustment for age and sex, the most significant indicators of the risk of having adenomas was:

• The number of generations in the whole family affected by either CRC or adenomas (OR for >2 generations = 2.16, p=0.0006).

• The pedigree type ("Amsterdam criteria" families versus other families = 1.76, p=0.02).

Screening individuals with > 2 generations affected by colorectal cancer (390 individuals, 60.6% of the screened population; adenoma prevalence = 24.9%), identified 97/144 of all adenomas (67.4%) and 5/7 cancers.

Screening individuals with a history of >2 generations affected by CRC or adenomas (431 individuals, 66.9% of those screened; adenoma prevalence = 30.0%) identified 112/144 (77.8%) of adenomas and all seven cancers (Table 4.7).

Multivariate Analysis

The results of the forward stepwise logistic regression multivariate analysis are shown in table 4.8. Age, sex and all eight family history variables were considered.

The variables selected by the procedure to enter the model were (in order of entry): age (p = 0.0001), sex (p=0.0002), and the number of generations in the family affected by either CRC or adenomas (p = 0.0006) (Table 4.8). For a given age group, the presence of one "unfavourable" condition, either male sex or > 2 generations affected by CRC or adenomas, about doubled the risk of finding adenomas, whereas the presence of both variables raised the OR to a value of 4.6 [OR = exp.(0.76 + 0.77)] (Table 4.9) .

Faecal Occult Blood Testing

Of 310 F.O.B.T. tests performed (compliance 32%), 5 were abnormal. When these patients were further investigated by colonoscopy, one patient was found to have a normal colon, one had an adenoma plus infective colitis, one had inflammatory bowel disease without polyps, one had a tubular adenoma in the ascending colon, one had multiple adenomas plus a Dukes A carcinoma in the ascending colon.

Between 1986-93, 40 patients who fulfilled the screening criteria for colonoscopy, requested F.O.B. testing while awaiting colonoscopy. Two tests were positive due to ulcerative colitis. 10 of 38 patients with negative F.O.B.T. tests had adenomas, one of which contained a focus of carcinoma in situ.

Dermatological Findings

One patient, who developed carcinoma aged 42 years and who belonged to family with a strong history of colorectal cancer, was found to have a sebaceous adenoma, and by definition had the Muir-Torre syndrome (Fig. 4.1)

Twelve patients were found to have solitary dysplastic naevi, and five patients were diagnosed with dysplastic naevus syndrome. A 53 year old woman was found to have a superficial spreading melanoma of the lower leg.

Population risk	1 in 50
One first degree relative affected	1 in 17
One first degree relative affected and one second degree relative affected	1 in 12*
One first degree relative under 45 years affected	1 in 10
Two first degree relatives affected	1 in 6
Dominant pedigree	1 in 2

Table 4.1 Lifetime risks of colorectal cancer in first degree relatives of patients with colorectal cancer (based on Lovett series (122)).

*Estimated from polygenic model.

No. with relatives affected by CRC only	451 (70.9%) from 355 families
No. with relatives affected by CRC and adenomas	185 (29.1%) from 81 families
No. belonging to families fulfilling "Amsterdam criteria" for HNPCC	127 (19.9%) from 69 families (15.8%)
No. with > 2 FDRs with CRC, or a relative < 45 years, or > 2 generations with CRC	245
No. with only second degree affected relatives (SDRs)	63
No. with one FDR only > 45 years	209

Table 4.2 Pattern of family histories of individuals referred to family cancer clinic.

Subjects with: - metaplastic polyps only = 29 (4.6%)				
- adenomatous polyps = 144 (22.9%)				
Pathological features in patients with adenomas	all patients (n= 144)	HNPCC* (n=34)	non-HNPCC* (n=110)	p**
Synchronous metaplastic polyps	21 (14.6%)	1 (2.9%)	20(18.2%)	0.04
Multiple (> 5) adenomas	7 (4.9%)	4 (11.8%)	3 (2.7%)	0.03
Dysplasia (moderate or severe)	20 (13.5%)	5 (14.7%)	15(13.9%)	NS
Villous or tubulovillous adenomas	15 (10.5%)	4 (11.8%)	11 (10%)	NS
Large (>10 mm) adenomas	18 (12.5%)	6 (17.6%)	12(10.9%)	NS

Table 4.3 Colonoscopy Findings in those Screened at Family Cancer Clinic;

*HNPCC/non-HNPCC = patients which fulfilled/did not fulfil the "Amsterdam criteria"; **HNPCC vs. non HNPCC.

NS = Not Significant (P>0.05)

Pathological features in patients with adenomas	all patients (n= 144)	HNPCC* (n=34)	non-HNPCC* (n=110)	p**
Adenomas only proximal to the splenic flexure	46 (31.9%)	16 (47.1%)	30 (27.3%)	0.03
Cancers in/with adenomas	7 (4.9%)	4 (11.8%)	3 (2.7%)	0.03
Significant findings (villous/tubulovillous, or large, or moderately/severely dysplastic/malignant polyp)	39 (27.1%)	12(35.3%)	27 (24.5%)	NS
Single diminutive (< 5 mm) adenoma	59 (41.0%)	13(38.2%)	46 (41.8%)	NS

Table 4.3 Colonoscopy Findings in those Screened at Family Cancer Clinic;

*HNPCC/non-HNPCC = patients which fulfilled/did not fulfil the "Amsterdam criteria"; **HNPCC vs. non HNPCC. Pearson c^2 test.

NS = Not Significant (P>0.05)

Variable	Category	Screened subjects	Patients with adenomas	OR	95% CI
Age	25-34*	179	16 (8.9%)	1	
	35-44	215	40 (18.6%)	2.33	1.25,4.32
	45-54	157	46 (29.3%)	4.22	2.27,7.84
	> 55	93	42 (45.2%)	8.39	4.35,16.19
χ^2 trend				51.8	
(P-value)				(0.0001)	
Sex	Females	379	65 (17.2%)	1	
	Males	265	79 (30.95)	2.05	1.41,2.99
χ^2				14.2	
(P-value)				(0.0002)	

Table 4.4 Odds ratio for adenomas by age and sex. OR = Odds Ratio; CI = Confidence Interval; * =Reference category

Age	Sex	Dukes	Site	HNPCC	Generations with CRC or Ads
34	M	A	Proximal	Y	2+
41	M	A	Proximal	Y	2+
48	F	A	Proximal	N	2+
49	M	A	Proximal	N	2+
52	F	A	Proximal	Y	2+
57	F	C	Proximal	Y	2+
63	F	A	Distal	N	2+

Table 4.5 Clinico-pathological features of cancers diagnosed.

Variable	Category	Screened subjects	Subjects with adenomas	OR	95%CI	χ^2 (P-value)
No of Relatives	1*	148	32 (21.6%)	1		0.10**
affected by CRC	2	231	47 (20.3%)	0.78	0.46,1.35	0.75
	3	138	38 (27.6%)	1.27	0.72,2.27	
	>4	127	27 (21.2%)	0.90	0.49,1.66	
No of FDRs	0*	83	16 (19.3%)	1		0.16**
affected by CRC	1	412	90 (21.8%)	0.91	0.49,1.71	0.69
	2	128	30 (23.4%)	0.71	0.34,1.50	
	>3	21	8 (30.1%)	1.28	0.42,3.94	
No of Generations	1*	254	47 (18.5%)	1		3.66
affected by CRC	>2	390	97 (24.9%)	1.49		0.056
No. of Relatives	1*	114	18 (12.5%)	1		2.86**
affected by CRC or	2	202	42 (20.8%)	1.21	0.63,2.29	0.09
Adenomas	3	141	41 (29.1%)	2.03	1.05,3.92	
	>4	187	43 (23.0%)	1.53	0.81,2.91	

Table 4.6 Age and Sex Adjusted Odd Ratio for Adenomas and CRC by Family History Variables.

CRC = Colorectal Cancer; Ads = Adenomas; FDR = First Degree Relative; *= Reference category;

** χ^2 trend; CI = Confidence Interval.

Variable	Category	Screened subjects	Subjects with adenomas	OR	95%CI	χ2trend
No of FDRs	0*	32	7 (21.9%)	1		2.04
affected by CRC or	1	399	80 (20.1%)	0.97	0.39,2.41	0.15
Adenomas	2	163	33 (20.2%)	0.77	0.29,2.01	
	>3	50	24 (48%)	2.34	0.81,6.75	
No of Generations	1*	213	32 (15.05%)	1		11.66
affected by CRC or	>2	431	112 (30.0%)	2.16	1.37,3.43	0.0006
Adenomas						
Pedigree	non-HNPCC* +	517	110 (21.3%)	1		5.08
	HNPCC	127	34 (26.8%)	1.76	1.08,2.86	0.02
No of Relatives	0*	305	79 (25.9%)	1		0.04
with early diagnosis	>1	339	65 (19.2%)	0.96	0.64,1.44	0.84
(< 50y) of CRC						

Table 4.6 Age and Sex Adjusted Odd Ratio for Adenomas and CRC by Family History Variables. CRC = Colorectal Cancer; Ads = Ads = Adenomas; FDR = First Degree Relative; *= Reference category; ** χ2 trend; CI = Confidence Interval.

	No.	(%) of total screened subjects	Patients with adenomas No.(prevalence)	Proportion of total neoplasia detected if the screening had only been performed in the group considered	
				Adenomas	Cancers
HNPCC*	217	(19.7%)	34 (26.8%)	34/144 (23.6%)	4/7
≥ 2 Generations affected by CRC alone	390	(60.6%)	97 (24.9%)	97/144 (67.4%)	5/7
≥ 2 Generations affected by CRC and Adenomas	431	(66.9%)	112 (30.0%)	112/144 (77.8%)	7/7

Table 4.7 Number of adenomas and cancers that would have been detected using the number of generations as screening criterion.

Variable	Category	Coefficient	OR	(95% CI)
Age	25-34*		1	
	35-44	0.94	2.56	(1.36,4.81)
	45-54	1.52	4.59	(2.44,8.36)
	≥ 55	2.28	9.76	94.96,19.22)
	χ^2 trend			55.46
	(p-value)			(< 0.0001)
Sex	Female*		1	
	Male	0.76	2.14	(1.44,3.19)
	χ^2			14.20
	(P-value)			(0.0002)
No.of Generations affected by CRC or Adenomas	1*		1	
	≥ 2	0.77	2.16	(1.37,3.43)
	χ^2			11.66
	(p-value)			(0.0006)

Table 4.8 Multivariate Analysis (Stepwise Logistic Regression)

*= Reference category, OR – Odds Ratio, CI = Confidence interval.

Age	Sex	No.generations CRC or Ads	Screened Subjects	Subjects with Ads (@)	Observed Prevalence	Predicted Prevalence	(95%CI)
25-34	F	1	35	3	8.6%	3.6%	(1.2,6)
	F	≥2	72	8 (1)	11.1%	7.4%	(3.4,11.4)
	M	1	14	1	7.1%	7.3%	(2.7,11.9)
	M	≥2	58	4 (2)	6.9%	14.5%	(7.5,21.5)
35-44	F	1	51	4(1)	7.8%	8.6%	(4.4,12.8)
	F	≥2	72	13 (2)	18.1%	16.9%	(10.7,23.1)
	M	1	33	6 (1)	18.2%	16.8%	(9.4,24.4)
	M	≥2	59	17 (5)	28.8%	30.4%	(21.4,39.4)

Table 4.9 Observed and Predicted Prevalences Based on the Model of Multivariate Analysis. CRC = Colorectal Cancer. Ads = Adenomas; M = Male ; F = Female; CI = Confidence Interval; @ = in brackets the number of patients with either a large adenoma (> 10 mm), a tubulo-villous or villous adenoma or a severely dysplastic adenoma.

Age	Sex	No. Generations CRC or Ads	Subjects screened	Subjects with @	Observed Prevalence	Predicted Prevalence	(95% CI)
45-54	F	1	31	3(1)	9.7%	14.4%	(7.6,21.2)
	F	≥2	64	17 (3)	26.6%	26.7%	(18.5,34.9)
	M	1	16	4(2)	25%	26.5%	(15.7,37.3)
	M	≥2	46	22(5)	47.8%	43.9%	(33.3,54.5)
≥55	F	1	19	4(3)	21.1%	26.4%	(15.6,37.2)
	F	≥2	35	13 (3)	37.1%	43.7%	(31.7,55.7)
	M	1	14	7(2)	50%	43.4%	(29.0,57.8)
	M	≥2	25	18 (8)	72%	62.4%	(50.4,74.4)

Table 4.9 Observed and Predicted Prevalences Based on the Model of Multivariate Analysis.

CRC = Colorectal Cancer. Ads = Adenomas; M = Male ; F = Female; CI = Confidence Interval; @ = in brackets the number of patients with either a large adenoma (> 10 mm), a tubulo-villous or villous adenoma or a severely dysplastic adenoma.

Discussion

It has been estimated that one in four individuals in the western world, will develop cancer at some stage in their lifetime (358). The majority of the population will have at least one relative affected by cancer at some stage, and by chance, some individuals will have many. In one survey in the USA, 6% of patients with cancer and 1.5% without, said that they had 3 or more relatives who had cancer (359). In a second survey, involving 200 consecutive patients attending an adult oncology clinic, 50% reported having at least one first degree relative with cancer (360). Dunlop has estimated that as many as a third of the population over the age of 40 years may have a single first degree relative with cancer of the breast, uterus or colon (361). Cancer is therefore an extremely common disease process, and clearly counselling all individuals with a family history of cancer would be a formidable undertaking. Furthermore, not everyone with inherited susceptibility will go on to develop cancer, and not every form of cancer will be due to inheritance. The prinicipal object of the clinic therefore, was to try and identify those individuals at most genetic risk.

It was the policy at the St Mark's Family Cancer Clinic to offer colonoscopy to those individuals with a family history consistent with a 1 in 10 chance or greater of developing colorectal cancer. In practice, the screening criteria were slightly more elastic as some extremely anxious individuals would only be reassured by a normal finding at colonoscopy.

Every effort was made to verify the cause of death in relatives by pathology and medical reports where possible. It has been noted elsewhere however, that most people recall the fates of first degree relatives with great accuracy (134).

The overall prevalence of adenomas in the present series was 22.4% (median age 41 years), and this compared with a rate of between 12-27% in other colonoscopy series where the individuals had at least one relative with colorectal cancer (124,125,135-138,362). In individuals who do not have a family history, adenomas are also

common after 50 years (337), but are relatively rare before this (125). Thus, those with a positive family history of colorectal cancer or adenomas appear to develop adenomas one decade earlier. With the exception of the youngest age band studied (25-34 yrs), the prevalence of adenomas in males was the same as that observed in females one decade later. The higher prevalence with increasing age and male sex was consistent with data from other studies involving persons with and without a family history. There was no sex difference in the size or morphology of adenomas identified. It can be seen from Table 4.3 that adenomas were more common, more often multiple and right-sided in patients belonging to families fulfilling the "Amsterdam" criteria for HNPCC, a characteristic feature of HNPCC. For reasons presently not understood, hyperplastic polyps were more common in those individuals who did not have a family history conforming to HNPCC, although there is general agreement that these lesions are not premalignant (332). Previous studies have shown that although hyperplastic polyps may increase in prevalence up to the 5th decade, they tend to be constant in frequency thereafter (336,338).

The limitations of the "Amsterdam" criteria are well recognised; extra-colonic malignancies are not taken into account and many small HNPCC families are not identified. In this study, if the "Amsterdam criteria" had been adopted for screening by colonoscopy, only 23.6% of adenomas and 4 of 7 cancers would have been detected. In an effort to refine the screening criteria, the St Mark's pedigrees were studied for more accurate predictive factors which might increase the adenoma and cancer detection rate. The only pedigree variable investigated previously has been the number of relatives (either FDRs only or FDRs and SDRs) with colorectal cancer (125,362) or colorectal cancer and adenomas (123). In all these studies, an increased risk of about two-fold was observed in subjects with > 2 versus 1 affected relatives. The predictive power of the number of generations has not previously been addressed. At the St Mark's Family Cancer Clinic, the most powerful pedigree predictor of adenoma prevalence identified was the number of generations (>2 vs. 1) affected by either CRC or adenomas, irrespective of i) the total number of relatives or FDRs

affected ii) early age of onset (<50 yrs) of cancer in the relative or iii) the presence of the "Amsterdam " criteria for HNPCC (Table 4.6). However, people with ≥2 generations affected by CRC or adenomas had more relatives with CRC than those with one affected generation only (mean number of FDRs and/or SDRs 3.1 and 1.6 , respectively, P<0.001), and a greater number of FDRs affected by CRC or adenomas (40.2% with > 1 FDRs in > 2 generation families vs. 23.8% with > 1 FDRs in one generation families, P = 0.001), indicating that the "generation" variable was not independent of the number of affected relatives and their relationship.

It can be concluded from data in this series that if colonoscopy had been offered to all individuals with ≥ 2 generations affected by CRC or adenomas, 70% of the colonoscopies would have been performed detecting 80% of the total number of adenomas and all 7 cancers (Table 4.7).

The fact that only one of the cancers had developed beyond Dukes stage A, suggests that screening in these cases, had probably improved the prognosis for these patients. With the exception of one cancer (in a 63 year old woman), all the cancers were proximal in distribution, and none was associated with distal adenomas. Therefore,it can be concluded that , for those at genetic risk of colorectal cancer, screening by total colonoscopy is more sensible than screening by flexible sigmoidoscopy (112,363).

Psychological Aspects

The general public's perception of the role of genetic factors in the aetiology of cancer may be underestimated. Linn et al studied 120 patients with advanced cancer (mixed site), and compared them with non cancer patients matched for age, sex and hospitalisation. Beliefs about the cause of cancer were measured at interview with a 10 item scale covering smoking, drinking alcohol, diet, inheritance, type of occupation, stress, medicine, water, environment, God's will, and 'other'. Interestingly, for the cancer patients, "God's will" and inheritance were listed among the top four "causes"(364). Taylor conducted a similar study among 79 women with breast cancer, and found that 26% believed they had inherited their disease (365). Even a distant family history of cancer may cause relatives lifelong, if unexpressed, anxiety about their own health and that of their children.

The majority of persons attending a family cancer clinic seek the answers to three main questions:

• Am I at increased risk of developing cancer relative to the person who does not have a family history?

• If so, what positive action can I take to reduce that risk?

• Are my children also at increased risk?

The arrival of specific genetic markers for colorectal cancer should answer all these questions for some people. For the remainder, estimation of risk will still largely depend on the taking of an accurate extended pedigree, and a certain amount of educated guesswork. Most of the persons attending the St Mark's Family Cancer Clinic, the "worried well", appeared to welcome the opportunity to discuss their concerns, and felt positive that they were taking action to reduce a perceived risk. A compliance rate for colonoscopy of 99% would support this impression.

Conclusions

• Targeted screening, using family history as a risk factor, has the potential to reduce the mortality and morbidity of colorectal cancer. A total of 644 colonoscopic examinations were required to diagnose 39 potentially "dangerous adenomas" and 7 cancers. It is difficult to estimate, how many dangerous adenomas or early cancers would have been detected by randomly screening an equivalent number of adults in the general population, but all studies to date support the premise that adenomas are more common in persons with a strong family history. Furthermore, only 42/644 individuals had a second colonoscopy, and it seems probable that the adenoma detection rate would increase on repeat screening.

• Including the "number of affected generations" variable in the screening criteria appears to increase the detection rate of potentially dangerous adenomas and early cancers, although this observation needs to be validated in other studies. It is likely that the screening criteria will be further refined with the discovery and wider availability of specific genetic markers, increasing the effectiveness of targeted screening.

• The psychology of inherited susceptibility to cancer is poorly understood, and requires further study. Qualitative factors such as relief of anxiety, reassurance and improved well being are notoriously difficult to quantify, but initial impressions suggest that family cancer clinics help relieve anxiety and are of psychological benefit.

• Public demand for cancer genetic services may be greater than expected. The establishment of family cancer clinics nation-wide should be carefully monitored, until the effectiveness of such clinics has been fully demonstrated, and major cost-benefit issues addressed. Existing clinics require long term funding, to support those individuals who have been informed that they are at a significantly increased risk of developing colorectal cancer. Self-referral should be permitted in certain

circumstances, but can be a recipe for an exponentially increasing endoscopy workload. For this reason, the advertising of family cancer clinics should be cautious and responsible.

Chapter 5

The Hereditary Mixed Polyposis Syndrome- a New Disorder?

It is an old experience that through her errors, nature often reveals secrets which would otherwise remain a closed domain" (366)

Introduction

The hereditary disorders, Familial Adenomatous Polyposis (FAP), Generalised Juvenile Polyposis (JP) and the Peutz-Jeghers Syndrome (PJS) seldom present little diagnostic difficulty as the polyps that characterise these syndromes are readily distinguishable on histological examination. Furthermore, both FAP and PJS have a recognisable phenotype. In the case of FAP, the patient usually has congenital hypertrophy of the retinal pigment epithelium (CHRPE) and multiple adenomas in the rectum, and in the case of the Peutz-Jeghers syndrome the patient usually has a history of mucocutaneous pigmentation in and around the mouth or on the fingers. Problems with classification can occur however, particularly when an individual has polyps of different histology and/or polyps with overlapping histological features. In most cases of these "mixed polyposis syndromes", there appears to be no hereditary component, and it remains to be determined whether these patients have an atypical form of a recognised polyposis syndrome, or a distinct clinical disorder. A literature search reveals, only two case reports describing a mixed polyposis syndrome with a possible familial basis, in which both a parent and child developed either "mixed" polyps or colorectal cancer. Gene localisation in these families using the technique of linkage analysis would prove extremely difficult on account of their small size (367).

This chapter will describe the clinical, pathological and genetic features of a large kindred, St Mark's Family 96, who appear to have a dominantly inherited predisposition to an atypical polyposis syndrome and early onset colorectal cancer. The natural history of this disorder is being documented, and attempts are being made to localise the responsible gene, using linkage analysis. For the time being, this syndrome will be referred to as the Hereditary Mixed Polyposis Syndrome (H.M.P.S.).

The Proband

A 28 year old man presented in 1956 to St Mark's Hospital, London, with a 6 month history of bright rectal bleeding and lower abdominal colic. Abdominal examination was unremarkable, but sigmoidoscopy revealed multiple "adenomatous looking" polyps in his rectum. A provisional diagnosis of familial polyposis coli (FAP) was made, on the grounds that several of the patient's close relatives had died at a relatively early age from colorectal cancer. The extra-colonic features commonly associated with FAP - such as sebaceous cysts and osteomas - were absent, and physical examination was otherwise unremarkable. A subtotal colectomy with ileorectal anastomosis was performed and the patient was kept under regular medical surveillance. The resected surgical specimen is shown in Figure 5.1. In contrast to FAP where the entire colon is usually carpeted with hundreds if not thousands of adenomas, only 6 polyps were identified in this specimen, 5 tubular adenomas and 1 juvenile-type polyp with overlapping histological features (Figs 5.2 and 5.3). Microadenomas, pathognomonic of FAP were absent. This patient has been followed up on an annual basis from 1956-93. He remains asymptomatic, but every so often develops further polyps in the rectum which require fulguration by diathermy.

Subjects and Methods

Other members of this kindred were ascertained by communication with the proband, and confirmation of disease status was obtained from pathology, medical and colonoscopy reports, operation notes, and death certificates. The number, site and histology of any polyps were carefully documented, as was the diagnosis of malignancy. Where possible, the polyps were classified by three specialist pathologists (Dr Ian Talbot, St Mark's Hospital, Professor Jeremy Jass, University of Aukland and Professor Stanley Hamilton, Johns Hopkins University, Baltimore) according to WHO criteria (356). Affected individuals were examined for skin pigmentation, skin cysts, lipomas, osteomas and CHRPE, all features commonly associated with other polyposis syndromes .

Results

St Mark's Family 96 has been followed up nearly 40 years, and over this period, the pedigree has been extended and updated (Fig. 5.4). It consists of 10 second generation, 35 third generation, 63 fourth generation and 42 fifth generation individuals. All surviving members are derived from the third, fourth and fifth generations, and updated clinical information has been obtained on 71 patients over the age of 21 years. The family originated in Lithuania and can be traced back to a marriage in 1897. They are now spread worldwide, living in the USA, the United Kingdom, Australia, Hong Kong, Netherlands, South Africa, Israel and Hawaii. Some members of the family died in the concentration camps between 1941-42, and possible survivors of this branch of the family are untraceable. Thirty three members (13 females, 19 males) are known to have developed either colorectal cancer or colonic polyps. In common with other more clearly defined polyposis syndromes, the Hereditary Mixed Polyposis Syndrome appears to be inherited in an autosomal dominant manner.

Patients presented at a median age of 40 yrs (range 23-65 yrs) with bright rectal bleeding, abdominal colic, altered bowel habit, anaemia or intestinal obstruction. 161 polyps have been examined and classified according to the WHO criteria of Morson and Sobin (2). The polyps, usually numbering fewer than 10 at initial examination, are distributed throughout the entire large bowel, as are the colorectal cancers. Polyp number and histology vary between patients, but essentially there are 6 types (Table 5.2):

1) tubular adenomas (Fig. 5.4)

2) villous adenomas

3) hyperplastic polyps

4) atypical juvenile polyps with mixed histological features (Figs 5.6, 5.7,5.8)

5) peutz-jeghers polyps and (Fig 5.9,5.10)

6) the flat adenoma (Fig.5.11)

One patient had multiple tubular adenomas < 1 cm. scattered throughout the colon, which were considered too numerous to count.

Six patients had "mixed" polyps in addition to other polyps, and one patient had "mixed" polyps only.

Eleven individuals with multiple polyps have undergone total colectomy and ileorectal anastomosis, and the remaining individuals with polyps are presently being screened and treated by colonoscopic polypectomy. Several unaffected members of the pedigree also undergo regular colonoscopies. Thirteen individuals have developed colorectal cancer (median age at diagnosis 47 yrs, range 32-74 years) and the first recorded case was in a woman in her 40s in 1913 (Table 5.1).

There have been two cases of breast cancer in women over the age of 70 years (II3, II8), but neither was found to have polyps at colonoscopy. One woman (II1), a non-smoker, developed bronchial carcinoma aged 57 years. It is not known whether this individual had colorectal polyps, but it seems likely that she was a gene carrier as 4 of her children developed multiple polyps and/or colorectal cancer. The possibility remains that her bronchial carcinoma was a actually a metastasis from a colorectal primary cancer.

There has been no family history of CHRPE, desmoid disease, osteomata or bone cysts. One patient had an epidermoid cyst removed from his face, and another was known to have a lipoma overlying his left scapula. These skin lesions are associated with FAP, but sporadic cases are frequent. The characteristic mucocutaneous pigmentation of PJS was absent from the family.

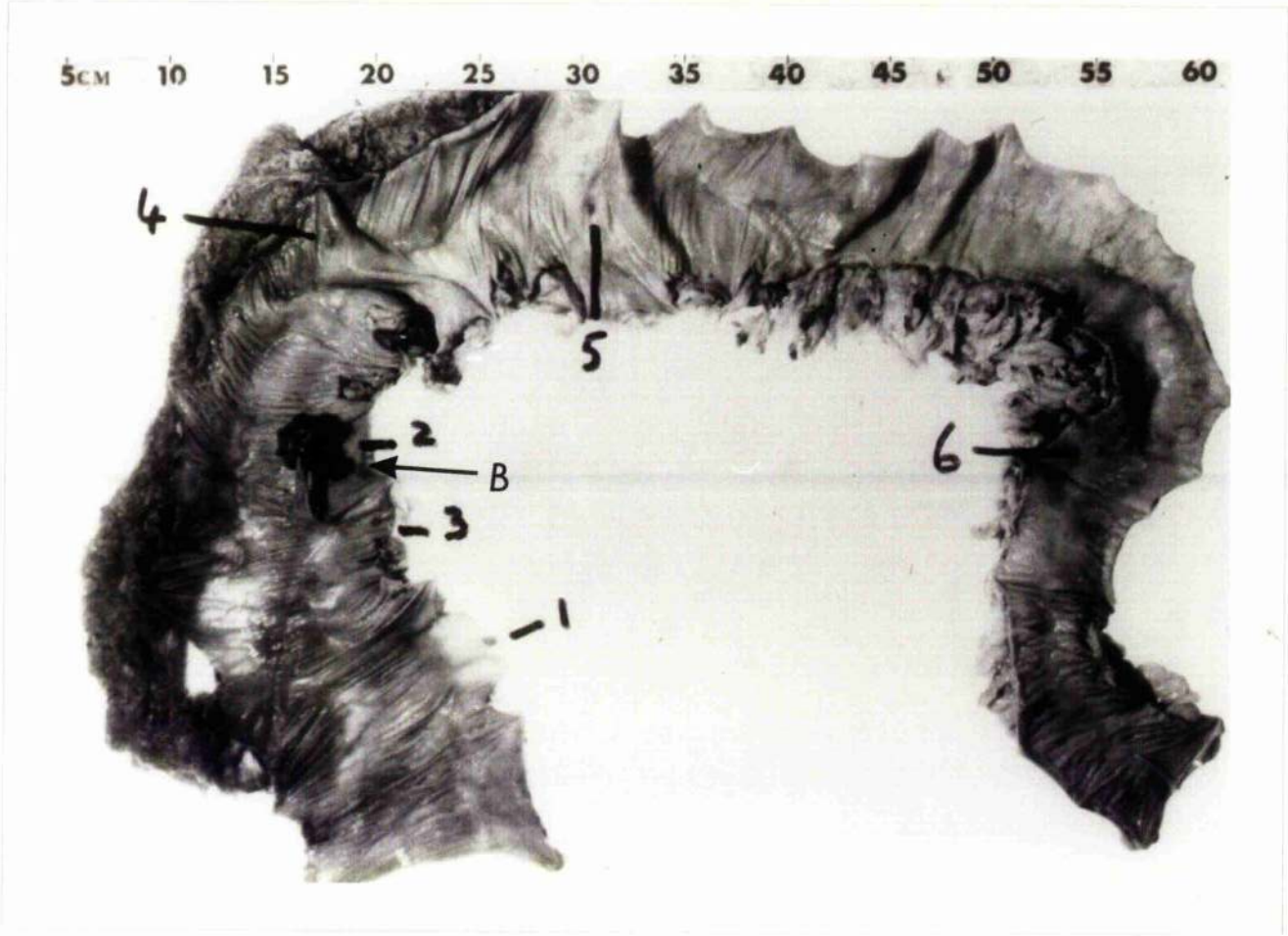


Fig.5.1 Colectomy specimen from 26 year old proband A). Note the relative paucity of polyps compared to FAP, and the large atypical polyp (B).

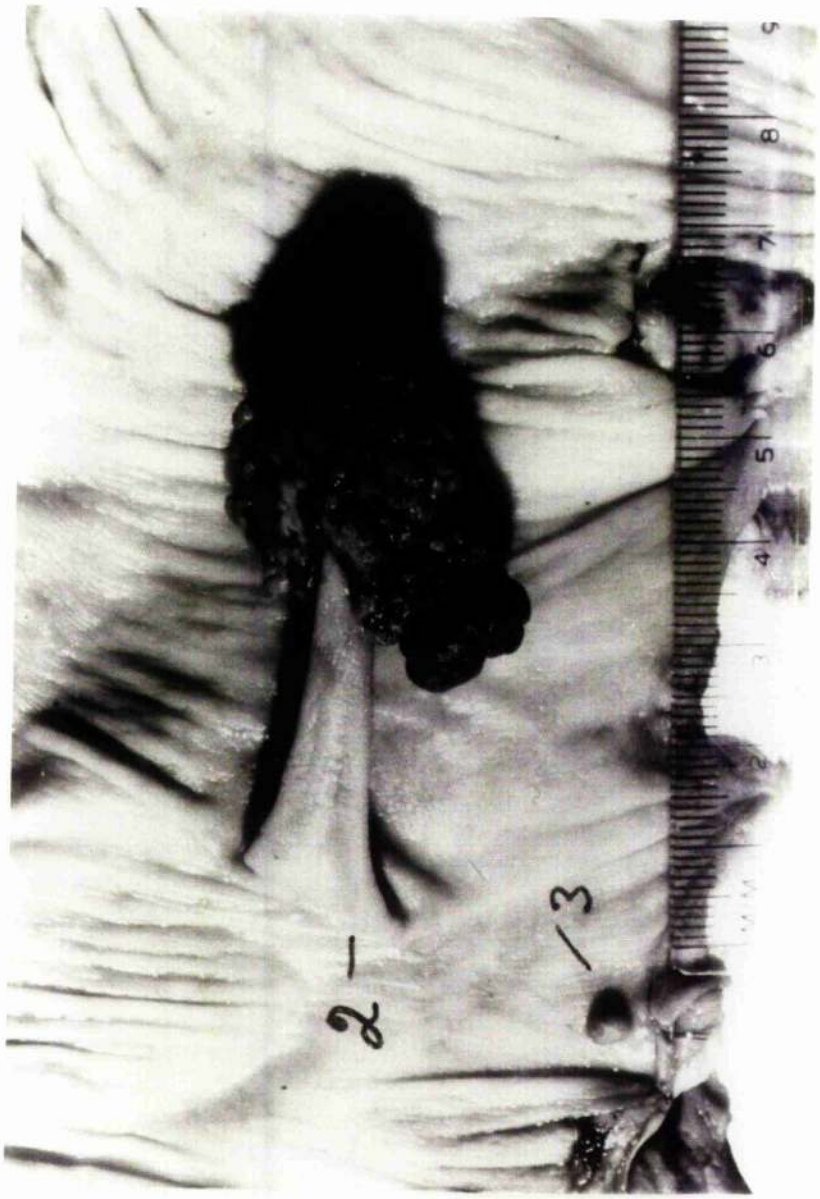


Fig. 5.2 Low power view of atypical mixed polyp (B)

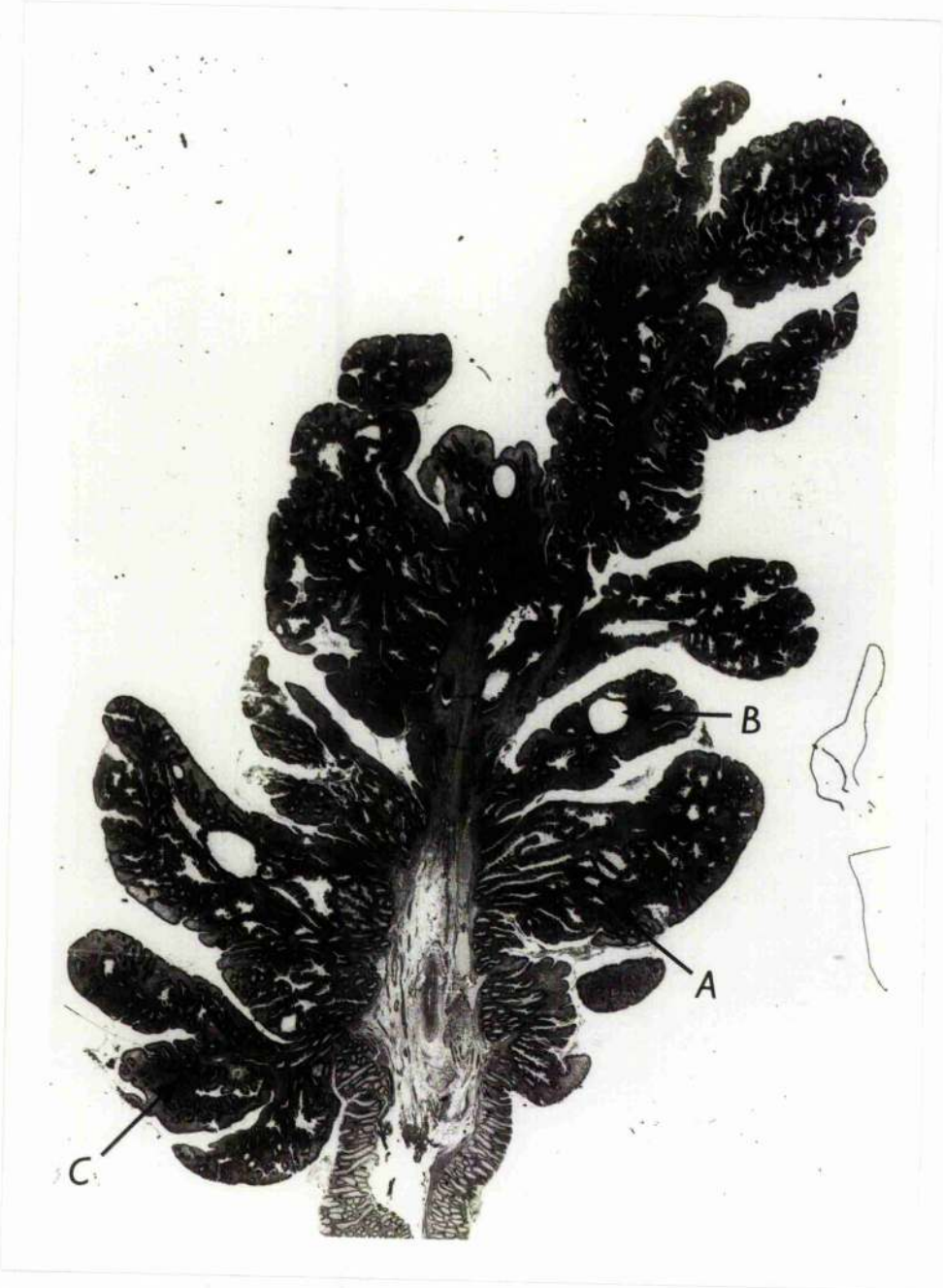


Fig. 5.3 A higher power appearance of atyical mixed polyp (X 7) A= hyperplastic area, B = juvenile area, C = adenomatous area.

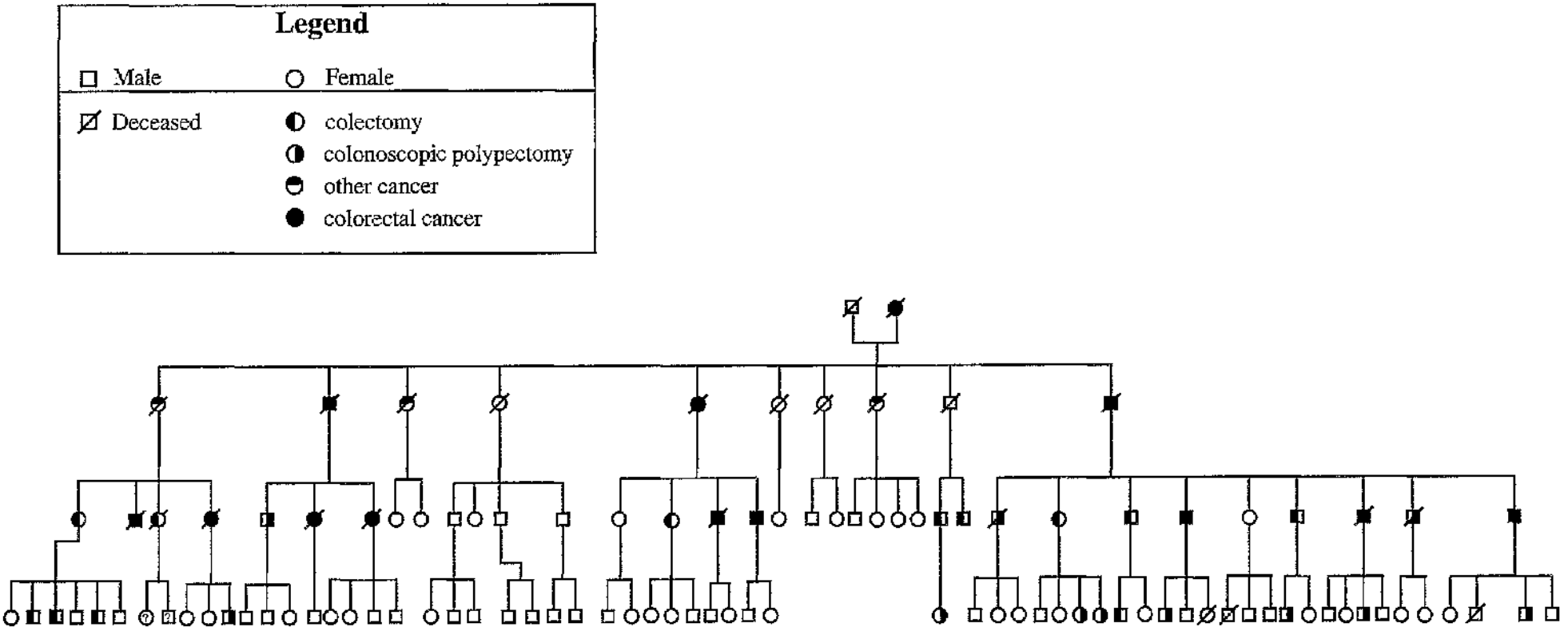


Fig 5.4 Pedigree of St Mark's Family 96

Fig. 5.5 Tubular adenoma (X 4)

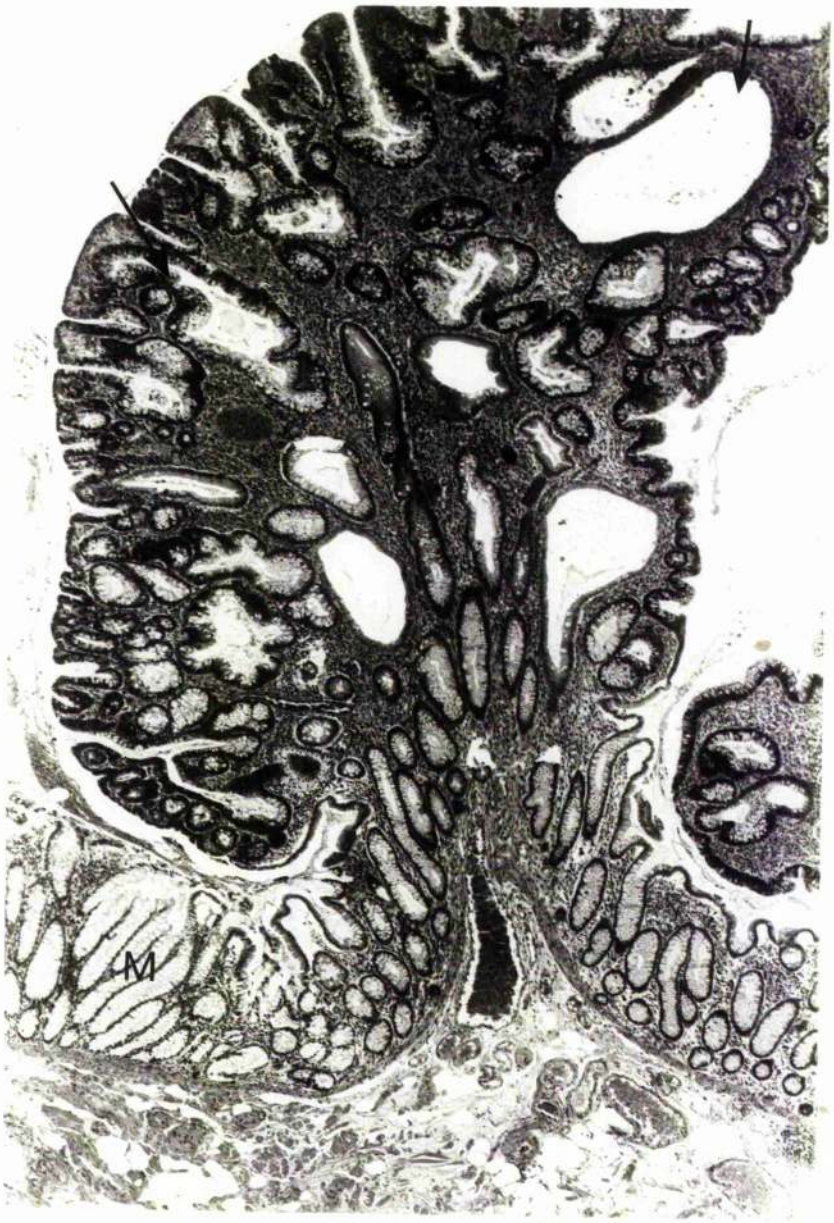


Fig 5.6 Polyp with irregularly shaped and focally dilated glands arising from mucosal surface (M),(X 40). Note, the hyperplastic area with characteristic papillary infolding (epithelial serration) adjacent to narrow tubular gland (*long arrow*) and juvenile area with abundant lamina propria and retention cystic space (*short arrow.*)

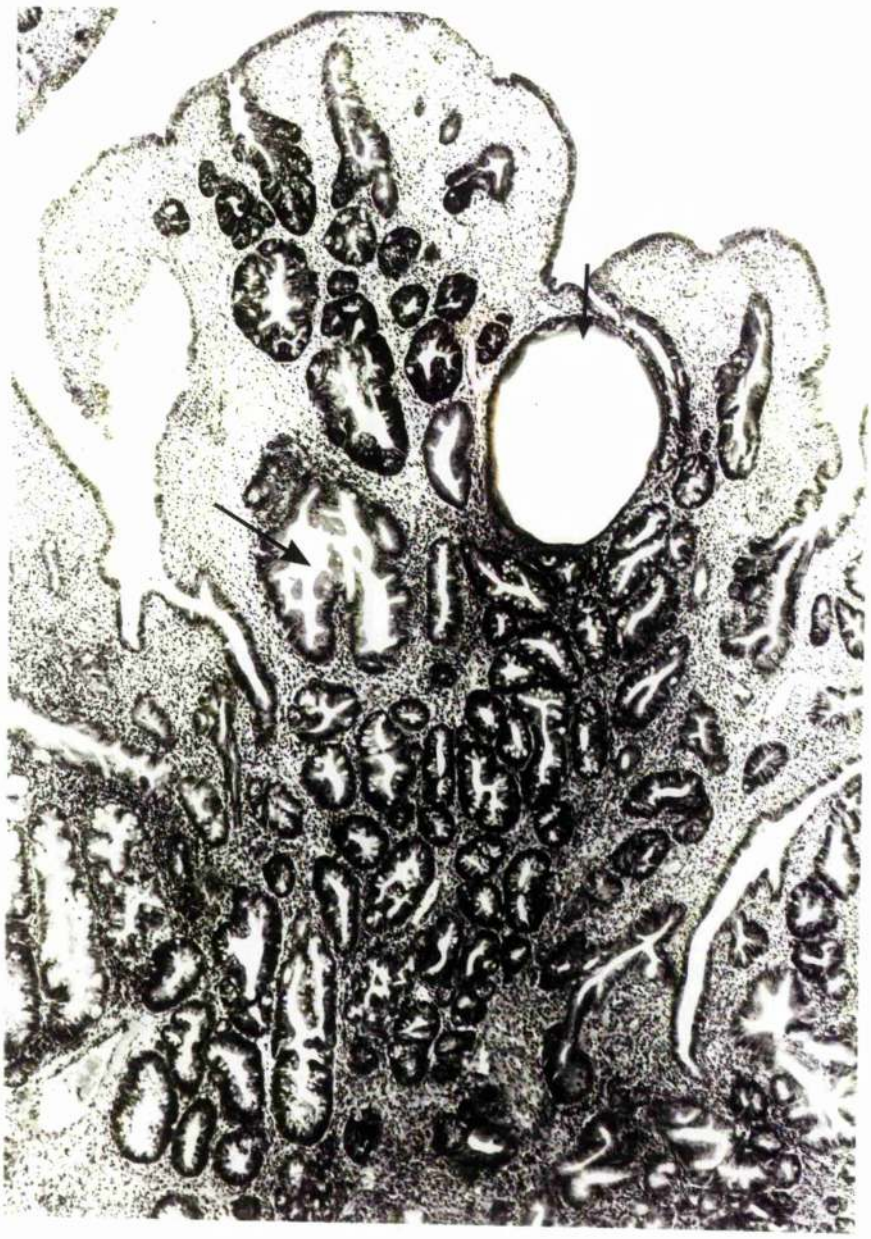


Fig. 5.7 A mixed polyp showing glandular crowding with mild epithelial dysplasia centrally. Superficially, there is focal gland dilatation (short arrow), and irregularly shaped glands (long arrow) (X 44).

Fig. 5.8 A mixed polyp showing severe epithelial dysplasia. The glands on the left exhibit nuclear enlargement, stratification and pleomorphism. Mitotic figures are readily identified (X 351)

Fig. 5.9 Part of a Peutz-Jeghers polyp showing typical branch like pattern (X 10).

Fig 5.10 High power of a Peutz-Jeghers polyp showing typical smooth muscle bundles (X 200)

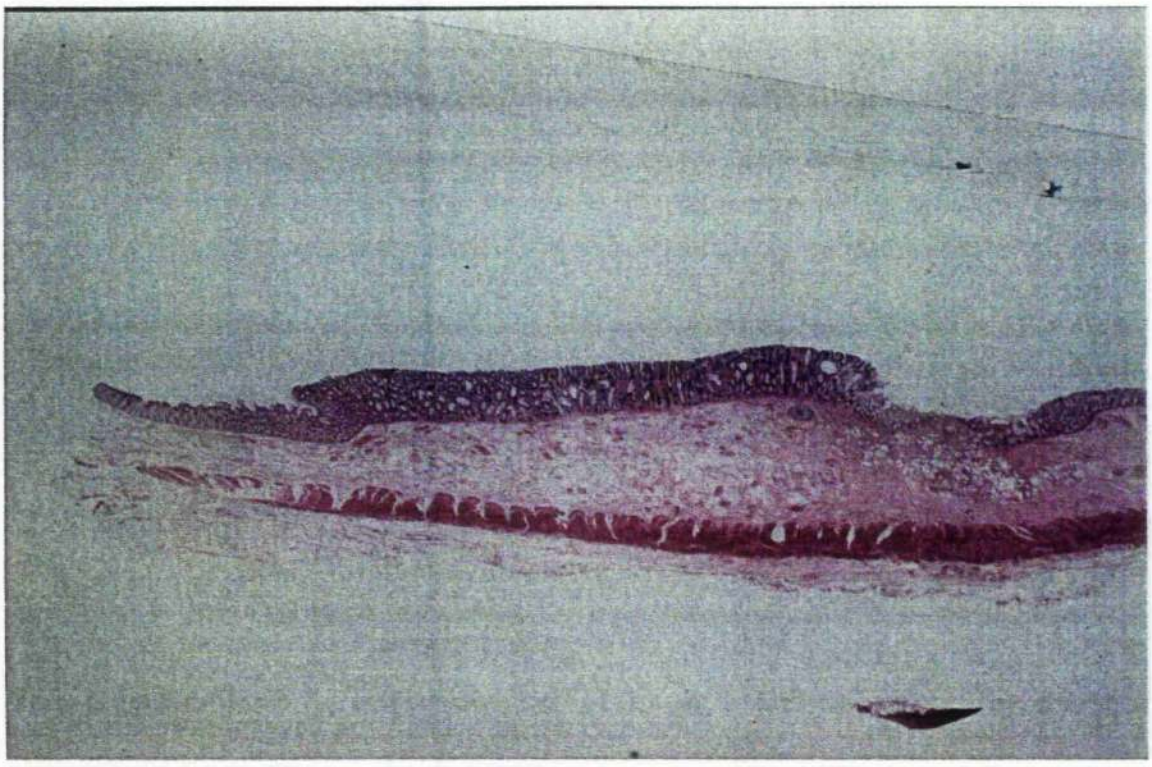


Fig 5.11 A flat adenoma found in the Hereditary Mixed Polyposis syndrome (x 5)

Patient	Location of Colorectal Cancers	Source	Age/Sex
I2	Ca Colon (Colostomy)	FH	40s/F
II2	Ca Colon (Colostomy)	FH	47/M
II5	Ca Sigmoid	MR	65/F
II10	Ca Rectum	FH	45/M
III 2	Ca Caecum	PR	47/M
III 4	Ca Caecum	MR	60/F
III 6	Ca Splenic Flexure	MR	74/F
III 7	Ca Transverse Colon	PR	49/F
III16	Ca Sigmoid Colon	MR	41/M
III17	Ca Rectum	MR	32/M
III30	Ca Caecum	PR	40/M
III33	Ca Transverse Colon	PR	58/M
III35	Ca Caecum	PR	51/M

Table 5.1 Colorectal Cancer Site/Character in St Mark's Family 96

FH = Family History, MR = Medical Report, PR = Pathology Report

Histology of polyp	No. identified/no. patients
Tubular adenomas	101/20
Villous adenomas	7/4
Hyperplastic Polyps	25/7
Juvenile type polyps with Mixed Features	25/7
Flat adenoma	1
Peutz-Jeghers polyps	2/1
Total	161/23

Table 5.2 Histology of polyps removed from affected members of St Mark's Family 96

Discussion

The recognition of polyposis syndromes is usually straightforward. The three most common disorders, familial adenomatous polyposis (FAP), Peutz-Jeghers' syndrome(PJS) and juvenile polyposis (JP), have been relatively well defined clinically, and the polyps associated with each have characteristic histological features. Furthermore, in the case of FAP, presymptomatic diagnosis is now possible using genetic markers (237-241).

Occasionally, however, the diagnosis of a polyposis syndrome is less clear-cut, when an individual has a mixed pattern of disease, presenting with polyps of different histology and/or polyps with "overlapping" histological features. It is unknown whether such "mixed" polyposes are variants of FAP, PJS or JP, or are truly distinct diseases. Adenomatous polyposis, for example, has been described in association with PJS polyps (368,369) and JP polyps (370-377), and there have been isolated case reports describing patients with all three types of polyp (378) as well as adenomas and metaplastic polyps (367,379,380). Furthermore, hyperplastic and adenomatous elements have been found within the same polyp (381-383), and exceptionally, juvenile polyps have been described which contain dysplastic epithelium (384-387), hyperplastic epithelium (388), areas of adenomatous proliferation (371,375,386-388) and even adenocarcinoma(75,387,388). Until the underlying germ-line and somatic mutations in these patients are characterised, it is impossible to determine whether mixed polyposis is a separate disease from the other polyposis syndromes.

There are four possible genetic explanations for mixed polyposis. First, an individual may have inherited mutations predisposing to more than one type of polyposis: this appears unlikely, but mixed polyposis *is* very rare and there may exist relatively common, undiscovered genes predisposing to colorectal polyps. Second, an individual may have inherited a mutation predisposing to one type of polyposis (most likely JP or PJS) and have acquired another type of polyp by somatic mutation. (Presumably, it is also possible to acquire more than one type of polyp by somatic

mutation alone). Third, all mixed polyposis lesions may progress at different rates through the same sequence (for example, hyperplastic -> juvenile -> Peutz-Jeghers' -> adenomatous -> adenoma -> carcinoma): the appearance seen at colonoscopy reflects the different stages of progression that polyps have reached. Here, a germ-line mutation must be assumed to predispose to the earliest lesion in the pathway. Fourth, a germ-line mutation may predispose to polyp formation in general: the subsequent somatic mutations determine a polyp's histological type. Possibilities three and four are both consistent with those cases in which a single polyp has "mixed" histology.

Family SM96 is inconsistent with the first and second of the above possibilities . The pattern of disease segregation does not fit well with the existence, for example, of both an *APC* mutation and a Peutz-Jeghers' mutation in the family. Alternatively, it would be necessary to invoke an unrealistically large number of somatic mutations if, for example, these had caused the adenomatous polyps seen in the family. Possibilities three and four are, however, plausible and fit well with the apparently Mendelian inheritance of HMPS.

The clinical features of HMPS suggests that it is unrelated to the hereditary non-polyposis colorectal cancer (HNPCC) syndromes. The number of colorectal polyps is not known to be increased in HNPCC and there are no reports of JP polyps in HNPCC. HMPS is also clinically dissimilar to FAP: family SM96 has fewer polyps, non-adenomatous polyps and little evidence of extra-colonic disease as compared with FAP.

The characteristic lesion of family SM96 is the juvenile polyp with mixed features, similar to those previously described in an "atypical" juvenile polyposis syndrome (372). HMPS may be a variant of JP, but differences exist between the two diseases. Firstly, the majority of polyps removed from members of SM96 have been tubular or villous adenomas (73%) with no features to suggest that they have arisen within pre-existing juvenile polyps. Indeed, some members have been found to have tubular

adenomas only. This contrasts with juvenile polyposis syndromes, where in a large histological review of 1032 polyps removed from such patients, only 21 (2%) were adenomas with no residual juvenile features (79). Secondly, patients with juvenile polyposis typically have between 50 and 200 polyps (78), although some juvenile polyposis patients do have fewer polyps of numbers similar to those found in some members of Family 96. Thirdly, the juvenile polyposis syndromes and HMPS differ at the age at presentation and development of colorectal cancer. Patients with generalised juvenile polyposis tend to present in the second decade and develop colorectal cancer in the third decade (78). In one series involving 87 patients with JP, 18 developed colorectal cancer at a mean age of 34 years (78). Affected members of family 96, on the other hand, tend to present in the fourth decade (mean age 40.8 years), and develop cancer much later (mean age 48.3 years).

Variable gene expression is a common feature of many autosomal dominant disorders, including the polyposis syndromes. In FAP, for example, some affected individuals develop life threatening desmoid disease or duodenal cancer, whereas others affected within the same family remain complication-free. The gene responsible for HMPS may play an important role in controlling the differentiation of the colorectal epithelium, and if so, this gene may be subject to modifying influences. Alternatively, the induction and cellular transformation of the polyps in family 96, may be under the control of mesenchymal elements (389,390). It remains possible, therefore, that mutations in the HMPS gene are also responsible for other polyposis syndromes, although the clinical features of family SM96 suggest that HMPS may be a distinct disease.

The Clinical Management of St Mark's Family 96

Members of Family 96 need to be kept under close medical surveillance, and central registration plays a vital role in keeping track of at-risk individuals. The age at which colonoscopy should be commenced and the frequency with which it should be performed have not yet been established, but some guidelines are emerging. The earliest age at which polyps have been diagnosed in an affected individual is 18 years, and this would appear a reasonable age therefore to commence screening. Although three-yearly colonoscopic surveillance is considered adequate for most members of the general population who develop "sporadic" colorectal adenomas (340), one individual developed 12 tubular colonic adenomas in a 2 year screening interval. For this reason biennial colonoscopy would seem most appropriate until further data on the rate of polyp growth in this family is available. A more limited examination of the bowel using the 60 cm fibre-optic sigmoidoscope would not be acceptable, as half the cancers diagnosed in Family 96 were proximal to the mid transverse colon.

The family described here raises several questions about the classification of polyposes, which can only be clarified by molecular genetic studies. Although the HMPS syndrome is likely to be extremely rare, identifying the gene responsible is important for the following reasons.

1) Predictive DNA testing would be possible for at risk presymptomatic individuals ; those not carrying the gene could avoid unnecessary colonoscopic surveillance, and be reassured that they had not passed the gene on to their children.

2) Germ-line mutations of the HMPS gene may be responsible for other polyposis syndromes, or may contribute to an increased risk of colorectal tumours in the general population.

3) Genes that are mutated in the germ-line in hereditary cancer syndromes are often mutated in the sporadic form of the disease. As colorectal cancer is believed to result from the stepwise accumulation of rate-limiting genetic mutations, the identification

of these mutations will be essential for determining the cause of colorectal cancer and for planning rational therapy for established disease.

As Garrod stated in 1924," Nature is nowhere accustomed more openly to display her secret mysterics than in cases where she shows traces of her workings apart from the beaten path; nor is there any better way to advance the proper practice of medicine than to give our minds to the discovery of the usual law of Nature, by careful investigations of cases of rarer forms of disease " (391).

Chapter 6

The Search for Colorectal Cancer Genes

Introduction

Until relatively recently, the isolation and identification of the genes responsible for inherited disorders, has been possible only in those rare diseases in which the biochemical fault has already been established. In this situation, a knowledge of the amino acid structure of the faulty protein permits cloning of the cDNA sequence and later, the gene itself. Recent advances in molecular biology, however, render it possible to isolate genetic mutations without any prior knowledge of the molecular defect responsible. The overall approach involves mapping of the responsible gene to its chromosomal location by linkage analysis, followed by isolation of the gene and characterisation of the mutations using a combination of finer resolution techniques. Formerly referred to as "reverse genetics", this process is now more accurately termed positional cloning (Fig. 6.1). Several steps are involved. Initially, data have to be collected on families which have been affected by the disorder under study, over two or three generations. An accurate diagnosis of the disorder then has to be made using consistent and objective criteria to separate normal from affected individuals. Following this, pedigrees are constructed and DNA from family members is analysed for linkage to a large panel of informative genetic markers which may span the genome. Once linkage has been established, flanking markers are then developed around the disease locus, so that the area of interest can be narrowed, and the region of DNA containing the diseased gene, isolated and cloned. The gene of interest can then be sequenced and a search can be made for mutations in affected family members. Diagnostic tests can then be developed for each family, and a search made for drugs or proteins which may minimise or totally correct the adverse effects of the faulty protein (392).

Principles

The technique of linkage analysis relies on the principle that genetic markers situated close to a disease locus will have an increased chance of being consistently co-

inherited with the disease at meiosis. Likewise, genes that are far apart on the same chromosome will have a higher probability of being separated by recombination. The first report of linkage can be attributed to the Baltimore geneticist, Thomas Hunt Morgan, who, in 1900, observed that eye colour in the fruit fly, Drosophila Melanogaster, was always co-inherited with the X chromosome, and concluded that the particle or gene responsible for eye colour resided on or was linked to this chromosome (393,394) In 1911, Wilson et al demonstrated that the gene responsible for colour blindness was also carried on the X chromosome (394), and in 1927, Haldane reasoned that if it was possible to map 50 or more characters, they could then be used as markers to predict whether children were likely to develop important genetic diseases (393). In 1955, Morton developed mathematical formulae for calculating linkage in humans (395), and in 1956, Edwards pointed out, that with sufficient genetic markers, it would be possible to perform prenatal diagnosis, and prevent the transmission of many autosomal dominant disorders (393).

In contrast to research on Drosophila, the establishment of linkage between two genetic loci in humans can be a painstaking business, involving a large number of observations on structured families. Elaborate mathematical methods are required to distinguish coincidence and significance, and the situation is further complicated by human behaviour. In general, human beings tend to have relatively small families, long generation times, often live long distances apart making ascertainment difficult, and often have far from ideal pedigree structures for statistical analysis.

The term used to describe statistical outcome of linkage in man, the LOD score, denoted (Z), is the logarithm of an odds ratio and represents the likelihood that two loci are linked at a given recombination fraction to the likelihood that they are not. Since LOD scores are to the base 10, a value of 3 represents a probability of 1000:1 that the observation did not occur by chance, a LOD score of -2 means that linkage is highly unlikely, and LOD scores between these values are inconclusive and require further evaluation. The formal unit used to measure the recombination fraction, i.e..

the probability of crossing over during meiosis, is the centimorgan (cM), with 1cM equal to 1% frequency of recombination (396). In physical terms this is equivalent approximately to 1 megabase of genomic DNA, although the physical distance will vary between chromosomes of different size. Once linkage is established, a more detailed genetic map can be constructed for other markers in that region using multi-locus analysis which is particularly useful for identifying double recombinants and determining the order of a set of linked markers (397).

Requirements

The presence of a large kindred with a well defined phenotype greatly facilitates the technique of linkage analysis. FAP is an ideal disorder for linkage analysis because a) the condition has a well defined phenotype which can be recognised early and treated effectively providing two to three generation families with a large number of living affected individuals for study and b) many families have been fully ascertained through polyposis registries. In other hereditary cancer syndromes however, such as HNPCC, the nature of the illness means that large kindreds with living affected members are a relative rarity (281,282).

Genetic Markers

A second requirement for linkage analysis is the availability of detailed genetic maps and a large panel of finely spaced genetic markers.

It has been known since Landsteiner discovered the ABO blood group system in 1900 that human beings may be polymorphic at the protein level (398), but it was not until the discovery of restriction endonuclease enzymes in the early 1970s that the full scale of genetic variation at DNA level became appreciated (399). Although only about 20 % of the entire human genome codes for proteins, the remaining DNA contains considerable sequence variation , and it is this treasury of variation between individals, that has proved so invaluable for exploiting linkage analysis.

Restriction endonucleases are prokaryotic enzymes which act as defence mechanisms for bacteria, by degrading foreign DNA, such as might derive from an invading bacteriophage. The bacterium which produces the particular restriction enzyme protects itself by methylating A or C residues within the restriction sequence. These enzymes also have the ability to cleave human DNA in a specific, accurate and highly reproducible manner, and it is this property which has largely been responsible for the present revolution in molecular genetic research.

Point Polymorphisms

The human haploid genome is about 3×10^9 base pairs long, and it is estimated that a base difference occurs between every 200 and 300 base pairs (400) This sequence variability can be identified by restriction fragment length polymorphism (RFLP) analysis, a method that detects single nucleotide polymorphisms based on restriction enzyme cleavage sites (401). A locus is said to be polymorphic if it contains two or more alleles and the frequency of the most common allele is less than 95% " (396). Degree of polymorphism, and its usefulness depends on the number and frequencies of the alleles present and the frequency with which the maternal and paternal alleles can be differentiated. Most RFLPs are distinguished by Southern Analysis, using radioactive labelled probes (Fig 6.2), but PCR-amplified DNA can also be cleaved with restriction enzyme and analysed after electrophoresis using ethidium bromide staining (Fig 6.3).

The total number of known RFLP loci is over 2000. Most loci, however, represent only two alleles with a heterozygosity lower than 50% , and consequently these loci are poorly informative in linkage studies (283).

Length Polymorphism

Scattered throughout the genome are tandemly repeated sequences whose copy number varies between people because of unequal recombination and replication slippage. A restriction fragment which contains the whole tandemly repeated block

will be different sizes in different people, and because these markers have several alleles, the chances are high, that all 4 chromosomes of an individual's parents will be individually identifiable. Two main types of length or repeating unit polymorphism have been identified, and these have been used to generate probes known as mini and microsatellite markers.

Minisatellite Markers

In 1984, Jeffreys and co-workers reported the discovery of hypervariable tandemly repeated minisatellite regions or VNTRs. The number of repeat core units may vary from 3-100 between different individuals, and if a restriction enzyme is chosen which cuts outside the repeat region, this variability can be observed as multiple alleles on Southern hybridisation (402). The mean heterozygosity of the known VNTR loci is over 70%, and VNTRs with heterozygosities of 98% have been reported (403). VNTR regions can lie close to mapped genes, but generally they are enriched in non coding regions of the genome, being most frequent at the telomeric ends of the chromosomes, where the number of recombination events is often increased (404-406). The stable inheritance of variable minisatellite fragments with the low population frequency of individual fragments makes them excellently suited for linkage analysis (Fig 6.4) (406).

Multilocus Probes

In 1985, Jeffreys described a short minisatellite area consisting of 33bp. nucleotide sequence which was repeated four times in the first intron of the myoglobin gene and from this region, he prepared a probe in which the 10-15 bp core sequence was repeated several times. When these minisatellite probes were hybridised to human DNA they detected several VNTR loci simultaneously, creating a unique pattern or "DNA fingerprint" for each individual. These multilocus probes can detect about 15-20 bands greater than 2 kilobases per individual on Southern analysis (407). Combined, it has been estimated that the probes 33.6 and 33.15 can detect

Combined, it has been estimated that the probes 33.6 and 33.15 can detect approximately 60 hypervariable loci, most of which will be unlinked and scattered throughout the genome (407). The disadvantage of using multilocus probes in linkage analysis, is that unlike single locus analysis, linkage data cannot be pooled between unrelated small pedigrees, since a different minisatellite allele is likely to be associated with the disease locus in each pedigree.

Microsatellite Markers

Microsatellite or (C-A)n repeats are short tandem runs of di-,tri-or tetrameric poly (CA), poly (GT) nucleotide sequences which occur frequently in mammalian DNA (408,409). Blocks of CA repeat units are found in every 30-60 kb. of DNA, and are distributed evenly in 5' and 3' untranslated regions and introns. It is estimated that there may be between 50,000 - 100,000 blocks of CA repeats scattered throughout the genome. If the sequences flanking these repeats are obtained, primers can be synthesised that allow amplification of the repeats by the polymerase chain reaction, and the fragments can then be resolved by polyacrylamide gel electrophoresis. Several hundred primer sequences have now been assigned to specific chromosomes for the identification of blocks of CA repeats (283).

Microsatellite markers have several advantages over conventional markers. Firstly, they are highly polymorphic due to length heterogeneity, with over 75% of meioses being informative (409). Secondly, analysis requires less than 20 nanograms of DNA per marker, as opposed to 5-10 micrograms for Southern analysis, and this means paraffin embedded archival histological material can be used for analysis. Finally, the alleles can be identified using non-radioactive fluorescent labelled primers and automated sequence analysis, enabling hundreds of microsatellite markers to be studied in a fraction of the time required previously (410).

Genetic Maps

The concept of a genetic map dates to 1911, when A.H. Sturtevant, an undergraduate in TH Morgan's laboratory, realised that linkage information could be used to determine the relative position of genes along a chromosome. His paper "The linear arrangement of six sex-linked factors in Drosophila, as shown by their mode of association" was the first linkage map published, and forms the basis of all modern linkage studies (401). In 1979, Solomon and Bodmer proposed the construction of a genetic map, based on evenly spaced RFLPS (411), and the following year, White and colleagues at the University of Utah reported the assignment of nearly 500 RFLP markers covering 17 chromosomes, based on linkage data from 59 different three generation families (401). In 1992, a generation linkage map was described consisting of 814 highly polymorphic microsatellite markers spanning 90% of the estimated length of the genome (412).

Data Analysis

Linkage analysis is one area of medical research that is almost totally dependent on powerful computer technology. Most linkage studies use either the LIPED or LINKAGE software programmes.

Limitations of Linkage Analysis

Phenocopy.

Phenocopy is a phenotype produced by environmental factors that mimics a genetically determined trait. In disorders such as retinoblastoma, or multiple endocrine neoplasia (MEN), the issue of phenocopy, seldom, if ever presents a problem, because such disorders are relatively rare and the chances that affected individuals within the same family will not carry the mutant allele are extremely small. However, in common malignancies, such as colorectal cancer it may be difficult to separate the hereditary cancer due to a high penetrance mutation, from the

"sporadic" cancer due to some other cause. Some families with multiple cases of colorectal cancer may arise as a result of chance clustering, and some patients with colorectal cancer in susceptible families may not carry the mutation responsible. This fact has major implications for linkage studies because misclassification of disease status within the pedigree can lead to a significant loss of LOD score (397).

Penetrance

Penetrance is the probability that a person carrying the gene for a dominant disease will manifest the signs and symptoms of the disease. In some forms of hereditary colorectal cancer, gene expression will be age-dependent so that young gene carriers may appear unaffected, contributing little linkage information. If the hypothesis that most colorectal cancer is due to the inheritance of a common low penetrant gene therefore, identifying the gene(s) responsible using conventional linkage analysis may prove difficult if not impossible.

Definition of Phenotype

Accurate definition of phenotype is crucial to any linkage study. This is not a problem in disorders with a clearly defined phenotype such as FAP, but can be troublesome in disorders such as HNPCC where the phenotype be less than typical, Defining the phenotype too broadly in colorectal cancers studies by classifying individuals with adenomas as affected could destroy or create spurious linkage if the individuals concerned do not carry the mutant allele. It is customary in this situation, therefore, to eliminate uncertainty by adopting only the narrowest definition of phenotype. Definition can then be expanded at a later date to include other features once linkage has been established.

Genetic Heterogeneity

Different genes in different families may cause the same disease, invalidating the summation of LOD scores in small families. This can be minimised by studying

mainly large families with many living affected individuals, an uncommon situation in the context of hereditary non-polyposis colorectal cancer.

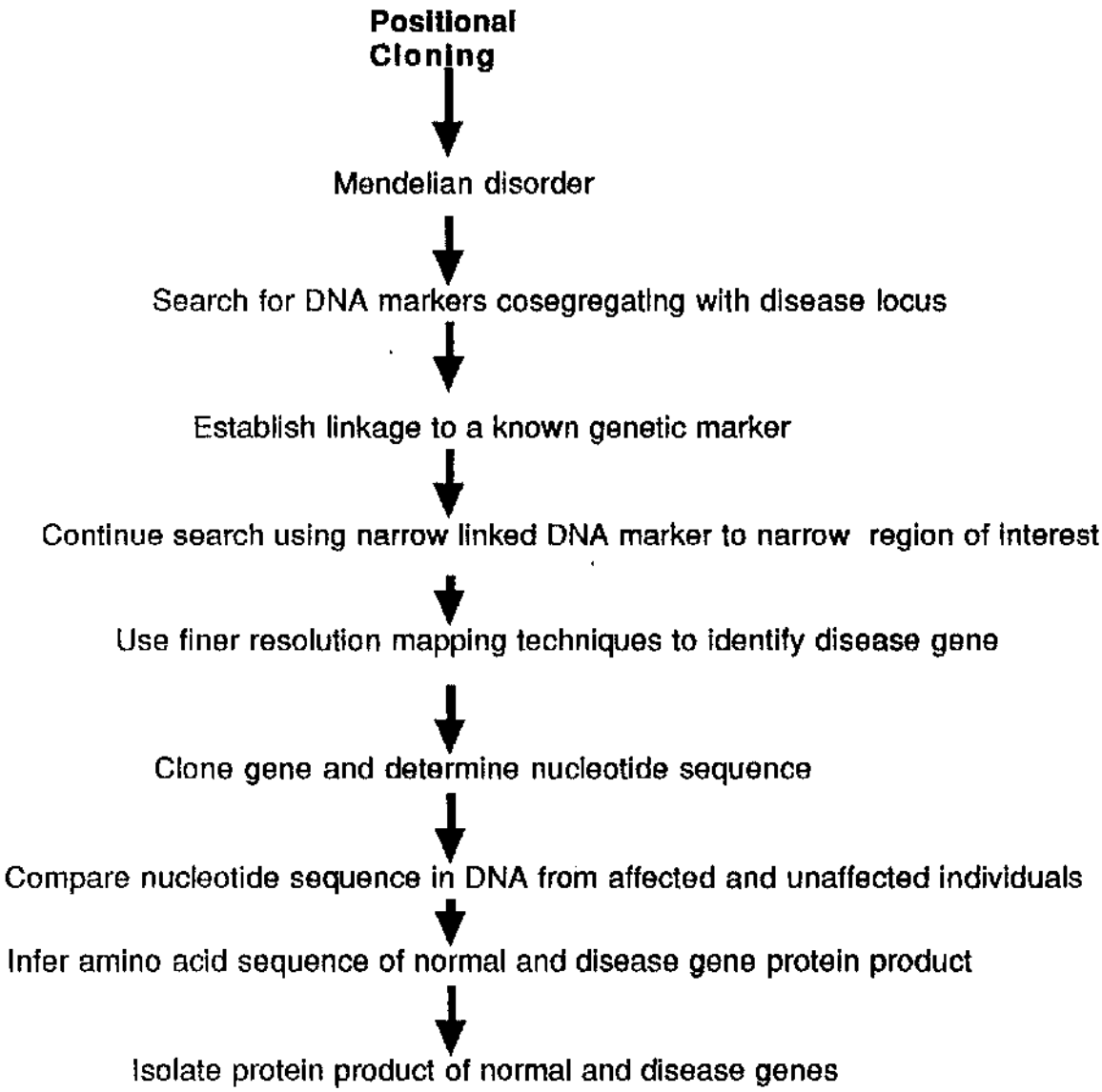


Fig 6.1 Schematic outline of positional cloning

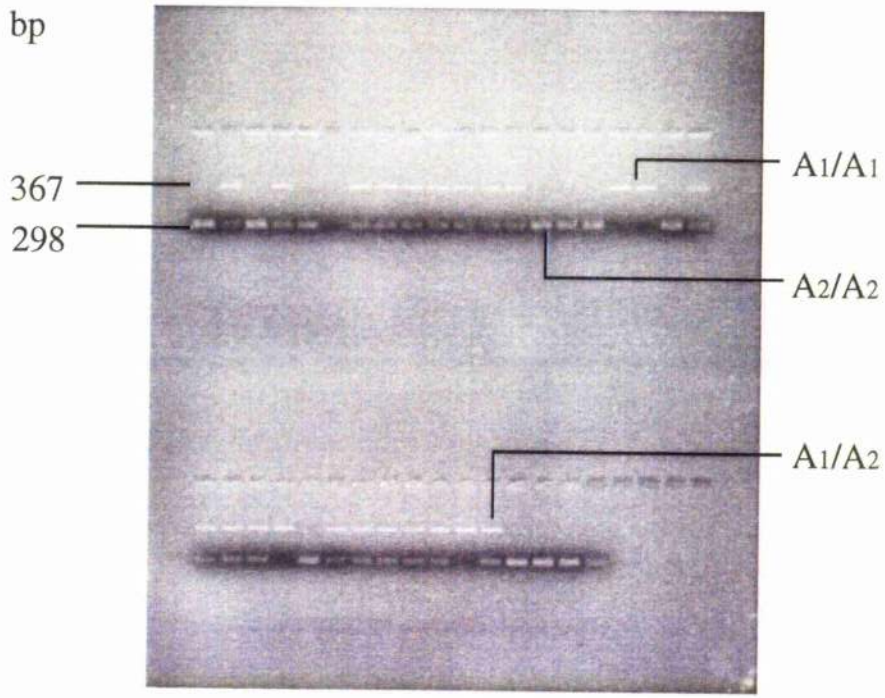


Fig.6.3 Restriction Fragment Length Polymorphism detected by cleavage of PCR products amplified within the THRB gene on chromosome 3p22-24

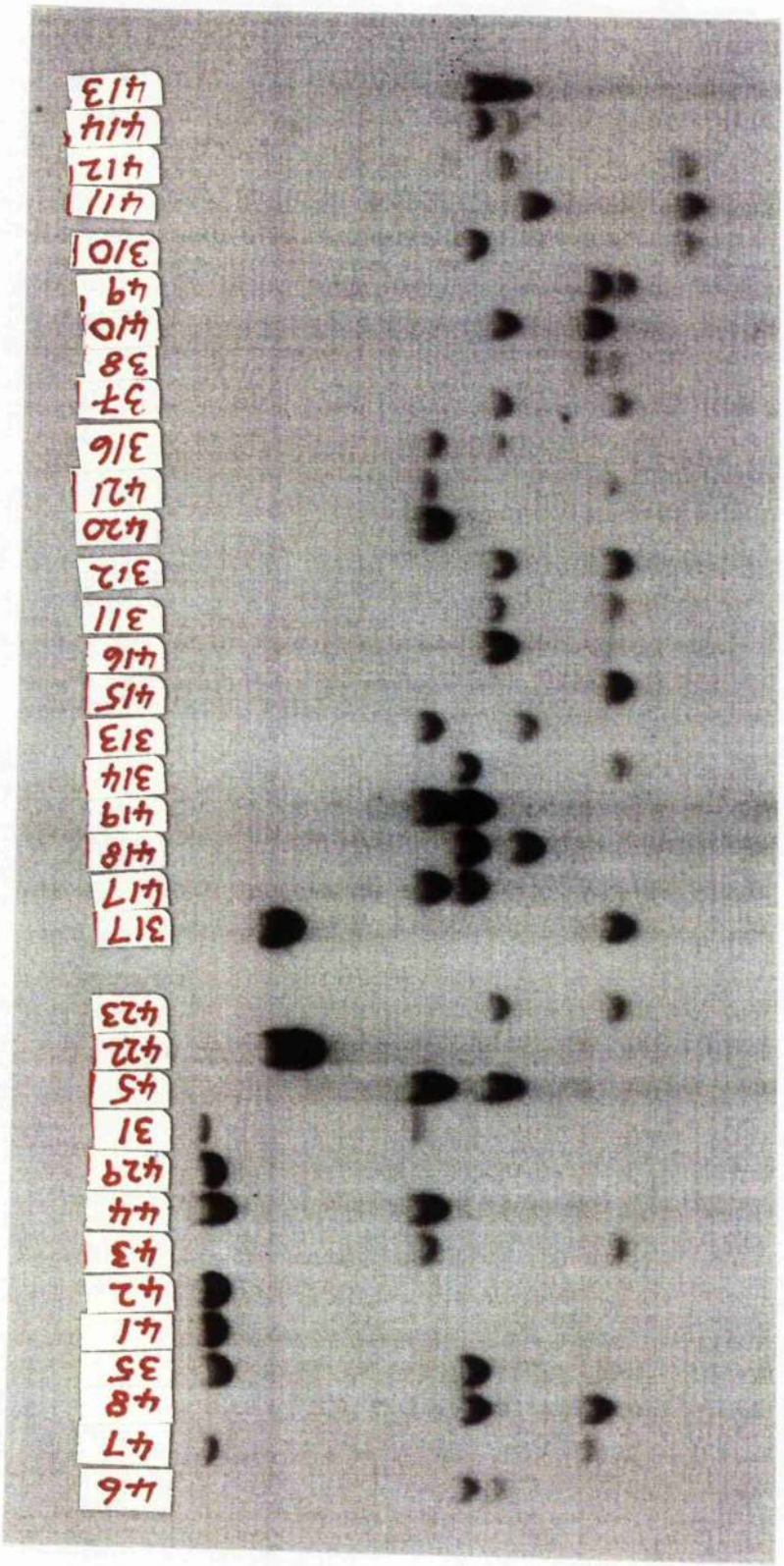


Fig.6.4 Autoradiographic bands detected by VNTR probe in DNA digested with *Hinf I* restriction endonuclease.

Chapter 7

A Linkage Study of St Mark's Family 96

Introduction

The purpose of this study was to localise the gene that predisposes St Mark's Family 96 to the development of atypical polyps and early onset colorectal cancer - the Hereditary Mixed Polyposis Syndrome discussed in Chapter 5.

St Mark's Family 96 was an ideal family for a linkage study because:

1) Affected individuals developed polyps at a relatively early age, and the phenotype in most cases could be readily identified.

2) The disorder appeared to have a high penetrance, and autosomal dominant inheritance.

3) The family had many living affected individuals and it was therefore possible to obtain blood specimens for DNA analysis.

4) The family were cooperative and enthusiastic about the project.

Linkage Strategy

Cytogenetic clues played a major role in the genetic mapping of both the retinoblastoma and APC genes. Such clues are rare, but when present, can significantly accelerate any linkage process. Before embarking on an exhaustive linkage study, therefore, a decision was made to examine the karyotype of an affected individual for any abnormality. Assume the karyotypes are normal, the next strategy would involve excluding linkage to the candidate genes, APC,MCC,P53 and DCC. Exclusion of linkage to the APC gene would almost completely rule out the HMPS as a variant of FAP. Following the exclusion of linkage to these loci, the next step would involve concentrating on those regions of the genome known to be associated with a high frequency of allele loss in a panel of colorectal cancer specimens, using when possible highly informative VNTR markers. Finally, an attempt would be made to identify an allele which was being consistently co-inherited with the disease, using a multilocus probe.

Such a study, would not localise the gene responsible per se, but if *all* affected individuals possessed a specific allele, and *all* unaffected members did not, then a test would be available which could identify those at risk.of developing polyps within the family.

All the linkage experiments were performed by the author between June 91-November 93, with the exception of the multilocus probe experiment, which was performed by Cellmark Diagnostics, ICI, Abingdon Science Park, Oxfordshire.

Patients and Methods

The section of St Mark's Family 96 used for the linkage study is shown in Fig 7.1. DNA was available from 37 individuals. All family members were drawn from the third and fourth generations. No tissue was available for DNA extraction from the first and second generation as the individuals concerned were deceased, and it proved

were considered too young for accurate assessment of disease status. Twelve family members lived in the United Kingdom, nine lived in South Africa, six lived in Israel, five lived in New York, three lived in Australia, one lived in the Netherlands and one lived in Hong-Kong. Blood specimens from individuals living in South Africa were collected by Professor Trefor Jenkins, Department of Human Genetics, University of Johannesburg.

Definition of Phenotype.

For the purposes of linkage analysis, any patients who developed polyps under the age of 50 years or colorectal cancer under the age of 55 years was considered a gene carrier.

Those patients who did not have polyps on colonoscopy examination by the age of 40 years were considered not to carry the gene.

Cytogenetic examination.

The chromosomes were prepared according to standard techniques (413). They were then G-banded using Wright's stain in 50% Sorenson buffer (diluted one part stain to three parts buffer). This was performed by Miss Tanya Jones, Cytogenetics Lab, ICRF.

Blood Collection

A 50ml Falcon tube containing 25ml of blood collection media was brought to room temperature and 25ml of fresh blood was added. The tube was then inverted to mix, and the sample was maintained at room temperature.

Sterile Separation of Lymphocytes

The contents of each blood collection bottle was poured into a 250ml flask and the blood bottle was rinsed with 4ml of RPMI/HEPES. 20 sterile glass beads were then added to the flask, followed by 0.6ml of sterile 1M calcium chloride through the foil

top of the flask, and the blood was defibrinated for 15 minutes at 250 r.p.m. on a gyratory shaker. 20ml RPMI/HEPES was then added to the flask and the defibrinated blood was carefully overlayed on two 50ml tubes each containing 14ml of Lymphprep (Nyegaard). The cells were then separated by centrifugation at 700g for 20 minutes. The interface between the 'Lymphprep' and the serum was removed and diluted 1:1 with RPMI/HEPES. The cells were then counted on a haemocytometer. The cells were then spun at 1000g for 10 minutes and the supernatant aspirated. The cell pellets were then frozen in 1ml of foetal calf serum plus 10% Dimethyl sulphoxide at -70^0 C.

Epstein-Barr Transformation of Lymphocytes

This was performed by Miss Cynthia Dixon (Cancer Genetics Laboratory, ICRF) according to the protocol of Pelloquin et al (414).

DNA Extraction

20 mls of blood was collected into EDTA (0.5ml of 0.5M EDTA pH 8.0). The solution was then transferred to a 50ml. Falcon tube and made up to 50ml with lysis buffer, and left on ice for 10 minutes until the red cells had lysed. The cell nuclei were then collected by centrifugation at 2000 rpm for 10 minutes at 4^0 C. The supernatant was drained off, and the residual pellet was resuspended in 3 ml of salt/EDTA buffer and vortexed. 150 microlitres of 20% SDS and 30 microlitres of 10mg/ml proteinase K was added. The solution was then left overnight in a water bath at 37^0 C. 3 ml of phenol/chloroform was then mixed with the solution for 5-10 minutes using the tube rotator, and then spun at 2000 rpm for 5 minutes at room temperature. The upper aqueous phase was removed and the procedure repeated. The aqueous phase was then extracted with 3ml of chloroform/isoamyl alcohol 25:1, using the same centrifuge procedure. 1/10 volume of 3M sodium citrate was added followed by 2 volumes of cold absolute alcohol, and the solution was hand mixed. The precipitated DNA was hooked out with a glass rod, rinsed in 70% alcohol, dried briefly, resuspended in1-2ml of T.E., and rolled overnight in the cold room at 4^0 C. A

dilution of the DNA, 10 microlitres:1000 microlitres, was made in distilled water, and the optical density (OD) was measured at 260 and 280 nm against a distilled water blank in a silica cuvette. The ratio of OD260nm/OD280nm should ideally be 1.8. The concentration of the DNA solution was calculated with the knowledge that a solution of DNA at 1mg/ml is equivalent to 200 OD units. A working solution of DNA concentration 200 micrograms/ml was made, and the remainder was stored as stock at -20^0 C.

For extraction of DNA from lymphoblastoid cell lines, approximately 10^8 cells were spun down at 1000 r.p.m. for 5 minutes, resuspended and then washed in PBS (1ml of cell culture contains about 0.5 X 10^6 cells). The solution was respun, and the cell resuspended in a salt/EDTA/proteinase K solution as described above. The procedure was then identical to that described for DNA extraction from blood.

Restriction endonuclease digestions

This was carried out in the buffers and at the temperatures recommended by the suppliers of the restriction endonucleases. The amount of enzyme used in each case was 1-6 fold over that required to complete the digestion in one hour under optimal conditions. Due to the inhibitory effect of the glycerol present within restriction enzyme storage buffers, on no occasion was this component allowed to constitute more than 10% of the reaction volume. Reactions were incubated for 1-2 hours for cloned DNA and for at least 3 hours for genomic DNA. After digestion was completed, approximately 1 μg of DNA was taken from each tube to check digestion and run with 1/10 loading buffer on a test minigel. If digestion was thought to be complete, the Gyrovap was used to concentrate the samples to about 25 microlitres or to dryness and water was added. If digestion was incomplete, more enzyme was added and reaction volume was incubated for a longer period. If digestion was still incomplete after this, the sample was re-extracted and precipitated. Incomplete digestions after this time were phenol/chloroform extracted and precipitated prior to a second digestion. Multiple digestions were conducted simultaneously if the reaction

conditions required by each enzyme concerned are similar. If not the DNA was ethanol precipitated after each restriction and resuspended in an appropriate buffer for a subsequent digestion. Aliquots of all digestions were separated on appropriate percentage agarose gels to determine the completeness of the digestion.

Agarose gel electrophoresis

This was performed using 0.7-1.4% agarose gels of 200 or 300 ml volume poured upon 20 x15 cm or 25 x 15 cm perspex trays respectively. Various plastic well-formers were employed to make gel slots. The gel was prepared in 1 x TAE (stock 50x). 3.2 grams of agarose were then added to 300 mls of TAE buffer, and microwaved for 5-10 minutes until gel had boiled and the agarose dissolved. When the gel was about 60^0C, it was poured evenly into the mould without making bubbles, and left to set for about 30 minutes. The gels were then submerged under a 1 cm depth of electrophoresis buffer and after loading the DNA in 20% (v/v) loading buffer, a constant voltage of 0.5-3 V/cm was applied. Ethidium bromide at 0.5 mg/ml was included in both the gel and the electrophoresis buffer to permit visualisation of the nucleic acids under ultraviolet light. A 2 kb marker was also loaded onto the gel and was allowed to migrate at least 9cm. on a 20 cm. gel. This was estimated by photographing a ruler by the side of the marker lane.

Southern Blotting

The marker track was cut off, and the gel was depurinated by immersion in 0.25M HCL for 10 minutes. The gel was then neutralised in distilled water. The gel was then placed into a blotting tray containing 0.4M NaoH with a double thickness of Whatman 3MM wick. The gel was then transferred to the surface of the blotting wick, and air bubbles removed using a plastic roller. A sheet of Hybond-N+ membrane and 4 layers of Whatman 3MM paper were then placed on the gel. The membrane was rolled flat to ensure good contact with overlying paper, and a stack of paper towels towels was placed on top of filter to a depth of about 10cm. The edges

of the gel were masked with parafilm strips. A plate of glass was then placed on top of the stack, and the apparatus was left for 4 hours to allow complete DNA transfer. When the membrane was removed it was rinsed several times in 2 x SSC.

Processing of Genetic Probes

Most probes were provided by the UK. DNA Probe Bank (I.C.R.F.,Clare Hall) at a concentration of approximately 0.5 mgs /ml, more than an adequate amount for restriction digestion. However, some probes were in short supply and required further transformation..

Plasmid Transformation

A 200 microlitre volume of frozen competent Escherichia Coli bacterial cells was allowed to thaw gradually on ice. The transformed DNA (plasmid plus insert) was then mixed with the competent cells and left on ice for 20 minutes, before adding calcium chloride. The mixture was allowed to stand at room temperature for 10 minutes. 0.7 mls of prewarmed L broth was added and incubated for shaking for 1 hour at 37^0 C. The transformed bacterial solution was then plated out on L-broth/ampicillin plates using a glass spreader sterilised with alcohol and flaming. The inoculated plates were then inverted and incubated overnight at 37^0 C. The plates were then removed and stored at 4^0 C to arrest bacterial growth. The incubated plates were viewed, colonies selected using a sterile loop and then transferred to 100 mls. of L-broth/ampicillin in sterile flasks and incubated in the shaker at 37^0 C. overnight.

Alkaline Lysis

10 mls of an overnight culture was added to 1 litre of L-Broth plus antibiotic (usually ampicillin 25-50 g/ml for pBR322 type plasmids or 100-150 g/ml for pBluescript, pTZ etc.). 250-500 mls of this culture was then grown at 37^0C. and shaken overnight. The culture was then cooled on ice, and the cells were spun down in GSA tubes at 3000 rpm for 10 minutes at 4^0 C. The supernatant was drained off and the cells were

10 mls of an overnight culture was added to 1 litre of L-Broth plus antibiotic (usually ampicillin 25-50 g/ml for pBR322 type plasmids or 100-150 g/ml for pBluescript, pTZ etc.). 250-500 mls of this culture was then grown at 37^{0}C. and shaken overnight. The culture was then cooled on ice, and the cells were spun down in GSA tubes at 3000 rpm for 10 minutes at 4^0 C. The supernatant was drained off and the cells were resuspended in 7.5 mls of ice cold GTE solution. 0.5 mls of lysozyme (10mgs/ml) was then added to the solution, and the cells were allowed to sit at room temperature for 10 minutes, following which 15 mls of 0.2M NaOH/1% SDS solution was added, and the solution was left on ice for 5 minutes. 10 mls of ice cold 5M KOAc pH 4.8 was then added and the solution left on ice for a further 15 minutes. The solution was then spun at 10,000 rpm for 5 minutes at 40C, and the supernatant drained into a 250 ml graduated cylinder. Isopropanol (0.6 of total volume was then added, the solution was transferred to a clean GSA tube, and stored at -20^{0}C. for 1 hour. The solution was then spun for 10 minutes at 6,000 rpm at 4^0 C, and the supernatant drained off.

Caesium Chloride Gradient

The pellet was resuspended in 10 mls of TE pH8, and the solution transferred to a SS34 tube. 1g/ml of caesium chloride was then added and allowed to dissolve, and when this was complete, 200 microlitres of Ethidium Bromide (10mg/ml)was added. The solution was then left on ice for 30 minutes, and then spun at 10,000 rpm at 4^0 C. for 10 minutes to remove most of the RNA. The supernatant was then transferred to a Ti80 quickseal tube, balanced and sealed. The solution was then then spun in the ultra-centrifuge at 55,000 rpm for 18-20 hours, then 40,000 rpm for 2 hours, and then stopped without a break. The band of interest was then aspirated using a 16 gauge needle attached to a 5 ml syringe, and added to an approximately equal volume of water saturated butanol. The plasmid solution was then extracted until no longer pink in appearance, and then separated from the caesium chloride over a large A50M

Preparation of Insert DNA

Approximately 2 g of plasmid DNA were digested with the appropriate restriction enzyme as described above, and then resolved on low-melt ultra-pure agarose gel over a 2-3 hour period at 60-90 Volts. Included on the gel was 1 g of a lambda-Hind III size marker. The relevant band was then identified under U-V light, cut out with a scalpel, placed in an Eppendorf tube and then weighed. Three times the gel weight of distilled water was added. The first time the probe was labelled, it was boiled for 7 minutes, and the solution was dispensed into convenient aliquots.

Filter Hybridisations

Filters were prehybridised in glass bottles containing hybridisation buffer plus heat denatured competitor DNA as required at 65^{0}C for a minimum of 2-4 hours.

Denatured double-stranded DNA probes were radio-labelled according to the oligo-labelling method of Feinberg and Vogelstein using ^{32}P dCTP (415). This involved boiling the probe for 3 minutes in an Eppendorf tube (after piercing the top of the cap), then cooling to 37^{0} C. if in agarose or on ice if not. OLB, BSA, ^{32} P-dCTP and Klenow were then added and the mixture was left to incubate at 37^{0} C. or at room temperature overnight. A typical labelling reaction would consist of:

34 microlitres of probe in agarose or less if very concentrated or

20-50 nanograms of pure DNA plus water to 34 microlitres.

10 microlitres of OLB

2 microlitres of BSA

3 microlitres of ^{32}P-dCTP

1 microlitre of Klenow fragment.

To remove unincorporated nucleotides, probes in 250 microlitres of 1M TES were passed through a Sephadex G-50 (fine) column prepared in a 1 ml syringe by centrifugation of the column for 5 minutes at 1000g. The degree of radionucleotide incorporation was assessed with a 1ml aliquot. After denaturation at 100°C for 5-8 minutes probes were added to hybridisation buffer and then added to the glass bottles. The process was then allowed to proceed at 65°C for 16 hours in a rotating hybridisation oven.

Removal of the non-specifically bound probe was achieved by successive washes at 65°C in 0.1% SDS buffers containing 2x SSC, 1x SSC and 0.1x SSC. Several further washes at this final stringency were applied, and when the filter had been washed down to less than 10 counts/second over its entire surface, an autoradiograph was set up.

Hybridisation with Competitor DNA

Probes containing highly repetitive sequences were incubated with human placental competitor DNA prior to hybridisation. 200mg of competitor DNA will be added to the probe and heated at 100°C for ten minutes, and then the solution was incubated for two hours at 65°C before being added to the hybridisation mixture (416).

Autoradiography

Filters were placed between sheets of polythene and exposed to Kodak X-ray film between intensifying screens in X-ray cassettes. A radio-opaque marker was included to help with orientation of film when it was developed. The autoradiographs. were exposed at -70°C. overnight or longer of alleles were not clearly visible.

Stripping of Filters

To enable re-examination of the filters by other probes the filters were stripped of hybridised probe by immersion in boiling 0.5% SDS solution. When the filters were "cool" in a radio-active sense, they were stored damp in polythene bags at 4° C.

Oligonucleotide Synthesis

Oligonucleotides were synthesised by the Human Genetic Resources Laboratory (ICRF, Clare Hall). The melting temperature (Tm) of the oligonucleotides was estimated by multiplying the number of A + T residues by 20C and the number of G + C residues by 40 C. and adding the two numbers (417).

Polymerase Chain Reaction

A 2ml PCR reaction mixture was made up, which was sufficient for 40 x 50 microlitre reaction volumes.

This consisted of:

1672	microlitres of $H_2 0$
40	microlitres of Primer A (0.5 mgs/ml)
40	microlitres of Primer B (0.5 mgs/ml)
40	microlitres of dNTP
8	microlitres of Taq Polymerase (40 units)
200	microlitres of Taq Polymerase Buffer (10X)

Approximately, 100ng of target DNA was added to a 50 microlitre aliquot of this reaction mixture and the reaction mix was covered with paraffin oil. The PCR reaction was performed on a programmable heating block (Programmable Dri-block, Techne). Exhaustive trials of PCR conditions for each target sequence were often required until the ideal conditions for specific amplification were obtained, but generally the reaction was denatured at 94^{o}C and reannealed at 8^{o}C less than the calculated Tm of the oligonucleotide primers, the polymerisation temperature was usually at 72^{o}C. Extension times varied between 0.5 and 4 minutes, depending on the size of the desired PCR product, and 30 cycles were usually performed. After

completion of the reaction a 10 microlitres aliquot will be run out on an agarose gel and a photograph taken of the ethidium-stained gel.

Construction of a 10% polyacrylamide gel

The following components were mixed in a fume cupboard:

30% polyacrylamide	44 mls.
10 X TBE	13.2 mls
Distilled water	75 mls
20% Ammonium persulphate	300 microlitres

120 microlitres of NNNN tetra-ethylenediamine (TEMED) were added prior to injection into the gel mould.

Construction of the gel mould

A mould was constructed between two thick glass plates and plastic spacers, sealed with silicon tape and held in place between metal clips. The freshly prepared gel solution was then injected into the mould via a 60 ml syringe, until completely filled, taking care to make sure all bubbles were removed. Sharks tooth combs were then inserted into the top of the gel and the gel was then left to polymerise at room temperature for approximately one hour. The silicon tape was then removed from the bottom of the mould, and the mould was placed in the electrophoresis apparatus (Life Technologies) and immersed in TBE buffer. The PCR samples were added to loading buffer, and then pipetted into spaces formed by comb, using a fine pipette tip. The power supply was connected and a constant power of 11 watts was used over a running time of 6 hours. Every effort was made to keep the running temperature of the gel at 55^0C.

Drying of Gel

The gel was then dried onto 3MM Whatman paper using a commercial drier and autoradiography was performed as previously described.

Data Management

Data generated by this work was stored on an Apple McIntosh LCII computer, and analysed using the ICRF Main Frame Computer. An appropriate LOCUS and PHENOLIB file were generated for the marker details, and a FAMILY file was constructed to include details of pedigree, disease status, and typing with respect to the markers for each individual. From this database, input files for the linkage analysis programmes of LIPED and LINKAGE were generated.

Buffers and Solutions

50x Electrophoresis Buffer	Tris base	242g
	Sodium acetate	20.5g
	EDTA	18.6g
	Glacial acetic acid to pH 7.8 to 1L with H20	
Hybridisation Buffer	Dextran Sulphate	10%
	Denhardt's solution	5x
	SDS	0.5%
	SSC	6x
	Tarula Yeast RNA	1mg/ml
Denhardt's Solution(50x)	Bovine serum albumin	5g
	Ficoll	5g
	Polyvinyl pyrrolidine	5g
	made to 500ml with H2O	
Loading Buffer	Bromophenol blue	0.25%
	Xylene cyanol	0.25%
	30% Glycerol in H2O	

Phenol	Phenol	500g
	plus 300ml of:	
	Tris-HCl pH 8.0	500mM
	Na2EDTA.2H20	10mM
	NaCl	10mM
	Hydroxyquinoline	800mg
Chloroform	Chloroform	96ml
	Iso amyl alcohol	4ml
SSC (20x)	NaCl	175.3g
	Sodium Citrate	88.2g
	adjusted to pH7.0	
	with NaOH and autoclaved	
TE	Tris-HCl pH 7.4	10mM
	Na2 EDTA.2H20	1mM
	autoclaved	
TES	Tris-HCl pH 7.4	10mM
	Na2 EDTA.2H20	1mM
	SDS	0.1%
TEN	NaCl	0.1M
	Tris-HCl pH 7.4	10mM
	Na2 EDTA.2H20	1mM

PBS	NaCl	10g
	KCl	0.25g
	KH2PO4	0.25g
	Na2HPO4	1.43g
	CaCl2	1.334g
	MgCl2	1.0g
	made up to 1L with H2O	
PCR Buffer (10x)	(NH4)2 SO4	166mM
	Tris-HClpH 8.8	0.67M
	MgCl2	67mM
	2 Mercaptoethanol	100mM
	Na2 EDTA.2H20	67mM
	BSA	1.7mg/ml
High Salt Buffer	Tris-HCl pH7.5	10mM
	MgCl2	10mM
	NaCl	100mM
Medium Salt Buffer	Tris-HCl pH7.5	10mM
	MgCl2	10mM
	NaCl	50mM
	Dithiothreitol	1mM

Media

L Broth	Bacto Tryptone	10gms
	Yeast Extract	5gms
	NaCl	10gms
	pH7.2 in 1L H2O	
Restriction Enzymes	New England Biolabs and	
	Boehringer Mannheim	
Blood Collection Media	RPMI 1640 HEPES Buffered Medium 200ml	
	3.3% Trisodium Citrate40ml	
	5mM Mercaptoethanol 2ml	
	25ml aliquotted into 50ml sterile blood bottles.	
Penicillin	5000 units/ml (Flow Laboratories)	

Oligonucleotide Primers

Locus	Primer Sequence
D8S88	TCCAGCAGAGAAAGGGTTAT (CA strand)
	GGCAAAGAGAACTCATCAGA (GT strand)
D8S133	CAGGTGGGAAAACTGAGGGA (CA strand)
	AGCAACTGTCAACATATTGCT (GT strand)
TP53P	GAAGAGCCTCGGTTATGGGTATACA (CR81)
	TCAGAAGGAAGTAGGAAGGACTCAG (CR82)
THRB gene	GCTAATCTAGAAATGTATTTACTATAGG(EA2R-
	TTTACTTCATGAAGCTGGCACG (EA2R-2)
LPL-Tet	ATCTGACCAAGGATAGTGGGATATA (GZ-14)
	CCTGGTTAACTGAGCGAGACTGTGTC (GZ-15)

Results

Cytogenetic Studies

A karyotype of affected individual III 17 (Fig.7.1) raised the suspicion of a constitutional deletion on chromosome 18p (Fig. 7.2). The karyotypes of three other affected individuals, however, were reported as normal, and it was concluded that the "deletion" probably represented a chromosomal polymorphism. Furthermore, two markers mapping to chromosomal region 18p11, (D18S32 and the Thymidylate Synthetase gene) were not linked to the disease locus (Figs.7.3,7.4).

Linkage Studies

A linkage study was carried out between the HMP syndrome locus and 77 marker loci spanning 16 chromosomes (Table 7.1). Details of the markers are readily available in the Genome Database(283).

Linkage was excluded in 34 genomic regions where a LOD score of -2 or less was calculated. In the remaining regions, the LOD score was calculated to lie between -2 and 1 and linkage could neither be confirmed or refuted. The most positive LOD score at zero recombination was 0.69 with marker D6S44, which is known to map to chromosome region 6p21-qter.

Linkage to the candidate genes, APC, DCC, hMSH2 and P53 was convincingly excluded with LOD scores at zero recombination of -2.38, -2.13, -2.05 and -6.25 respectively(Figs.7.5-7.13).

Linkage to a highly informative tetranucleotide repeat marker within the LPL gene on chromosome 8p, the third highest region of allele loss in a panel of colorectal cancer specimens, was also excluded with a LOD score of -5.20 at zero recombination (Fig.7.14).

Hybridisation with the multi-locus probe 33.15 revealed a band that was present in 10/16 unaffected individuals, but which was only present in 1/17 affected individuals (Fig 7.15)

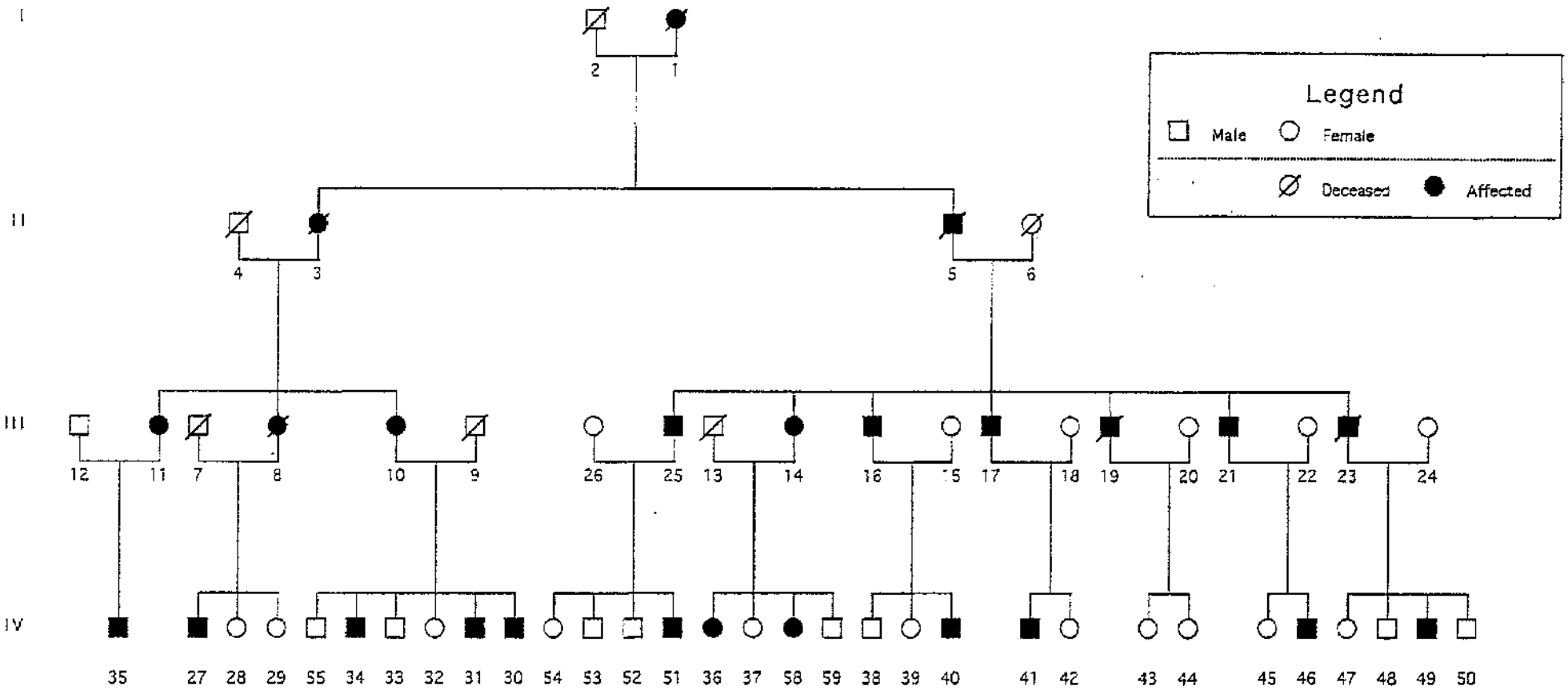


Fig. 7.1 Section of St Mark's Family 96 pedigree used for linkage study.

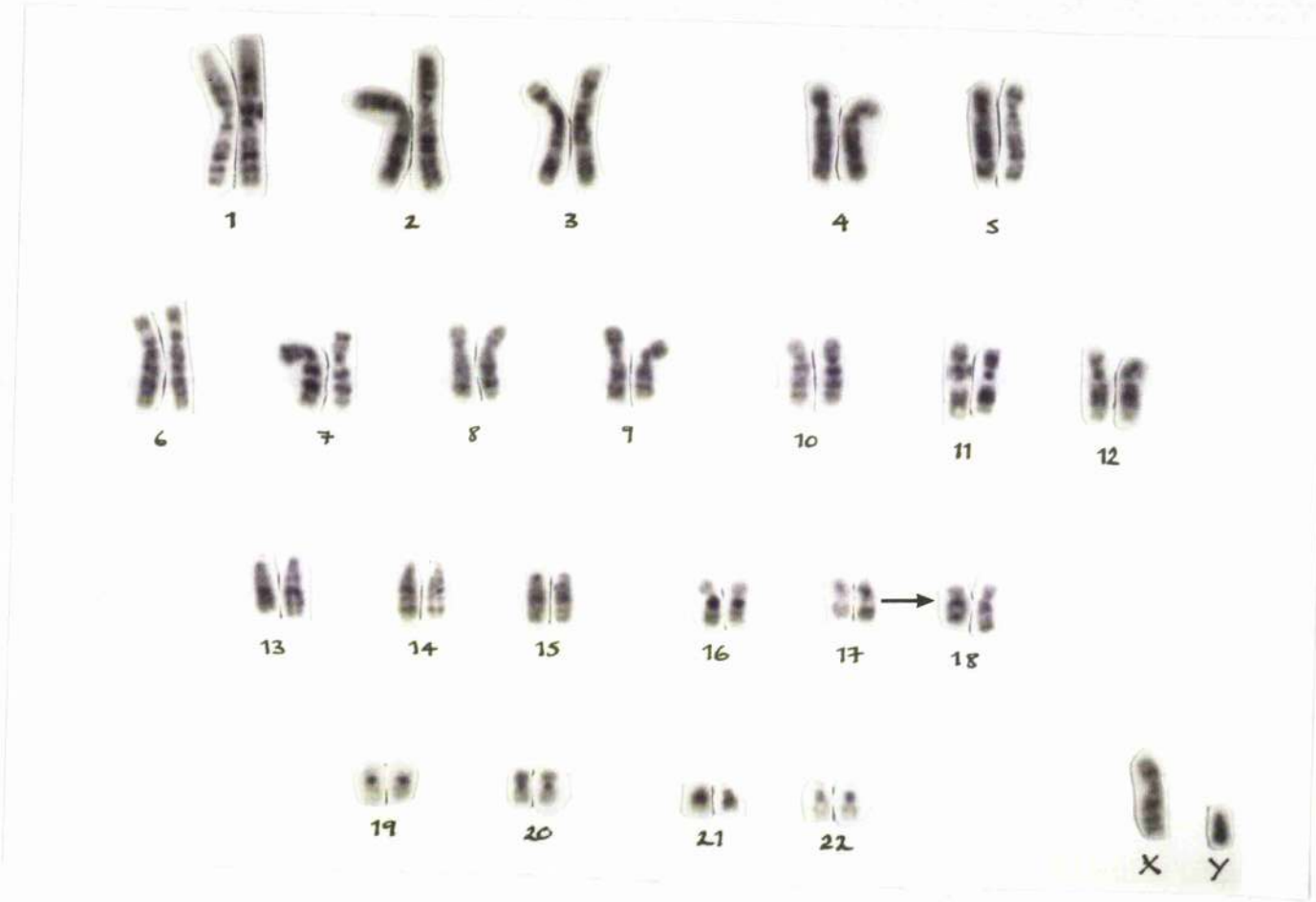


Fig. 7.2 Karyotype from affected individual 3.7 demonstrating polymorphism on chromosome 18p (arrow)

Family Member

Alleles

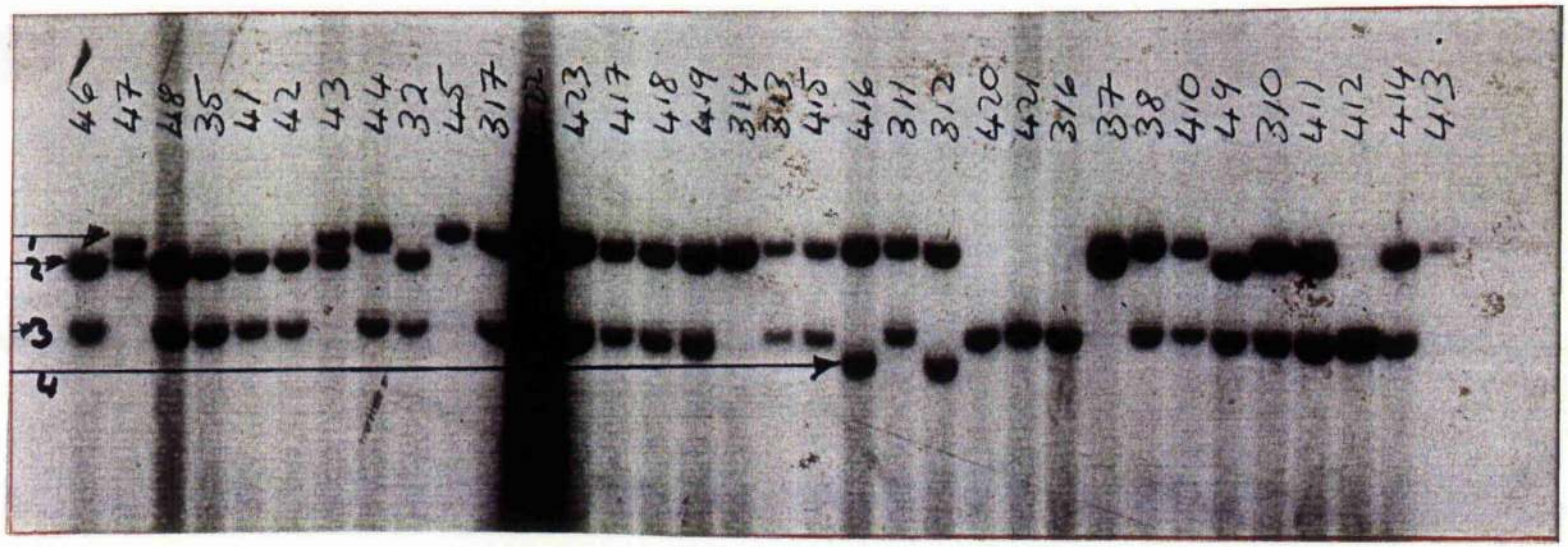


Fig.7.3 Autoradiographic bands detected by VNTR probe PMS 615 (D18S32) on chromosome 18p11.3 in DNA from St Mark's Family 96 digested with Hinf1 restriction endonuclease.

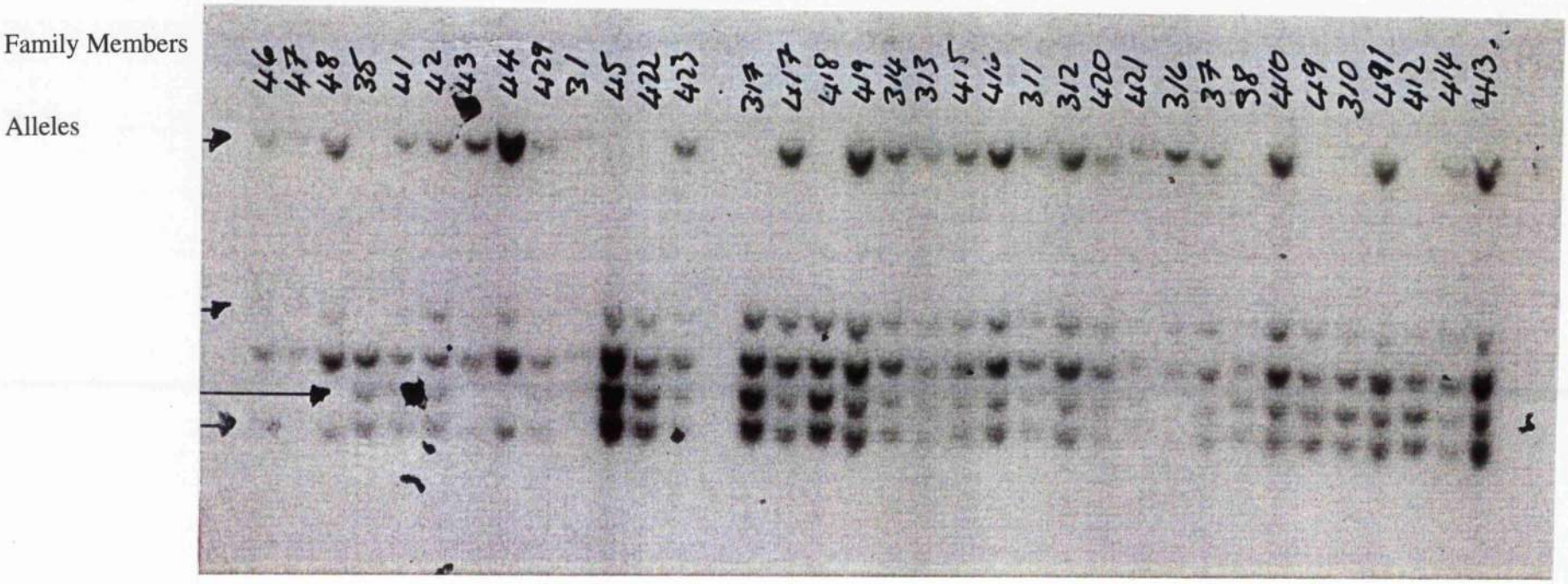


Fig.7.4 Restriction Fragment Length Polymorphism detected by Thymidylate Synthetase cDNA probe in DNA from St Mark's Family 96 digested with MSP1 restriction endonuclease.

Family Member

Alleles

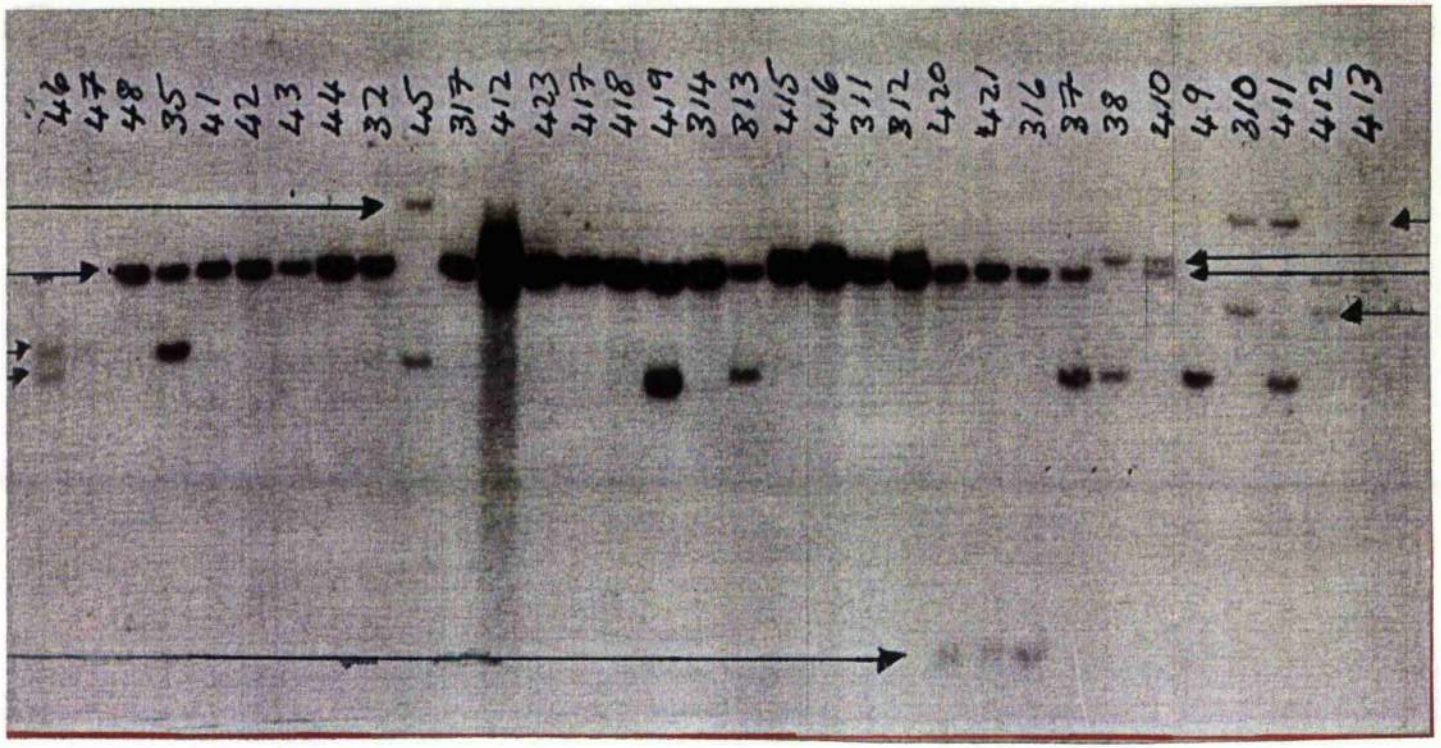


Fig.7.5 Autoradiographic bands detected by VNTR probe PMS 8 (D5S43) in DNA from St Mark's Family 96 digested by Taq 1 restriction endonuclease.

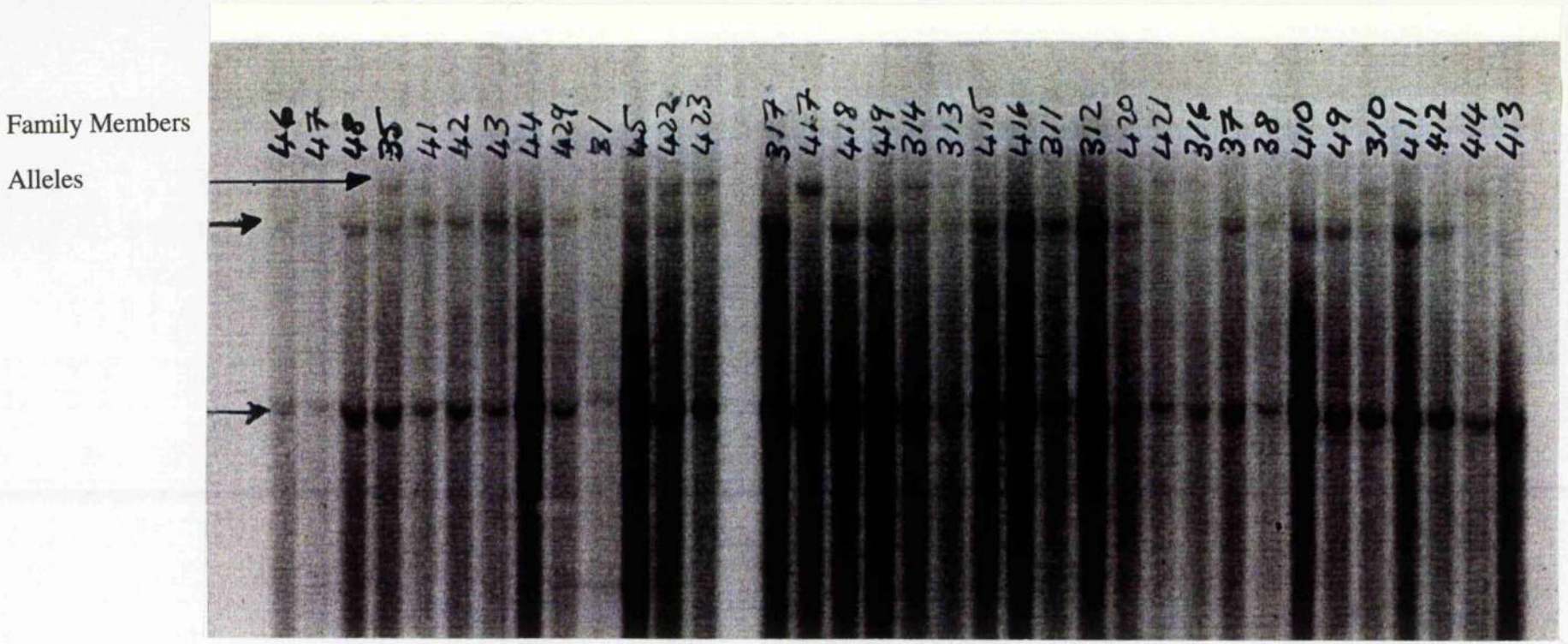


Fig. 7.6 Restriction Fragment Length Polymorphism on chromosome 5q21 detected by probe MC5.61 (D5S84) in DNA from St Mark's Family 96 digested by MSP 1 restriction endonuclease

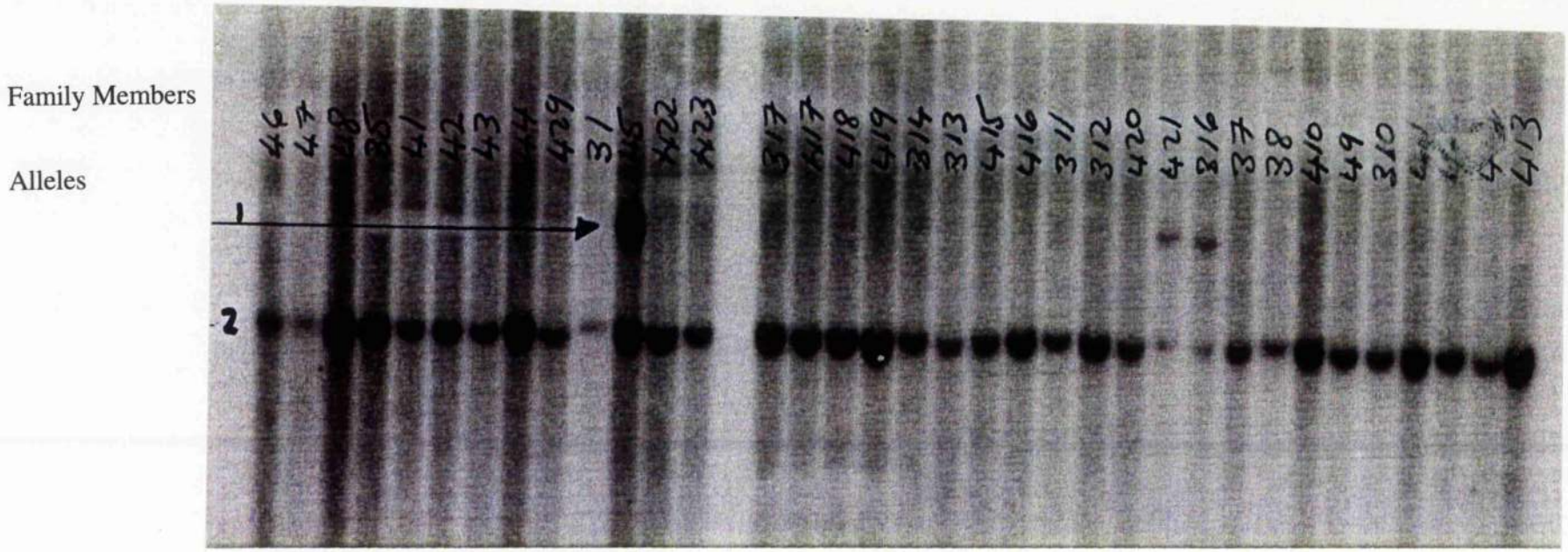


Fig.7.7 Restriction Fragment Length Polymorphism detected by probe EF5.44 (D5 S135) in DNA from St Mark's Family 96 digested with Msp 1 restriction endonuclease.

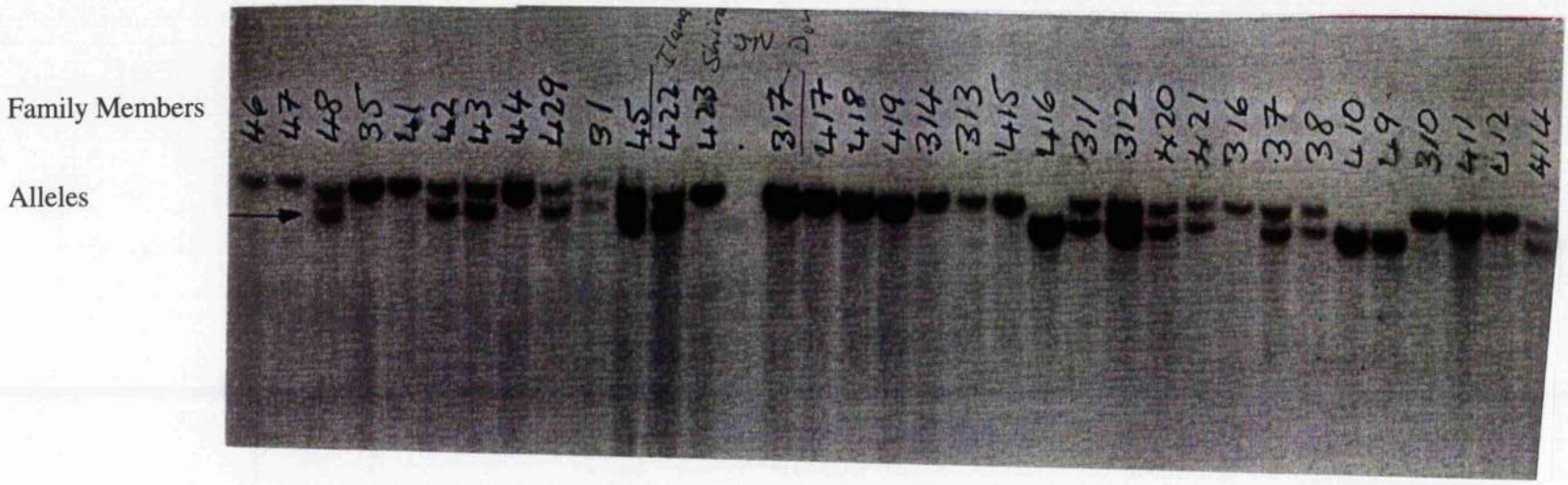
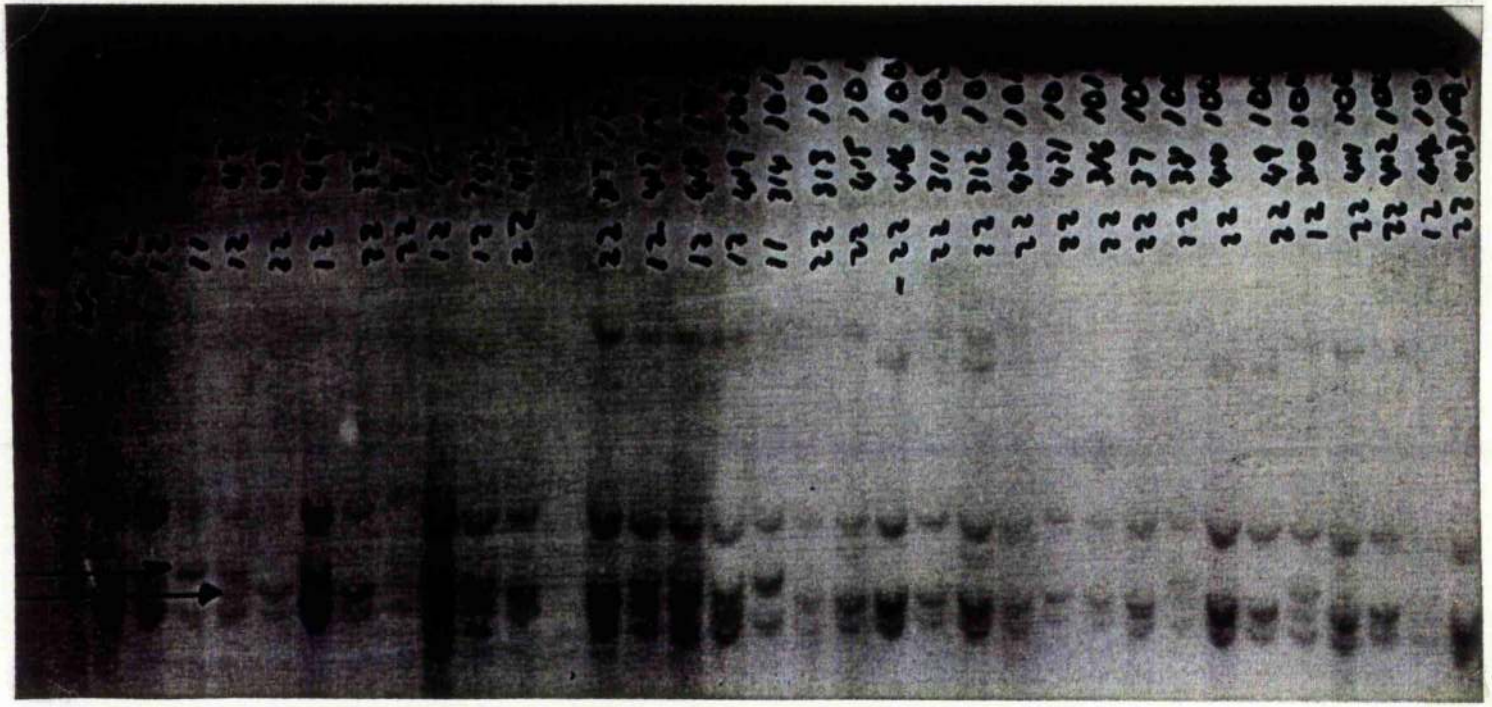


Fig. 7.8 Restriction Fragment Length Polymorphism detected by probe YN5.48 (D5S81) in DNA from St Mark's Family 96 digested with Msp 1 restriction endonuclease.

Family Member



Alleles

Fig.7.10 Restriction Fragment Length Polymorphism detected by probe SW15 (MCC) in DNA from St Mark's Family 96 digested with Msp 1 restriction endonuclease.

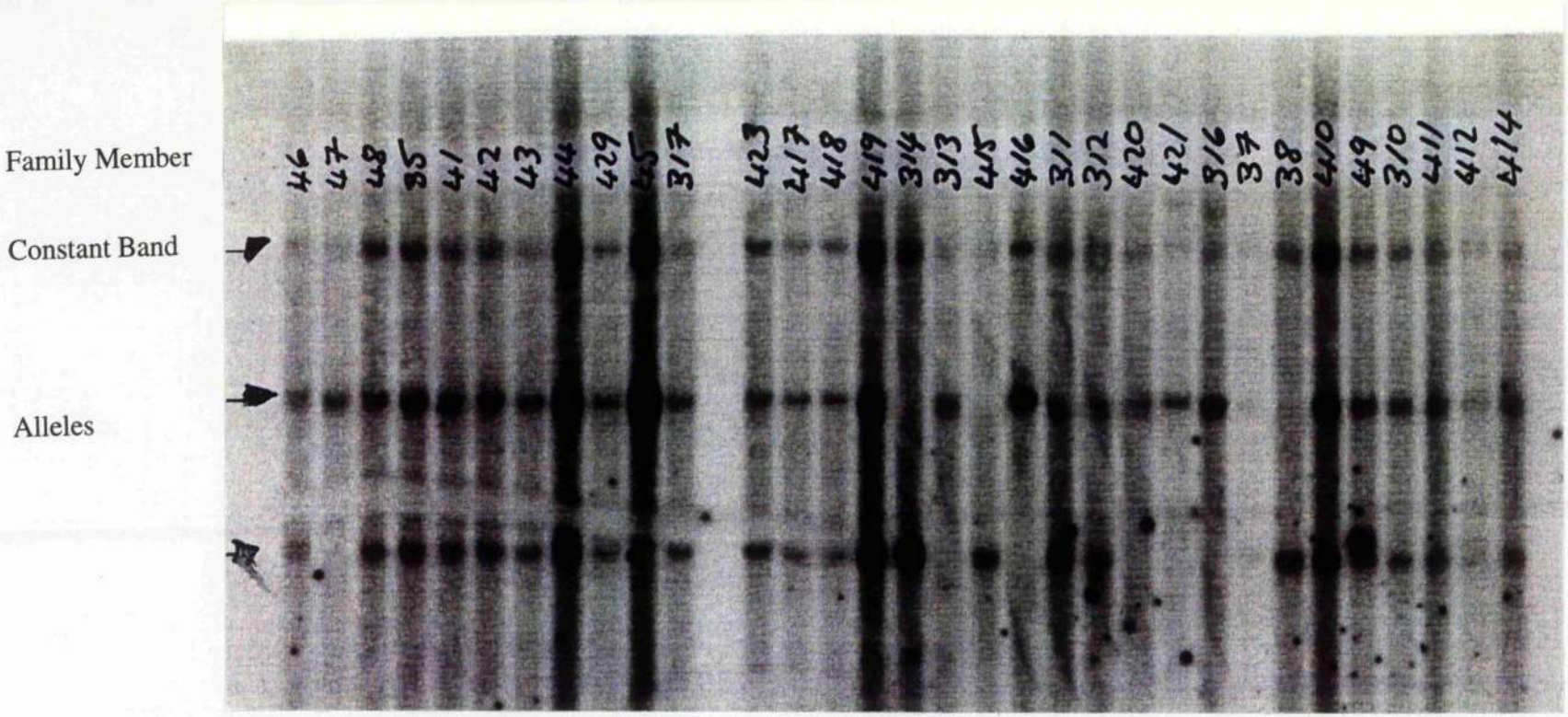


Fig.7.11 Restriction Fragment Length Polymorphism detected by probe FB40 (APC) in DNA from St Mark's Family 96 digested with Msp 1 restriction endonuclease.

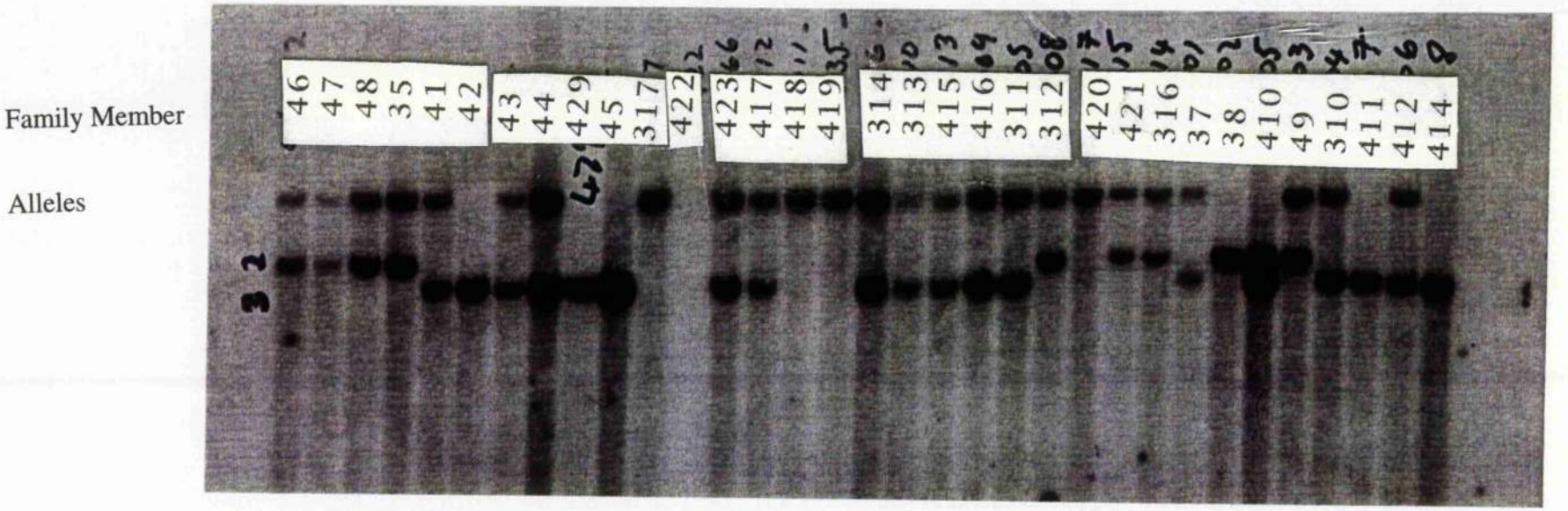


Fig.7.12 Restriction Fragment Length Polymorphism at the DCC locus detected by probe OLVII E10 (D18S8) in DNA from St Mark's Family 96 digested with Msp 1.

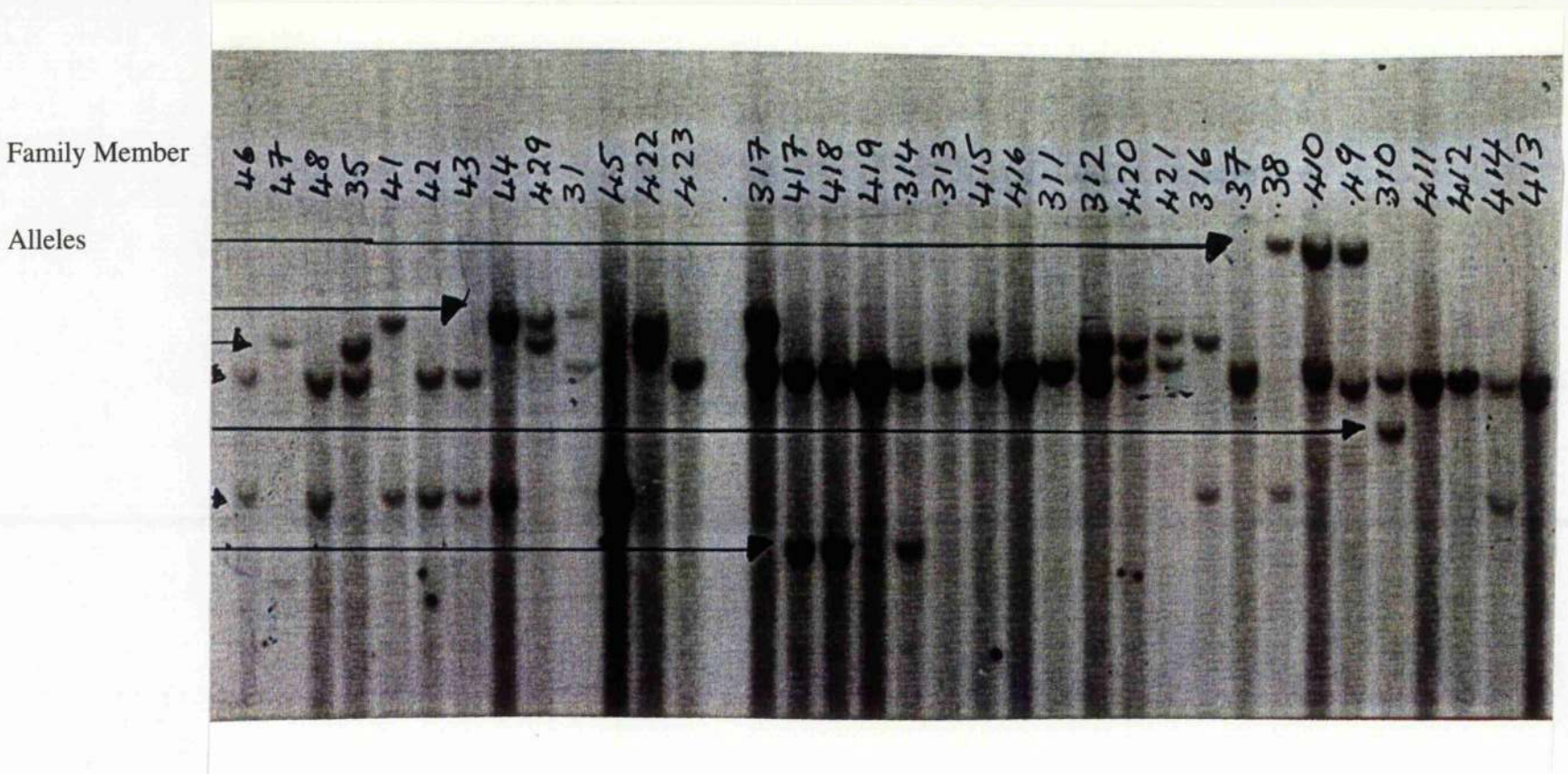


Fig. 7.13 Autoradiographic bands detected by probe p144 D6 (D17S34) in DNA from St Mark's Family 96 digested by Hinf 1 restriction endonuclease.

Family Member

Alleles

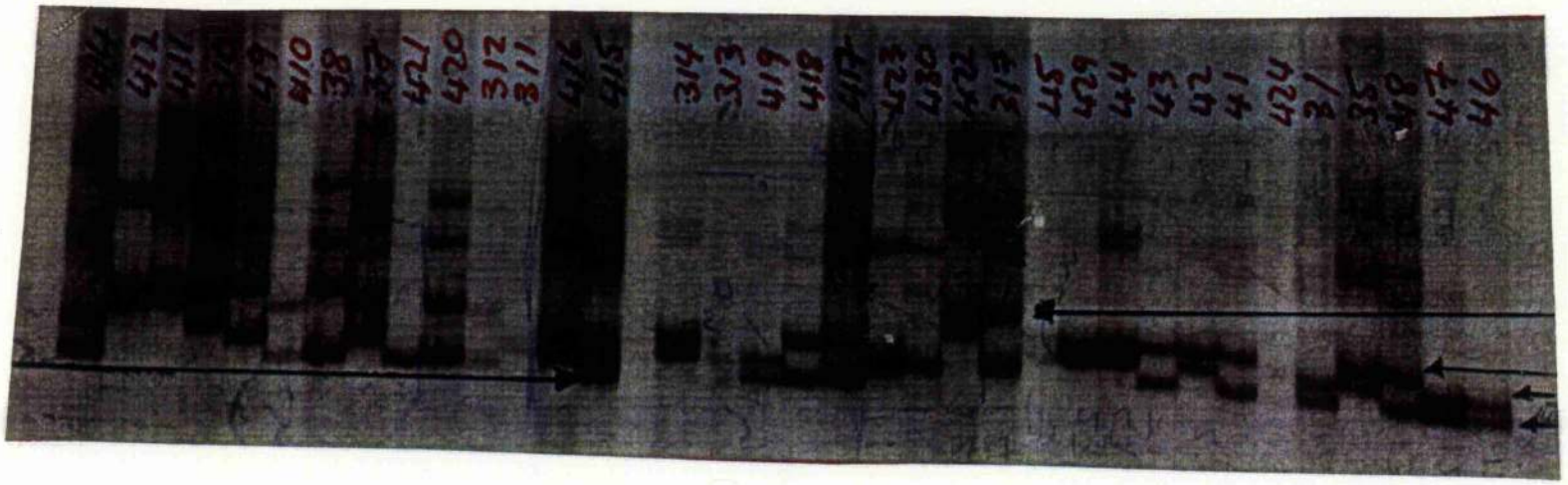


Fig. 7.14 Autoradiographic bands showing 5 alleles detected by LPL tetranucleotide marker on chromosome 8p in DNA from St Mark's Family 96.

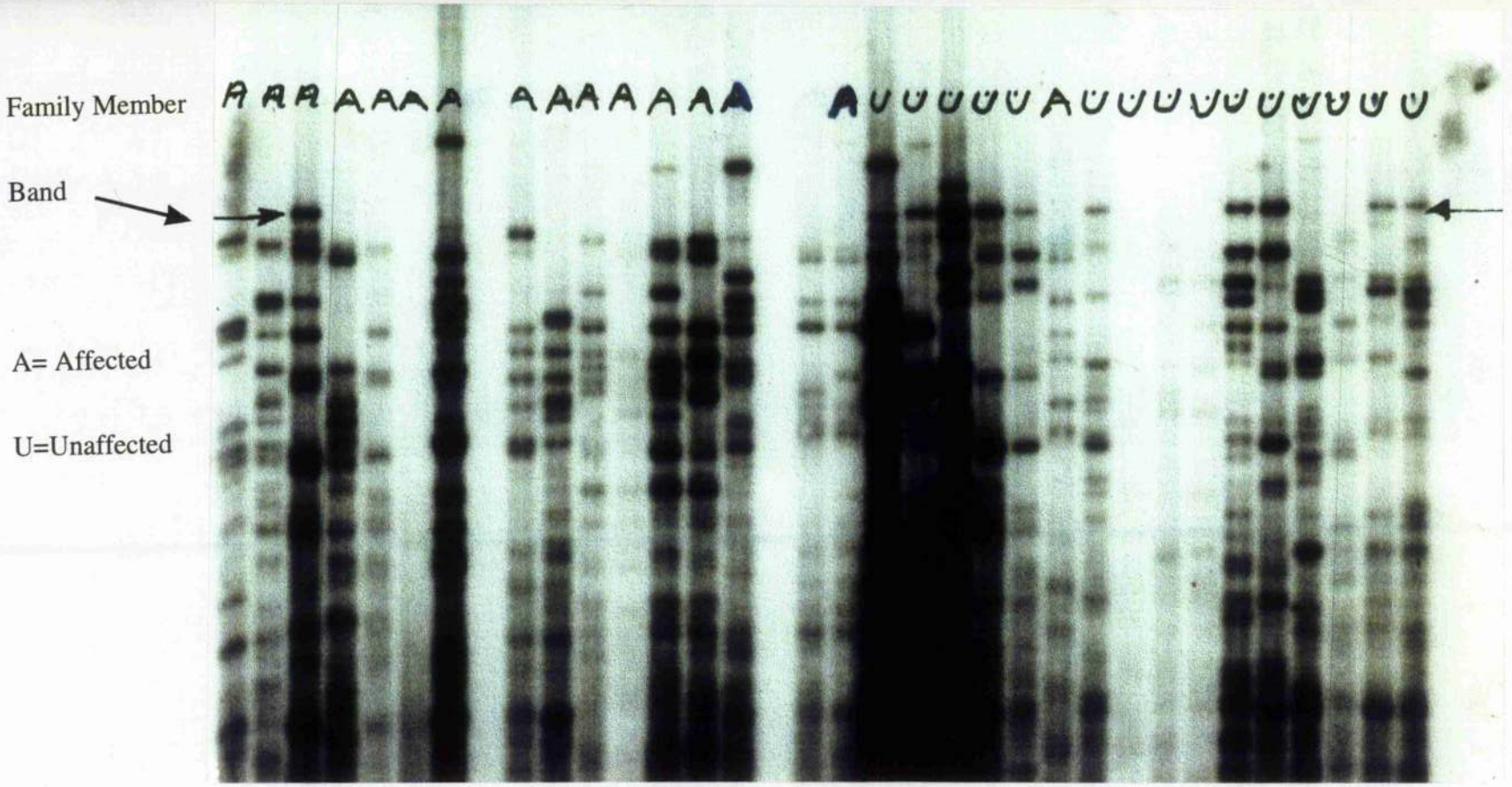


Fig.7.15 Fingerprinting autoradiograph showing bands detected by multilocus probe 33.15 in DNA from St Mark's Family 96

Table 7.1 Two-point LOD Scores between disease locus and each of 77 genetic markers tested.

		LOD score at recombination fraction Ø of						
Marker	**Locus**	**0.00**	**0.05**	**0.10**	**0.15**	**0.20**	**0.30**	**0.40**
D1S105	1	-4.18	-2.02	-1.37	-0.90	0.57	-0.19	-0.05
D1S57	1p35-p32	-2.05	-1.50	-1.04	-0.68	-0.41	-0.11	-0.09
D1S62	1p32	-2.33	-1.32	-0.86	-0.60	-0.42	-0.19	-0.06
D1S58	1q31-q32	0.27	0.18	0.12	0.06	0.03	0.02	0.003
D1S66	1q21-q31	0.26	0.20	0.15	0.11	0.08	0.03	0.001
D1S61	1q21-q31	-6.2	-2.47	-1.56	-1.05	-0.70	-0.28	-0.08
D1S7	1p35-p33	-INF	-4.3	-2.88	-1.87	-1.10	-0.41	-0.10
D1S85	1p32-p22	0.40	0.31	0.24	0.17	0.12	0.05	0.01
D2S44	2p	-INF	-2.59	-1.44	-0.89	-0.59	-0.32	-0.17
D2S50	2q	-4.46	-1.36	-0.75	-0.34	-0.09	0.10	0.06
D2S123	2p15-p16	-2.05	-1.72	-1.15	-0.76	-0.49	-0.17	-0.03
D3S42	3q21-qter	-3.82	-0.75	0.O2	0.38	0.54	0.49	0.24
THRB	3p22-p24	-1.35	-1.15	-0.70	-0.41	-0.22	-0.04	-0.02

Marker	Locus	0.00	0.05	0.10	0.15	0.20	0.30	0.40
D4S10	4p16.3	-1.90	-0.69	-0.43	-0.28	-0.20	-0.10	-0.04
D4S124	4p	-7.2	-2.7	-1.4	-0.7	-0.36	-0.04	0.00
D4S13	4p15-q21	-2.75	-1.43	-0.90	-0.59	-0.39	-0.16	-0.05
D5S98	5q15-q21	-1.82	-1.04	-0.69	-0.47	-0.31	-0.13	-0.04
D5S43	5q35-qter	-3.71	-1.02	-0.67	-0.34	-0.01	0.12	0.14
APC-FB40	5q21-q22	-2.38	-0.58	-0.35	-0.24	-0.17	-0.07	-0.01
D5S135	5q	0.10	0.07	0.05	0.04	0.02	0.01	0.003
D5S81	5q21-q22	-2.23	-0.53	-0.30	-0.20	-0.13	-0.04	0000
D5S84	5q21-q22	-2.00	0.06	0.20	0.23	0.20	0.11	0.025
MCC	5q21-q22	-2.40	-0.40	-0.20	-0.11	-0.07	-0.03	-0.02
D5S347	5	-13.2	-4.64	-2.61	-1.56	-0.54	-0.21	-0.13
D6S44	6p21-qter	0.69	0.55	0.42	0.31	0.22	0.09	0.02
PIM	6p21	0.15	0.07	0.04	0.02	0.01	0.00	000
D6S133	6q27	-INF	-1.52	-0.56	-0.15	0.04	0.12	-0.02
D7S439	7p	-9.24	-3.33	-2.15	-1.43	-0.96	-0.36	
D7S370	7pter-p14	-0.63	-0.37	-0.15	-0.04	0.005	0.02	0.017

Marker	Locus	0.00	0.05	0.10	0.15	0.20	0.30	0.40
NEFL	8p21	0.63	0.51	0.41	0.03	0.02	0.11	0.03
LPL-TET	8p22	-5.20	-3.33	-2.24	-1.63	-1.12	-0.58	-0.21
D8S133	8p	-0.49	-0.27	-0.14	-0.06	-0.02	0.04	0.07
D8S88	8	-2.97	-2.19	-1.63	-1.16	-0.77	-0.26	0.00
D9S10	9q34.3	-1.52	-1.07	-0.75	-0.54	-0.39	-0.21	-0.85
D9S7	9q34	-INF.	0.06	0.16	0.15	0.12	0.05	0.00
D10S90	10q26	-1.30	-0.84	-0.48	-0.02	-0.09	0.02	0.03
D10S92	10p15	-1.95	-0.39	-0.01	0.15	0.02	0.18	0.07
D10S25	10q26	-0.26	0.10	0.31	0.40	0.42	0.31	0.13
D10S32	10pter-p13	-1.27	-0.53	-0.21	-0.05	-0.03	0.07	0.06
D10S13	10q21-q23	-0.06	-0.03	-0.15	-0.08	-0.05	-0.04	-0.03
RPB3	10q11.2	-1.46	-1.12	-0.82	-0.57	-0.37	-0.12	-0.02
CD20	11q12-q13	-1.3	-0.93	-0.75	-0.57	-0.40	-0.16	-0.03
D12S42	12	-INF.	-5.29	-3.33	-2.27	-1.56	-0.67	-0.19
D12S40	12	-4.46	-1.36	-0.75	-0.34	-0.09	0.10	0.06
D15S86	15	-1.56	-1.13	-0.69	-0.39	-0.19	-0.01	0.00

Marker	Locus	0.00	0.05	0.10	0.15	0.20	0.30	0.40
D17S34	17p13.3	-9.14	-4.27	-2.15	-1.14	-0.36	-0.11	-0.02
D17S180	17q12-q21	-2.13	-1.27	-0.70	-0.48	-0.26	-0.11	-0.03
D17S308	17q21-qter	-6.94	-3.52	-2.50	-1.73	-0.78	-0.29	-0.06
D17S579	17	-2.17	-1.79	-0.79	-0.37	-0.05	0.04	0.03
TP53CA	17p13.1	-6.25	-2.27	-0.76	-0.01	0.46	0.39	0.15
D17S5	17p13.3	-5.22	-1.48	-0.24	0.16	0.33	0.24	0.10
D17S28	17p13.3	0.68	0.66	0.55	0.44	0.24	0.11	0.04
D18S8	18q21.3	-2.13	-1.80	-1.22	-0.80	-0.51	-0.18	-0.04
D18S32	18p11.3	-4.48	-1.69	0.29	0.45	0.46	0.29	0.04
D18S7	18q11.1-11.2	-1.66	-0.26	-0.10	-0.04	-0.02	0.00	0.00
D18S3b	18q11.3	-1.85	-0.79	-0.51	-0.34	-0.23	-0.08	-0.02
T.S.	18p11.32	-INF.	-0.68	-0.34	-0.18	-0.10	-0.04	-0.01
D22S164	22	-0.52	-0.50	-0.28	-0.02	0.14	0.24	0.15
D22S163	22	-1.23	-1.02	-0.57	-0.28	-0.12	-0.09	000

Discussion

Before the advent of automated sequence analysis and microsatellite markers, linkage analysis was often a painstaking and laborious process. Restriction Fragment Length Polymorphic Markers often provide little linkage information and the number of readily available VNTR markers was limited. The whole undertaking was further complicated by the sheer size of the human haploid genome, estimated to be 3 billion base pairs long.

The presence of a cytogenetic clue can greatly accelerate the linkage process. In St Mark's Family 96, the finding of a possible deletion on chromosome18p11.3 in the karyotype of individual III-7 was considered sufficiently interesting to study genetic markers in this region for linkage, while awaiting the results of confirmatory cytogenetic studies in other affected family members. Disappointingly, negative LOD scores excluding linkage were obtained for two markers, D18S32 and the thymidylate synthetase cDNA, which mapped to this region (Figs 7.3,7.4). Furthermore, high resolution banding karyotypes of three other affected members were found to be normal suggesting that the observed "deletion" was a polymorphism.

At the commencement of this project, the APC gene had been localised but not cloned. Six useful markers were readily available for linkage analysis in this area (D5S98, D5S43, D5S81, D5S84, D5S135, SW15), and all yielded negative LOD scores with the exception of D5S135 which gave an ambiguous LOD score of 0.1 at zero recombination (Figs 7.5-7.10). Subsequently, when the APC gene was cloned, linkage to an intragenic marker FB40 gave a LOD score of -2.38 at zero recombination, demonstrating convincingly that HMPS is not an atypical variant of FAP (Fig.7.11).

Linkage to other candidate genes, namely DCC (Fig.7.12), P53 and neighbouring region (Fig 7.13) and hMSH2 was also excluded. At the time of this study, other

genes which predispose to HNPCC, the hMLH1, PMS1 and PMS2 genes had not been identified.

Regions of high allele loss in DNA extracted from colorectal cancer specimens include the short and long arm of chromosome 6, the short arm of chromosome 8, and the short arm of chromosome 1. All readily available markers were tested for these regions, including a tetrameric microsatellite repeat marker for the LPL gene mapping to 8p21 (Fig 7.14), but again no linkage was demonstrated.

The complexity of DNA fingerprinting detected with multilocus probes usually precludes its use in linkage analysis studies between families. However, within a very large kindred such as St Mark's Family 96, a single band can be assumed to represent a single locus. If a band is then found to segregate with the disease phenotype, theoretically it would then bepossible, to isolate and clone the band DNA to make a locus specific probe which could confirm or refute linkage. The allele pattern obtained with probe 33.5 was particularly interesting, in that the vast majority of affected patients appeared to have a missing band, as illustrated in (Fig 7.15). Unfortunately, this observation was not all or none in nature and probe 33.5 could not be used,therefore, for predictive testing.

In any linkage analysis study, even the most advanced technology is of little value in the presence of inaccurate pedigree information. In one experiment involving a highly polymorphic VNTR marker, it appeared in one sibship, that a son had inherited a rare allele not possessed by either of the parents. This raised questions about the accuracy of the remainder of the pedigree, possible non-paternity and the mislabelling of DNA samples. It subsequently transpired that both the father and son in the sibship in question had an identical name, and that blood samples had been mislabelled. When this situation was clarifed, inheritance with this and several other VNTR and microsatellite markers was found to be Mendelian, thus strengthening confidence in the accuracy of the pedigree.

The localisation of the gene responisble for disease in St Mark's Family 96 was a highly ambitious project that was not achieved within the two year time limit of this research project. Nonetheless, it has been shown unequivocally that the Hereditary Mixed Polyposis syndrome is not a variant of familial adenomatous polyposis, and linkage to other candidate genes, notably p53, MCC, DCC, hMSH2, NM23, and 8p21-22 has been excluded. Interestingly, the highest LOD score obtained in this study with the marker (D6S44) which maps to chromosome 6p21-qter (see below).

Postscript

Following this period of research, Dr Huw Thomas and Sally Cottrell from the Cancer Genetics Laboratory, ICRF, took the DNA samples from St Mark's Family 96 to the Genethon Genotyping Laboratory in Paris. This laboratory has robotic sample and reagent handling facilities capable of setting up multiple PCR reactions for genetic markers spanning the entire genome. Sixteen filters from 256 microsatellite markers were made, which were then hybridised back at the ICRF laboratories, Lincoln's Inn Field, London, using one of the PCR primers as a probe in the Amersham ECL direct labelling and detection system. After hybridising 84 of these, a LOD score of 2.2 was obtained with the marker D6S283 and efforts were then focused on the region 6q16-21. A significant LOD score of 3.45 was obtained with the marker D6S301 mapping to 6q21. Analysis of recombinants and multipoint linkage analysis suggests that the HMPS locus lies in a 4-cM interval containing the D6S283 locus and flanked by markers D6S468 and D6S301.

Thus, the hereditary mixed polyposis syndrome is a distinct autosomal dominant disorder linked to a mutated gene on chromosome 6q21.

Chapter 8

A Search for Attenuated Familial Adenomatous Polyposis in a Cohort of Patients with Colorectal Cancer

Introduction

A less severe, but phenotypically distinct variant of FAP, known as attenuated adenomatous polyposis coli (AAPC), has been described, in which patients develop a relatively low number of adenomatous polyps in the colon (usually less than 100) (418). The number of polyps in AAPC tends to vary markedly within members of the same family, complicating recognition of the phenotype. Like FAP, these patients retain an increased risk of developing colorectal cancer, but the average age at diagnosis is 15 years later than in its classical form, and 10 years earlier than in individuals with"sporadic" colorectal cancer. A series of linkage studies in AAPC families have now mapped the mutant alleles to the APC locus. In total, four distinct mutations in the APC gene have now been identified in seven unrelated AAPC families . These mutations differ to those observed in classical FAP in that they are located very close to one another, in exons 3 and 4 nearer the 5' end of the APC gene (419,420).

A variety of methods are now available to detect point mutations in genes. The technique of Single Strand Conformational Polymorphism (SSCP) analysis relies on the fact that single stranded DNA has a folded conformation as a result of intra-strand base-pairing. The electrophoretic mobility of these sequences is dependant on these secondary structures, which in turn are determined by the nucleotide sequence of the PCR products synthesised from the region of interest. If the PCR products are electrophoresed on denaturing polyacrylamide gels, and if the secondary structure of the single stranded products is altered by a mutation, a mobility shift will result, which can be observed as a band shift on the autoradiograph. The position of the single nucleotide change is then confirmed by sequence analysis, using the PCR primers as sequencing primers (421).

The purpose of this study was to investigate whether a small subset of 'sporadic" colorectal cancer might be due to mutations in exons 3 and 4 of the APC gene. SSCP

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dryers, and autoradiographed. The sample with an altered band was sequenced with the Sequenase 2.0 system (USB, Cleveland,USA) with a single strand direct PCR product immobilised on streptavidin beads (Dynol,Oslo,Norway). The PCR product was generated with one biotinylated primer from the original DNA sample exhibiting the altered band.

Ologonucleotide Primers

Exon 3	Primer 1- ATGATATCTTACCAAATGATATAC
	Primer 2- TTATTCCTACTTCTTCTATACAG
Exon 4	Primer 1- TACCCATGCTGGCTCTTTTTC
	Primer 2- TGGGGCCATCTTGTTCCTGA

Results

SSCP analysis of exons 3 and 4 failed to demonstrate any convincing evidence of a mutation in the constitutional DNA of any of the 18 patients examined (Figs 8.5 and 8.6). Although there was a suggestion of a band shift in lane 8 of Fig 8.6, sequence analysis revealed that this was artefactual with no evidence of a nucleotide alteration.

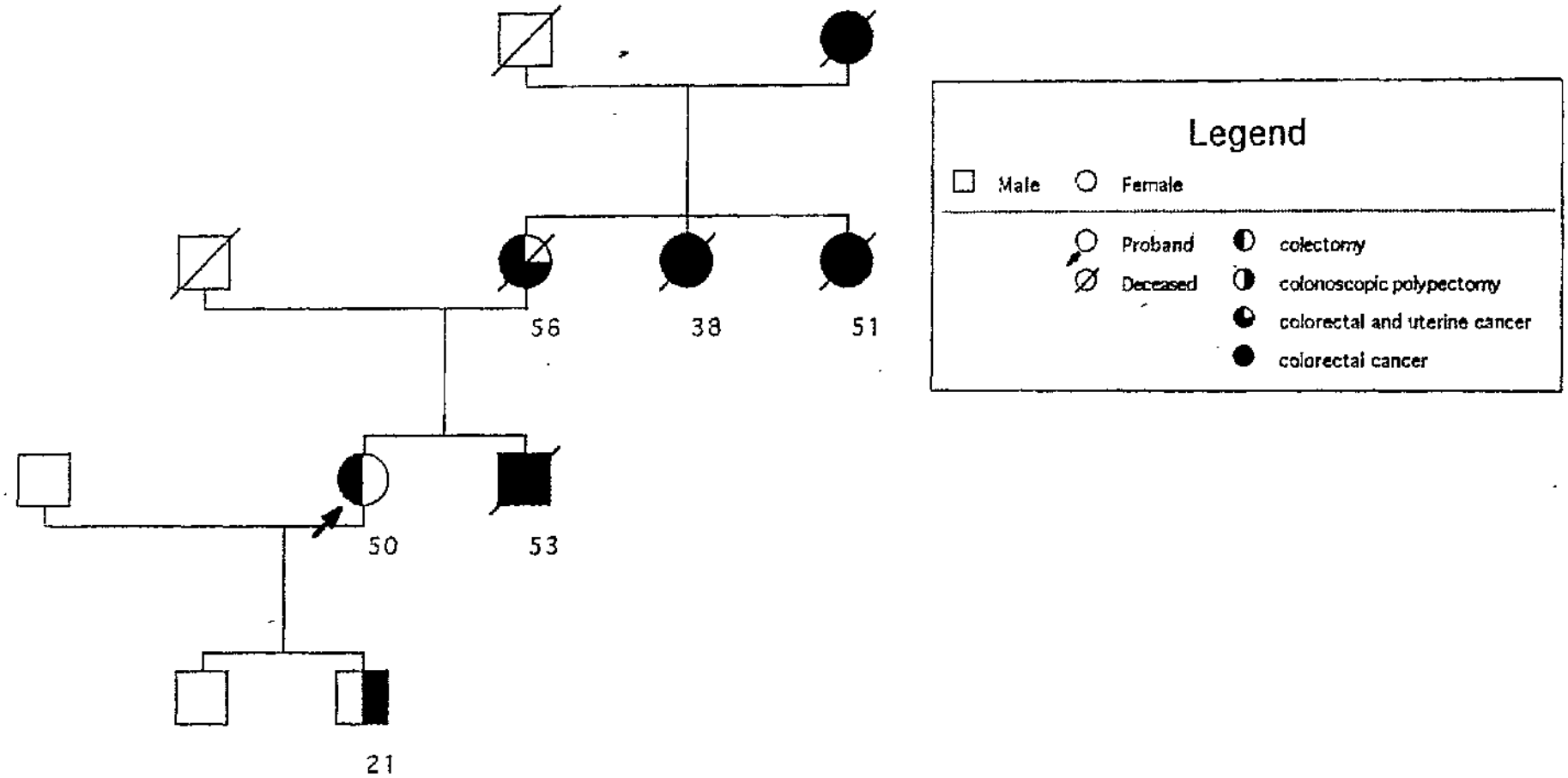

Fig. 8.1 Pedigree of St Mark's Family "G"

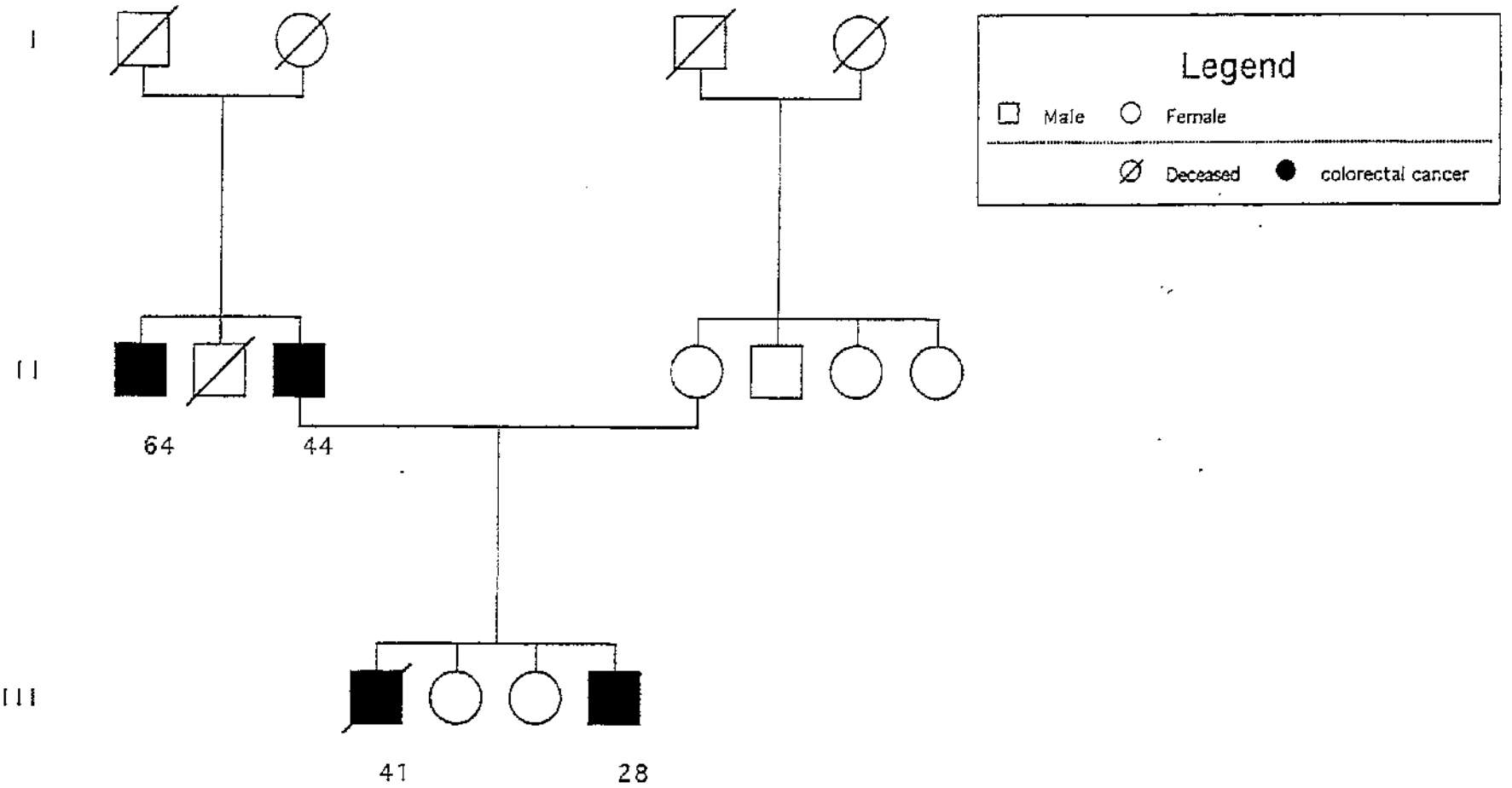

Fig. 8.2 Pedigree of St Mark's Family "P"

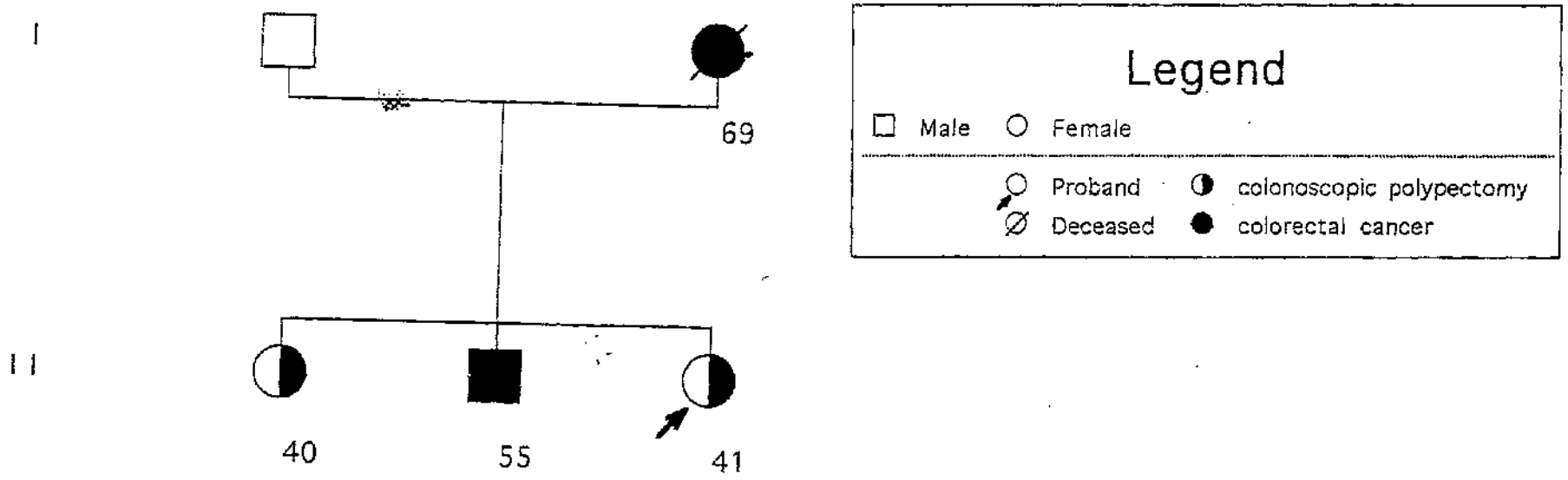

Fig. 8.3 Pedigree of St mark's Family "W"

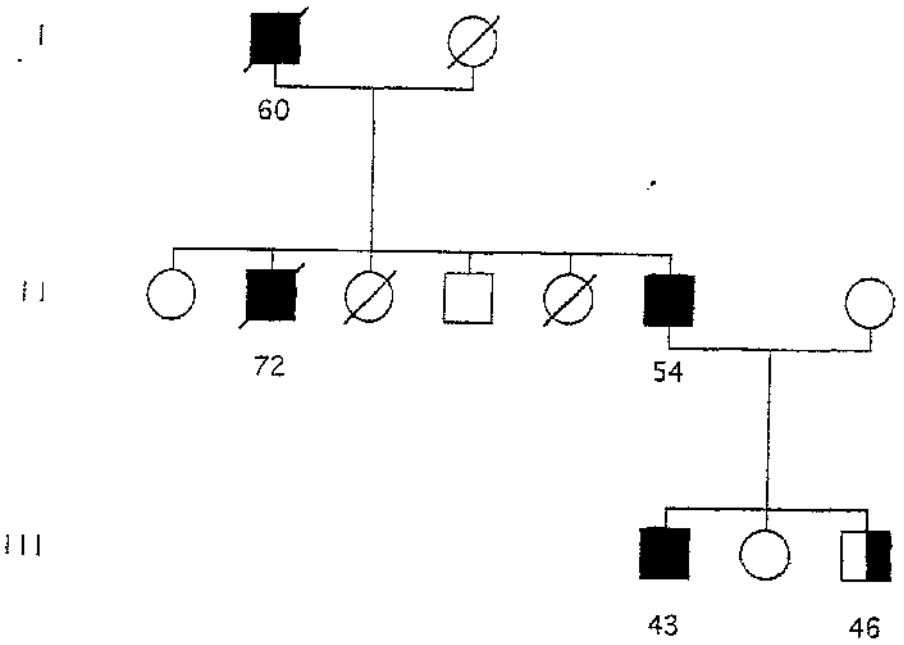

Fig.8.4 Pedigree of St Mark's Family "S"

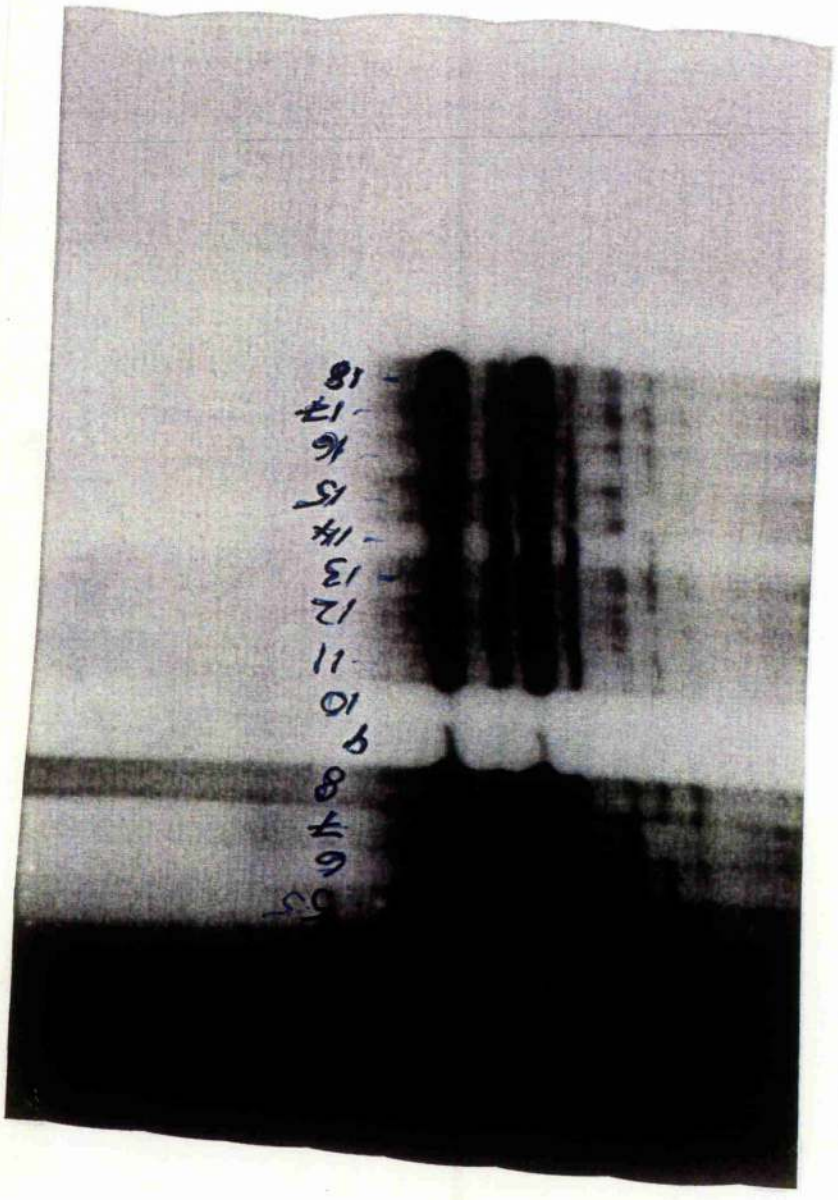

Fig.8.5 SSCP autoradiograph of Exon 3 of APC gene

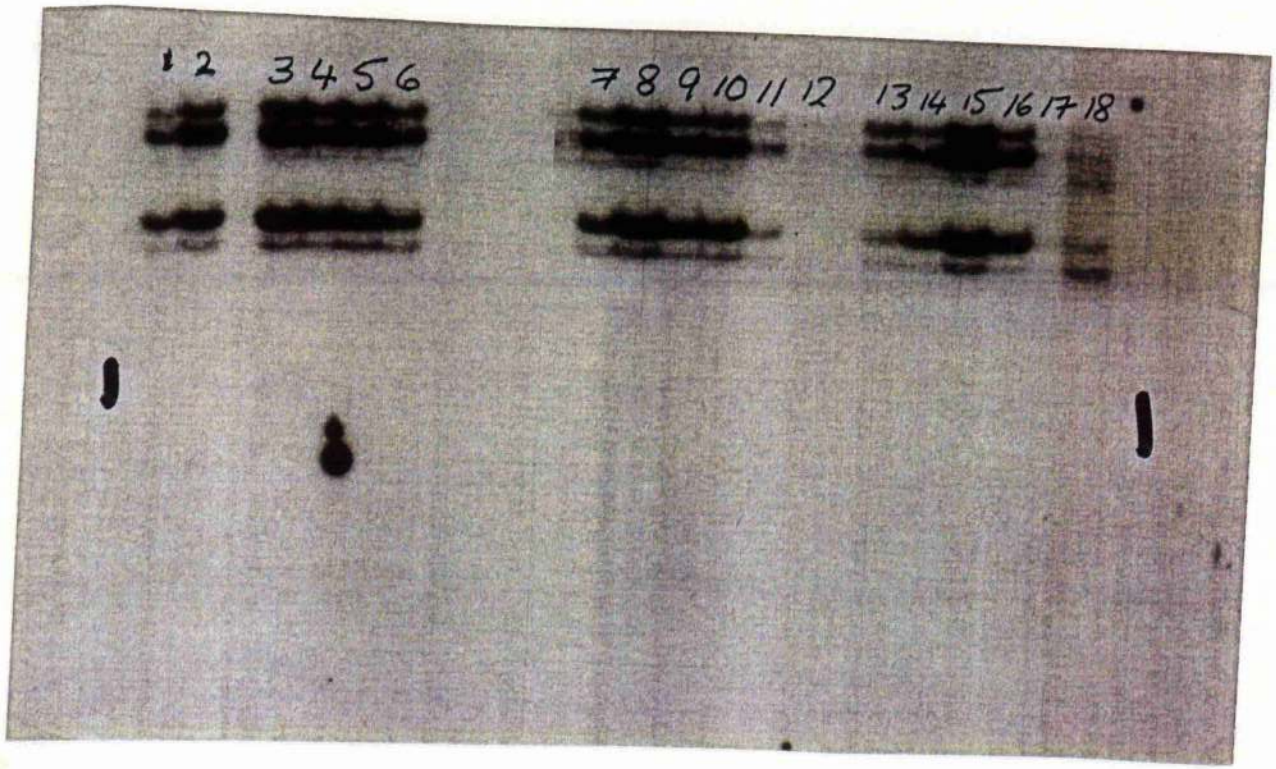

Fig.8.6 SSCP autoradiograph of Exon 4 of APC gene

Patient	Age	Sex	Pathology
1	46	F	Carcinoma of Caecum
2	40	F	Carcinoma of Rectum
3	44	M	Carcinoma of Caecum
4	69	F	Carcinoma of Sigmoid
5	50	F	50-100 Adenomas
6	51	M	Carcinoma of Rectum
7	50	F	Carcinoma of Rectum
8	52	F	Carcinoma of Sigmoid
9	34	F	Carcinoma of Rectum
10	40	F	Carcinoma of Rectum
11	47	M	Carcinoma of Rectum
12	26	F	Carcinoma of Rectum
13	46	M	10 Adenomas
14	43	M	Carcinoma of Rectum
15	41	F	6 Adenomas
16	35	M	Carcinoma of Rectum
17	27	F	Carcinoma of Rectum
18	40	M	Carcinoma of Caecum

Table 8.1 Clinico-pathological Features of Study Population.

Discussion

It has been speculated that AAPC may be under-recognised in the general population, accounting for a higher percentage of colorectal cancer than is generally appreciated. The patients in this study either developed colorectal cancer at a relatively early age or were found to have relatively few adenomas in the presence of a family history of CRC, and it might be anticipated that germ-line mutations in exons 3 or 4 of the APC gene might be present in some members of this cohort. No mutations in exons 3 or 4 were detected by SSCP, although the study population was small, and this perhaps precludes any firm conclusions about the "subset" hypothesis. Some of these patients may have germ-line mutations in other parts of the APC gene, in the MCC gene nearby or even in one of the mismatch repair genes which had not been localised or cloned at the time of this study.

Several possibilities exist to explain why a mutation at the beginning of the APC gene may predispose the individual to less adenomas than is seen in mutations affecting exon 15. Olschwang has suggested that the two groups of mutations may define a functional boundary within the APC gene that determines phenotypic differences between APC and AAPC families (422). Most of the mutations identified in classical FAP have been found in exon 15 and are of the " knock-out " or nonsense variety, in which no useful gene product is produced. This product however may retain the capacity to homodimerise or complex with the normal APC gene product, causing almost total abolition of function. A missense mutation right at the start of the APC gene on the other hand, may generate a stop codon, leading to the formation of a very short peptide which is unable to complex with the APC protein, or alternatively to no protein product. In this event, only 50% of APC protein would be available for cell utilisation (222,423), leading to uncontrolled proliferation of the colorectal epithelium and formation of polyps. Affected individuals in the same family, however, can exhibit markeds variation with regard the number of adenomas they develop in the

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Chapter 9

Psychological Aspects of Familial Adenomatous Polyposis and Attitudes to Predictive DNA Testing

" Life is not just a matter of holding a good set of cards, but of playing a poor hand well"

Robert Louis Stevenson

Introduction

The cloning of the APC gene now means, that in a high percentage of cases, presymptomatic and even prenatal diagnosis can be offered for FAP using either linkage or mutational analysis (238,424). An at-risk individual who does not have the genotype segregating with the disease within the family has a very low risk of developing FAP, and this risk becomes even less if an integrated approach combining the presence of CHRPE and linked markers is adopted (425). Where the mutation has been identified in the family, a direct test for this can be offered to at-risk relatives, with a very high degree of accuracy. Two questions now need to be addressed.

What do people with FAP think the best age to offer predictive DNA testing for FAP in their at-risk children is?

How acceptable is antenatal testing and selective termination of pregnancy for FAP for couples at risk of having affected children?

The answers to these questions will undoubtedly be influenced by psychological factors, perception of disease severity and philosophical, personal and religious attitudes to predictive DNA testing and termination of pregnancy. In turn, the perception of the severity of the disease and its subjective effects, are likely to be strongly affected by the individual's previous experience of FAP. In haemophilia, it has been shown that a woman's perception of the pros and cons of prenatal diagnosis is strongly influenced by personal experience of the course of the disease in closest male relatives (426).

This study was initiated before predictive DNA testing for FAP was readily available and was still a relatively new concept. Its purpose was to assess the psychological impact of FAP and patient attitude to predictive testing.

Patients and Methods

Sixty-three patients with FAP who attend the out-patient department of St Mark's Hospital, London on a regular basis, were invited to participate in the study and 62 (33 male, 29 female) agreed. The median age of the patients was 38 years (range 19-67 years). Each patient had undergone prophylactic colectomy. 55 patients had an ileorectal anastomosis, 4 patients had a restorative proctocolectomy with ileoanal pouch formation, and 3 patients had a panproctocolectomy with formation of a permanent end ileostomy. 29 patients had children, of whom 10 had children in whom FAP had been diagnosed. The remainder had children whose disease status was still uncertain.

The Modified Illness Effects Questionnaire

Psychological responses were elicited in a semi-structured interview using the Modified Illness Effects Questionnaire (MIEQ), a 20 item scale that assesses the individual's perception of how the subject's illness interferes with, or affects personal and social behaviour (Table 9.1). The statements were specifically designed to assess feelings of anxiety, depression, relationship disturbance, preoccupation with illness and alienation with the health professions. The MIEQ was based on a previously validated Illness Effects Questionnaire (427) which has been shown accurately to assess cognitive depression (428). It was modified and tailored to assess emotional problems specifically anticipated to be relevant to FAP patients. Level of psychological distress was quantified using a scoring system, ranging from zero, correlating with no distress, to 60, correlating with maximum distress. The statements were read to the patients who were asked to respond ; the same clinician interviewed all 62 patients. Only 4 possible response options -agree, strongly agree, disagree and strongly disagree-were offered to the patients to minimise " fence-straddling", and several statements were polarised e.g. , " Having polyposis is the biggest difficulty in my family" or "Having polyposis has helped me get more out of

life". Each patient was given the opportunity to discuss their illness openly, and any additional anxieties were documented.

Patients were also asked if they understood the chances of their children inheriting polyposis.

Attitude to Predictive DNA Testing

The following questions were employed in a semi-structured interview by the same interviewer:

If you knew it was possible to diagnose FAP in the early weeks of pregnancy, would you ask your doctor if you could have the antenatal test, and if so, why?

If the test showed that the unborn baby was affected by polyposis would you proceed to termination of pregnancy, and if so, why?

If it was possible to diagnose polyposis in early childhood by performing a blood test, would you want your own children tested at:

a) Birth b) Infancy c) 10-14 years d) never e) don't know?

At what age do you think children should be introduced to the concept of polyposis?

Statistical Analysis:

This was performed using the Mann-Whitney U test and Chi squared test with Yates correction.

Results

The Effect of FAP on Relationships

Fifty patients (80%) stated that FAP caused no tension between themselves and other members of their family, while thirty-four (54%) felt that FAP had actually drawn the family closer together. Twelve patients (19%) felt that a significant degree of tension had been created between family members, and five (12%) directly attributed the disintegration of their families to FAP. Two girls in their late teens ran away from home because they thought they would develop FAP, and one patient stated that her sixteen year old son would leave a room whenever the disorder was mentioned. Another woman's husband left home, the day she told him of her diagnosis. Four patients volunteered that relationships outside the family had been discouraged by prospective in-laws when the genetic and premalignant nature of FAP was explained to their partner.

Satisfaction with Medical Care

Twenty patients (32%) felt that the doctors looking after them did not understand what it was like to suffer from FAP. Five patients (8%) were resentful that unnecessary suffering had been endured as a result of delayed diagnosis in other hospitals, or their illness mismanaged as they perceived it, expressing feelings of insecurity when being treated by doctors other than those associated with St Mark's Hospital.

Preoccupation with Illness and Anxiety

Twenty-five patients (40%) stated that polyposis was always at the back of their mind, and twenty patients (32%) stated that Polyposis made them very concerned about the future.

Depression

Twenty patients (32%) stated that having polyposis occasionally made them feel depressed, but only three (4%) stated that it often made them depressed.

Hobbies and Jobs

Eight patients (12%) felt that post-operative side effects of colectomy significantly interfered with their work, jobs or hobbies. One patient treated by subtotal colectomy and ileorectal anastomosis, experienced problems of diarrhoea postoperatively and was sacked by his employer because of frequent visits to the toilet. Another patient gave up his job as a postman because frequency of diarrhoea episodes made it impractical for him to continue.

Guilt and Blame

Six out of ten individuals with affected children spontaneously volunteered that they had strong guilt feelings about passing on the gene.

The response of the study population to the statements in the MIEQ is shown in Table 9.1, and the correlation of MIEQ scores with variable studied is shown in Table 9.2.

Attitudes to Prenatal DNA Testing

Forty patients (64.5%) stated that they would request prenatal testing if it was possible to diagnose polyposis in the early weeks of pregnancy; of these, fifteen (24%) (7 men, 8 women) stated that they would proceed to termination of pregnancy if the unborn baby was affected.

Of the 15 patients who said they would opt for prenatal testing and termination of pregnancy if the unborn baby was diagnosed as likely to be affected, 2 (13%) were under 30 years. Of the 39 who would not consider termination, 23 (59%) were under 30 years ($p < 0.01$).

Individuals who stated that they would opt for termination had significantly increased MIEQ scores relative to those who would not ($p < 0.005$).

Six of the 15 (40%) of the patients who said they would opt for termination had affected children compared to 4/39 (10%) who would not ($p < 0.01$).

Ten of the 15 patients (66%) who would opt for termination had at least one FAP related death in the family compared to 13/39 (33%) who did not ($p < 0.05$).

These results are summarised in Table 9.3.

Reasons volunteered for opting for prenatal testing and termination were as follows:

Six patients who had previously refrained from having children for fear of passing on the polyposis gene, felt that the option of prenatal testing would enable them to consider planning a family, which they would otherwise not have done.

Six patients who had affected children stated that they felt very guilty about passing on the polyposis gene, and felt that had the prenatal option been available, a great deal of unnecessary suffering might have been prevented.

Three patients who had children of unknown disease status said that they were very worried about the possibility of passing on the faulty gene, and felt that prenatal testing could have removed this chronic source of worry from their lives.

Patient opinion on the most suitable age to introduce the subject of polyposis to their children.

51/62 patients discussed this issue. The results are summarised in Table 9.5.

Table 9.1 Response of Subjects to Statements in Modified Illness Effects Questionnaire;

SD = strongly disagree, D = disagree, A = agree, SA = strongly agree

1) *Having polyposis has caused tension between myself and at least one member of my family.*

Response	SD	D	A	SA
Total	44	6	7	5
Men	22	3	3	5
Women	22	3	4	0

2) *Having polyposis has drawn me closer to some members of my family.*

Response	SD	D	A	SA
Total	19	9	13	21
Men	11	7	9	6
Women	8	2	4	15

3) *Having polyposis has affected my decision to have children*

Response	SD	D	A	SA
Total	21	17	15	9
Men	9	8	9	7
Women	12	9	6	2

4) *Having polyposis prevents me from enjoying life to the full.*

Response	SD	D	A	SA
Total	45	12	1	4
Men	24	7	0	2
Women	21	5	1	2

5) *Having polyposis has helped me to get more out of life, and has put other problems in perspective.*

Response	SD	D	A	SA
Total	24	12	6	20
Men	12	7	5	9
Women	12	5	1	11

6) *Having polyposis makes me worry about the future.*

Response	SD	D	A	SA
Total	21	21	6	14
Men	10	10	4	9
Women	11	11	2	5

7) *Having polyposis isn't such a big deal*

Response	SD	D	A	SA
Total	14	15	14	19
Men	5	9	5	14
Women	9	6	9	5

8) *The thought of polyposis is always at the back of my mind.*

Response	SD	D	A	SA
Total	12	25	11	14
Men	7	12	4	10
Women	5	13	7	4

9) *Having polyposis is not as bad as having some other conditions.*

Response	SD	D	A	SA
Total	1	4	40	17
Men	0	1	18	14
Women	1	3	22	3

10) *Having polyposis occasionally causes me to get depressed.*

Response	SD	D	A	SA
Total	13	29	17	3
Men	7	15	10	1
Women	6	14	7	2

11) *Having polyposis often causes me to get depressed.*

Response	SD	D	A	SA
Total	36	23	1	2
Men	19	13	0	1
Women	17	10	1	1

12) *Coming to hospital at regular intervals for a check-up is stressful.*

Response	SD	D	A	SA
Total	15	28	6	13
Men	8	19	3	3
Women	7	9	3	10

13) *Doctors do not understand what it is like to have polyposis*

Response	SD	D	A	SA
Total	13	23	11	15
Men	6	13	8	6
Women	7	10	3	9

14) *Having an internal examination when I come to hospital is stressful.*

Response	SD	D	A	SA
Total	6	26	12	18
Men	4	15	7	7
Women	2	11	5	11

15) *Coming to hospital for regular check-ups makes me feel emotionally dependant on other people*

.Response	SD	D	A	SA
Total	33	21	8	0
Men	18	10	5	0
Women	15	11	3	0

16) *Having an operation for polyposis was the worst thing that ever happened to me.*

Response	SD	D	A	SA
Total	16	22	8	16
Men	11	12	5	5
Women	5	10	3	11

17) *Having an operation for polyposis has left me with symptoms which significantly affect my quality of life.*

Response	SD	D	A	SA
Total	21	27	10	4
Men	13	15	4	1
Women	8	12	6	3

19) *Because of polyposis, I don't work as well at my job, work or hobbies.*

Response	SD	D	A	SA
Total	36	18	4	4
Men	17	12	2	2
Women	19	6	2	2

20) *All things considered, polyposis is the biggest difficulty in my life*

Response	SD	D	A	SA
Total	34	20	3	5
Men	16	14	2	1
Women	18	6	1	4

Variable	Number positive	MIEQ Scores		Number negative	MIEQ Scores		p value
		Median	Range		Median	Range	
Family History of FAP	51	18.5	4-32	11	17	10-28	0.76
Good knowledge of how FAP is inherited	50	18	4-32	12	27.5	18-49	0.0001
Perceived medical mismanagement	5	31	19-49	57	19	4-35	0.006
Family history of FAP related death	23	23	12-49	39	18	4-28	0.0001
Previous experience of ileostomy within the family	9	30	19-49	53	18	4-19	0.003

Table 9.2 A summary of correlation of MIEQ scores and variables studied

	Those who said they would opt for selective termination of pregnancy	Those who would not consider termination of pregnancy	Undecided
Number	15	39	8
Age of patients			
< 30 years	2 (13%)	23 (59%)	5 (63%)
30-50 years	5 (33%)	10 (26%)	3 (38%)
> 50 years	8 (53%)	6 (15%)	
No. who had children	9 (60%)	20 (51%)	
No. who had one affected child	4 (26%)	2 (5%)	
No. who had more than one affected child	2 (13%)	2 (5%)	
No. who had an extracolonic complication themselves	2 (13%)	1 (2.6%)	
No. who had FAP related death in family	10 (67%)	13 (33%)	1 (12.5%)

Table 9.3 A summary of features in relation to patient attitude to prenatal testing and termination of pregnancy.

Age of Child(years)	Number of Patients
Under 10	3
10	20
11	2
12	6
10-12	7
13-14	13
Over 14	1

Table 9.4 Patient opinion on most suitable age to inform children about polyposis.

Discussion

Although, the psychology of some genetic diseases has been well documented, as has the emotional trauma associated with a cancer diagnosis, surprisingly little is known about the psychological impact of FAP on affected individuals and their families. An FAP patient has to cope with both the genetic and cancer aspects, so clearly FAP could have a devastating effect on the lives of some patients. Indeed an increased incidence of suicide in this disorder has been noted by some authors (15,429).

The newly diagnosed FAP patient has many conflicting emotions to cope with. Firstly, the majority of patients diagnosed by screening in a family with other affected relatives, are asymptomatic at diagnosis, and cannot be expected to find it easy to adopt the "sick role" associated with symptomatic illness. Accepting that colectomy is necessary may cause resentment, and denial may become a dominant feature of the coping response. Secondly, patients with FAP may be confronted with the possibility of cancer when they are children. For most people, cancer continues to have alarming connotations, creating considerable uncertainty and fear and this may be exacerbated by memories of cancer death or prolonged illness within the family. Finally, the patients will facethe possibility of passing the polyposis gene on to their own children. This may become a source of agonising choices and guilt, particulalry if the patients have suffered some serious complications of FAP. Taken in combination, these factors might be expected to reduce self esteem and cause depression .

Being an hereditary disorder, it might be anticipated that FAP would generate emotional tension within the family unit. Affected children might blame their parents for inheriting the gene, and parents might feel guilty about passing the gene on. Surprisingly, the majority of patients in this study stated that FAP had either drawn the family closer together or caused no tension, an effect observed in non-hereditary illness (430). A significant minority (20%) however felt that tension had been created

between family members, and the fact that two individuals felt that their families had disintegrated as a result of FAP, illustrates the devastating effect this illness can have in some cases. FAP may also have an effect on inter-personal relationships outside the family, as four patients stated that their prospective in-laws had discouraged the relationship when the genetic and premalignant nature of FAP was raised, illustrating the often underestimated stigmatising effect of genetic disease. Complicating the issue further is the ambivalent attitude of some FAP patients to having children. Hence, relationship problems may only be experienced by a minority, but when they do, they may have far reaching consequences.

Most patients in this study did not perceive themselves as being depressed. Nonetheless, there was a significant degree of illness preoccupation and concern about the future, suggesting that FAP directly affects emotional well being.

The presence of a family history of FAP per se did not significantly raise psychological distress ($p = 0.76$), but there was a strong correlation between distress and FAP related death with the family ($p = 0.0001$). Previous unpleasant experience of an ileostomy within the family, or a perception of receiving inadequate or inappropriate medical treatment at some stage, was also significantly associated with higher MIEQ scores (Table 9.2)

It has now become routine practice at St Mark's Hospital to refer all FAP patients or at-risk individuals for professional genetic counselling. At the onset of this study, some affected individuals had not received such counselling, and nearly 20% of the those interviewed did not understand the manner in which FAP is inherited. Some patients believed that only boys could be affected, while others thought that they could not pass on the gene again if they already had an unaffected child. A poor understanding of the mode of transmission of FAP clearly aggravates emotional distress, as all the patients in this category had significantly raised MIEQ scores ($p =$

000.1). This illustrates that even disorders with an apparently straightforward mode of transmission may be difficult for patients to understand.

Attitudes to Predictive Testing.

Individuals with FAP may wish to know the results of their children's predictive tests, in order to spare those found to be at low risk unnecessary screening procedures and to relieve personal guilt and uncertainty. Adults at risk of developing FAP are clearly concerned about their own risk of developing cancer, and this may explain the high uptake of DNA testing in this group which at St Mark's Hospital approaches 100%. By comparison, the uptake of predictive testing in Huntington's Disease (HD) may be less than 20%, this difference being due to the fact that people consider FAP treatable, whereas there is currently no treatment for HD (431,432).

With regard to prenatal testing, although 40 patients (64%) in this study stated that they would make use of prenatal testing if it was available, only 15 (24%) said that they would consider termination of pregnancy if the unborn baby was affected. This finding is similar to a previous report of a cohort of people with FAP, where 15/25 (60%) affected individuals said they would consider prenatal testing, but only 4(16%) would consider the termination option (433). Individuals who were over 30 years, who already had an affected child or had a history of FAP related death in the family were more likely to state that they would opt for prenatal testing and termination, and it is possible that these patients perceived FAP differently as a result of the emotional distress they associated with the disorder. In practice, none of affected individuals attending the St Mark's Polyposis Clinic in the last 4 years has requested prenatal testing, although only 2/62 interviewed, have yet to be in a position to request this service. A marked discrepancy between statement of intent and actual uptake in clinical practice has been observed in Huntington's disease, and it is possible that this also may occur in FAP (434). Factors influencing patient opinion may change over a

period of time. Most patients under 30 years in this study do not know yet whether their children are affected, and more complete ascertainment of at-risk individuals by polyposis registries should reduce death from colorectal cancer. The prenatal option may be taken up by the small group of patients who had previously refrained from having children for fear of passing on the gene.

There was a widespread feeling among the interviewees that children should not be told about polyposis until they were "old enough to understand ". Most parents considered between 10-12 years to be the most appropriate age to introduce the subject, as they did not feel it was fair to "spring" the diagnosis at the often emotionally turbulent time of puberty. However, it is interesting that 58/62 (93%) wished to test their children at birth and withold this information from them for a decade.

The predictive testing of children for adult onset disorders is a complex issue. Present consensus is that it cannot be justified ethically or morally for disorders such as Huntington's Disease where symptoms are rare in childhood and there is no treatment (435). In the case of FAP however, diagnosis before 18 years is frequently necessary to prevent the onset of colorectal cancer, and screening in most centres, already begins before the legal age of consent. It is thus logical to test them at least by the time clinical screening would be instigated.

A majority of parents (58/62) stated that they would request testing of their children at birth/infancy if it was available, and this finding was consistent with a previous report from Germany, where most parents wanted testing performed before the age of 10 years (436). Reasons given included 1) removing ambiguity about the disease status of their children, which some parents felt was a chronic source of stress and 2) satisfying parental curiosity. Medical management before puberty however is unlikely to be altered by the information predictive testing provides, and it is difficult therefore to envisage any clear advantage for the child. Furthermore, early testing may distort parent-child or sib-sib relationships, damage the child's self esteem,

remove the child's right to decide about testing at a later age and generate unwarranted anxiety about unrelated symptoms. Who is to judge what is in the best interests of the child in this situation, and what limits, if any, should be placed on parental autonomy?

Conclusions

In the majority of cases, FAP would appear to have a fairly minimal impact on the everyday life of the patient. However, in a significant minority (20%), a diagnosis of FAP can have a devastating effect on psychological well-being. Factors which might be important include previous unpleasant experience with an ileostomy, history of cancer death within the family, a poor understanding of the mode of transmission of FAP, delayed diagnosis and perceived medical mismanagement.

Hopefully, with earlier diagnosis, the widespread establishment of polyposis registries, improved genetic counselling and an increased awareness of genetic illness in general by general practitioners, it might be possible to prevent some of this distress in the future, and identify those who require additional psychological support.

The majority of patients stated that they would not consider prenatal testing and termination of pregnancy under any circumstances, but a significant minority (25%), felt that they would take up such testing. However,in practice, nobody has yet taken up this option.

Although, most parents stated that they would like their children to have DNA testing at birth or in infancy, in general such testing should probably be deferred until the age at which endoscopic surveillance was to commence- about 10 years- unless clinical symptoms suggest early onset of FAP polyps which can occasionally occur before 10 years. However, there are as yet no data to support any view regarding the optimum age to perform predictive testing.

Careful long term assessment and documentation of the impact of predictive testing will be needed in FAP, as in the rapidly increasing number of other single gene disorders for which DNA testing is now available.

Chapter 10

The Prognostic Value of Allele Loss in Colorectal Cancer

" Malignancy is essentially a clinical concept; in research it becomes a menace if it implies an indivisible, invariable entity or quality..... It is not enough to label a tumour malignant; it is necessary to specify which characteristics, and the degree of each of them, that make it malignant " (437)

Introduction

The ideal prognostic indicator in colorectal cancer would be one which was able to predict with a high degree of accuracy the likelihood of survival after " curative" surgery. Such a marker would enable patients to be polarised to favourable and poor prognostic groups, and statistical benefit for new treatment modalities could be demonstrated in studies with fewer patients over a shorter time period. Identifying such an indicator has proven an elusive goal. Dukes' staging, described over sixty years ago, remains the mainstay of prediction in everyday practice (438) yet predicts by implication only, the presence of distant occult metastases, now recognised as the principal determinant of survival in this malignancy (305). The Jass pathological scoring system provides more accurate prognostic information for rectal cancer in some hands (439), but still suffers from problems of subjective interpretation (440).

The idea that molecular markers may be valuable in assessing cancer prognosis was first proposed in the 1970s (441,442). Most interest has centred on the value of DNA ploidy, cell cycle kinetics and immunohistochemical detection of oncogene products, but these parameters have yielded conflicting and contradictory results, and are not considered yet reliable enough for routine clinical practice (443-447).

The predictive value of tumour suppressor gene inactivation, on the other hand, measured by loss of heterozygosity (LOH) or allele loss on Southern blot analysis, has received relatively scant attention, being the subject of only 5 reports in the literature (179,448-450). These studies have focused on chromosomal regions 17p, 18q and 5q which contain genes directly implicated in colon carcinogenesis (148).

The purpose of this study was to investigate further the prognostic significance of allelic deletion at the P53, APC DCC and Nm23 loci in colorectal cancer.

Materials and Methods

Material for this study was obtained from 70 patients with colorectal cancer admitted to St Mark's Hospital, London between 1987 and 1990. It included 40 male and 30 female patients with a median age of 65 years (range 27-103 years). Follow up was based on regular clinical examination, liver ultrasonography and computerised tomographic scanning when indicated. Median observation time was 36 months (range, 2-66 months). Six patients, who lived abroad, were lost to follow-up, and one patient, who died from a pulmonary embolism on the second post-operative day, were excluded from the study. Tumours were located proximal to the mid-transverse colon in 8 cases, in the sigmoid in 23 cases, and in the rectum in 32 cases. Ten percent of the tumours were Dukes' stage A, 43 % stage B, 30% in C1, and 16% in C2. 55% fell into Jass staging system groups III and IV, and the remainder into groups I and II.

Tumour tissue and normal mucosa was obtained from each patient at surgery, snap frozen and stored in liquid nitrogen at -70^0 C. Necrotic and non neoplastic tissue was removed from the tumour samples as completely as possible, and tissue adjacent to the tumour sample from which the DNA was extracted was sent for histological confirmation of malignancy.

DNA Extraction.

Tissue specimens were bathed with liquid nitrogen and ground to a powder. High molecular weight DNA was extracted from each tissue specimen using phenol-chloroform and precipitated with ethanol as previously described.

Southern Blot Analysis

This was performed as previously described.

Genetic Probes

Six probes were chosen to investigate LOH at the APC, DCC, P53, and NM23 loci. These were: YNZ22 (D17S5), YNH37.3 (D17S28) P144D6 (D17S34), OLVIII 10 (D18S8), FB40 (D5S135) and Nm23-H1 (D17S308).

Densitometric Scanning of Autoradiographs

When LOH was not obvious on visual inspection, LOH was quantitated by scanning of the autoradiograph using an imaging analysis system (Imaging Research Inc.) consisting of an IBM PC AT computer linked to a Sony CCD video camera and running BRS2 image analysis software. From the scans the ratios of the two alleles in tumour material to those in normal matched tissue were estimated (T1/T2 divided by N1/N2) (125). A cut-off level of 50% or more reduction in intensity of the bands was taken as evidence of LOH.

Clinical Data

This was obtained by reviewing clinical and out-patient records. In some cases survival data was obtained by contacting the patient's general practitioner.

Statistical Analysis

To determine whether any of the genetic abnormalities detected were related to the clinical variables, tables of these parameters were constructed, and Chi square tests (or Fisher exact tests where numbers were small) calculated. To determine whether allelic deletion was prognostic for survival, Kaplan-Meier curves were drawn and the log rank test was calculated for all the gene abnormalities and the clinical features. For the purpose of analysis, tumour site was coded as Rectum, Sigmoid or Other, Dukes' stage as A, B, C1 and C2, and site of metastasis as liver or other. Those variables with a p value of less than 0.1 were put into a stepwise Cox regression model to determine which, if any, was independently prognostic for survival. The end date for analysis was taken as February 93.

Results

Genetic Alterations in Tumours

This study included 63 colorectal cancers. In total 25 patients developed distant metastases (16 liver, 6 peritoneum, 1 lung, 2 brain). Overall median survival of this group was 21 months (range 2-70 months). Hepatic metastases, were diagnosed at the time of surgery in 12 patients; 2 of which are alive and well at 47 and 70 months.

All the patients were informative for at least one marker, and of these 37 (58.7%) demonstrated LOH. A molecular genetic profile for tumour 529 which was informative for all 6 markers is shown in Fig. 10.1. .Ninety-eight per-cent of patients were informative for a marker on 17p. LOH was seen more commonly on 17p (48.3%) than at the APC locus (38.8%), but was relatively infrequent at the DCC locus (38.7%). No LOH was observed at the Nm23 locus. (Table 10.1). Only 5 tumours had LOH at the loci on chromosomes 5q, 17p and 18q (Fig 10.1). On chromosome 17p, the highest percentage of LOH was observed with probe YNZ22, the marker in this study lying most close to the p53 gene. No difference was observed in the molecular genetics of those with metastases diagnosed at operation and those with occult metastases.

Genetic Alterations in Relation to Clinical Variables.

There were 48 patients with Dukes' B or C tumours who were informative for a 17p marker, and who did not have evidence of distant metastases at the time of surgery. Twenty-two patients had 17p LOH and of those 8 (36%) developed metastases. Twenty-six patients retained the 17p alleles, and of these 5 (19%) developed metastases ($p = 0.003$)

LOH on chromosome 17p was also found to be significantly associated with Dukes' stage and Jass score. LOH at chromosomes 5q and 18q was not found to be associated with any of the clinical variables studied (Table10.2).

Genetic Alterations in Relation to Post-operative Survival

Univariate analysis of survival was performed for each of the genetic abnormalities and Kaplan-Meier curves were constructed. 17p LOH had prognostic significance (Fig 10.2), as did age, Dukes' staging and Jass score (Table 10.3). LOH at the 5q and18q loci was not related to survival (Figs.10.3,10.4). The tumour of the patient who survived 47 months, exhibited LOH at both the 17p and 18q loci, while the tumour of the patient alive and well at 70 months retained 17p, but was uninformative at the18q locus.

Prognostic value of Jass scoring system versus Dukes' staging

In this small series, the Jass scoring system proved to be slightly better at predicting prognosis than Dukes' staging ($p < 0.0001$ vs. $p = 0.0006$ respectively) (Figs.10.5,10.6)

Multivariate Analysis.

Age, Jass score and LOH 17p were found to have a p value < 0.02 on univariate analysis for survival, and were therefore entered into Cox's regressional model. In this analysis only age and Jass score were independently prognostic (Table 10.4)

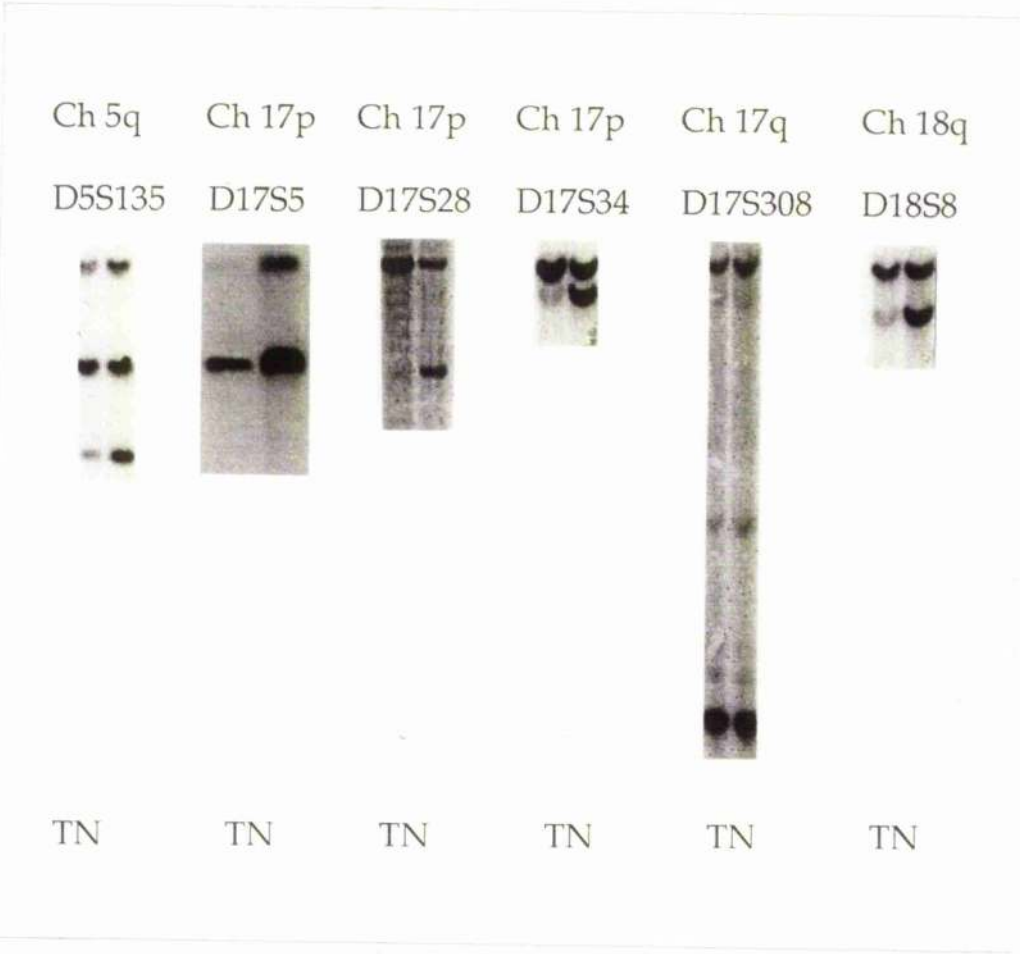

Figure 10.1 The Molecular Profile of Tumour 529. T = Tumour, N = Normal.

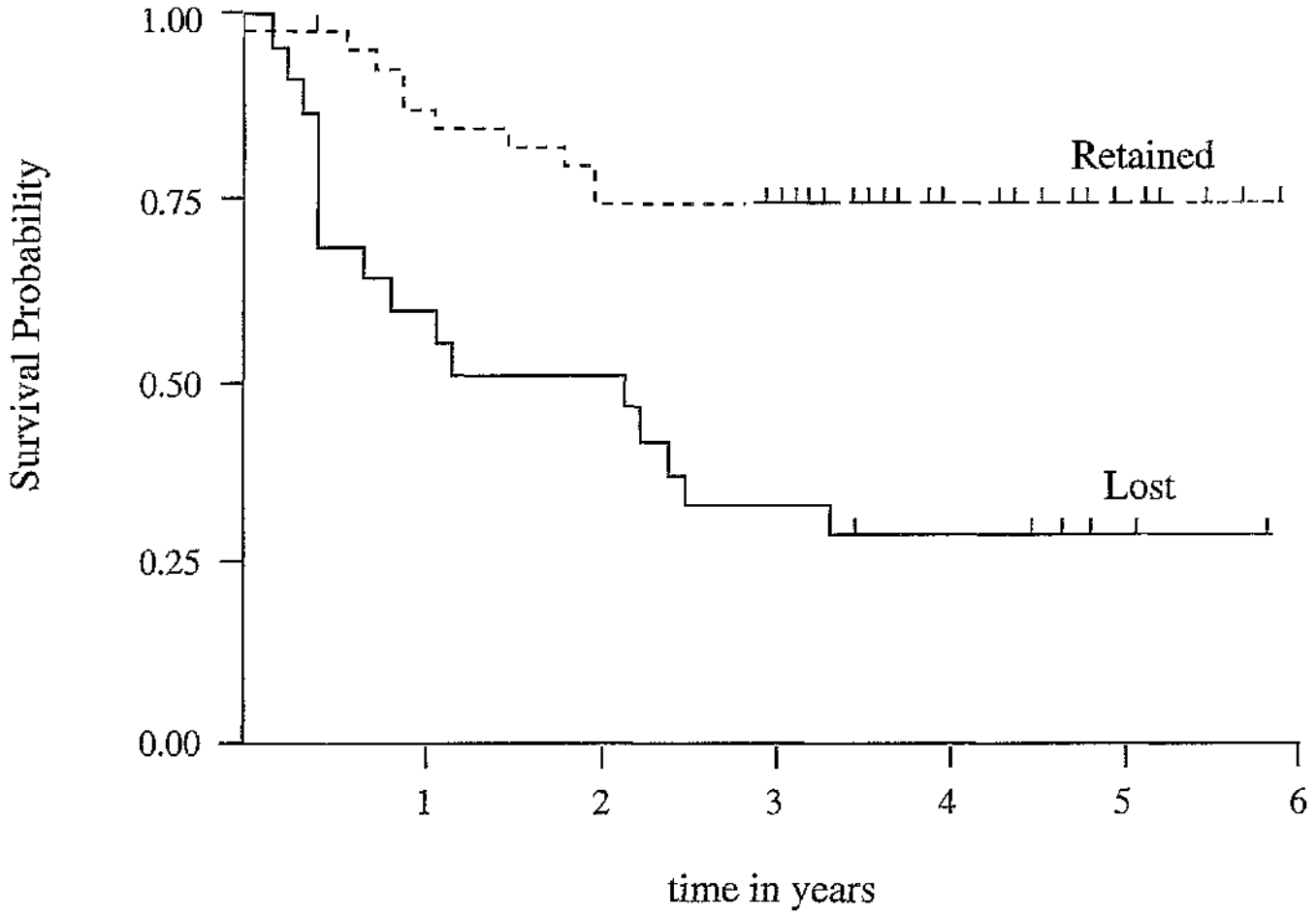

Fig. 10.2 Kaplan-Meier Survival Curve for Patients whose Tumours Retained and Lost Heterozygosity at the 17p13 Locus.

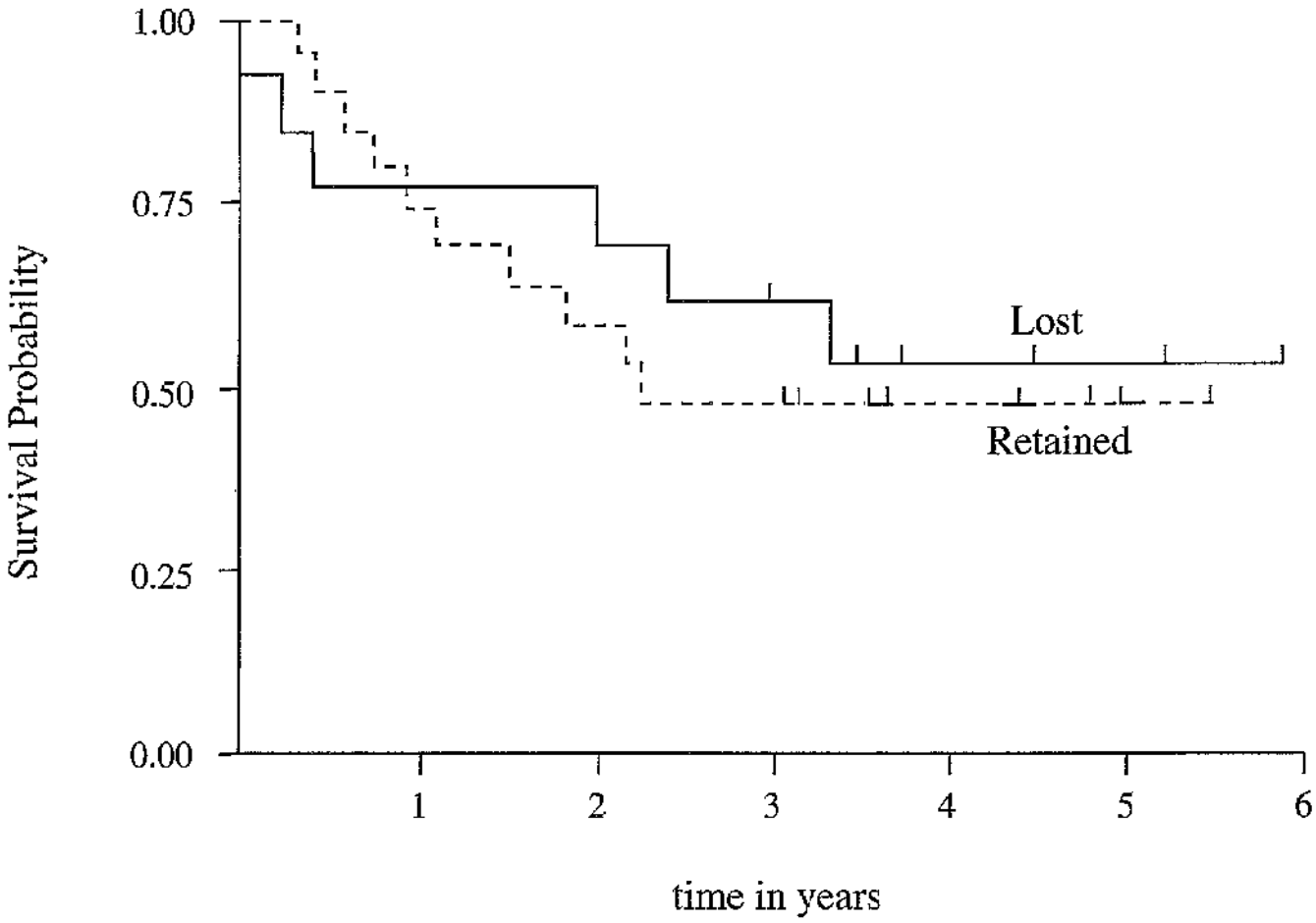

Fig 10.3 Kaplan-Meier Survival Curve for Patients whose Tumours Retained and Lost Heterozygosity at the 5q21 locus

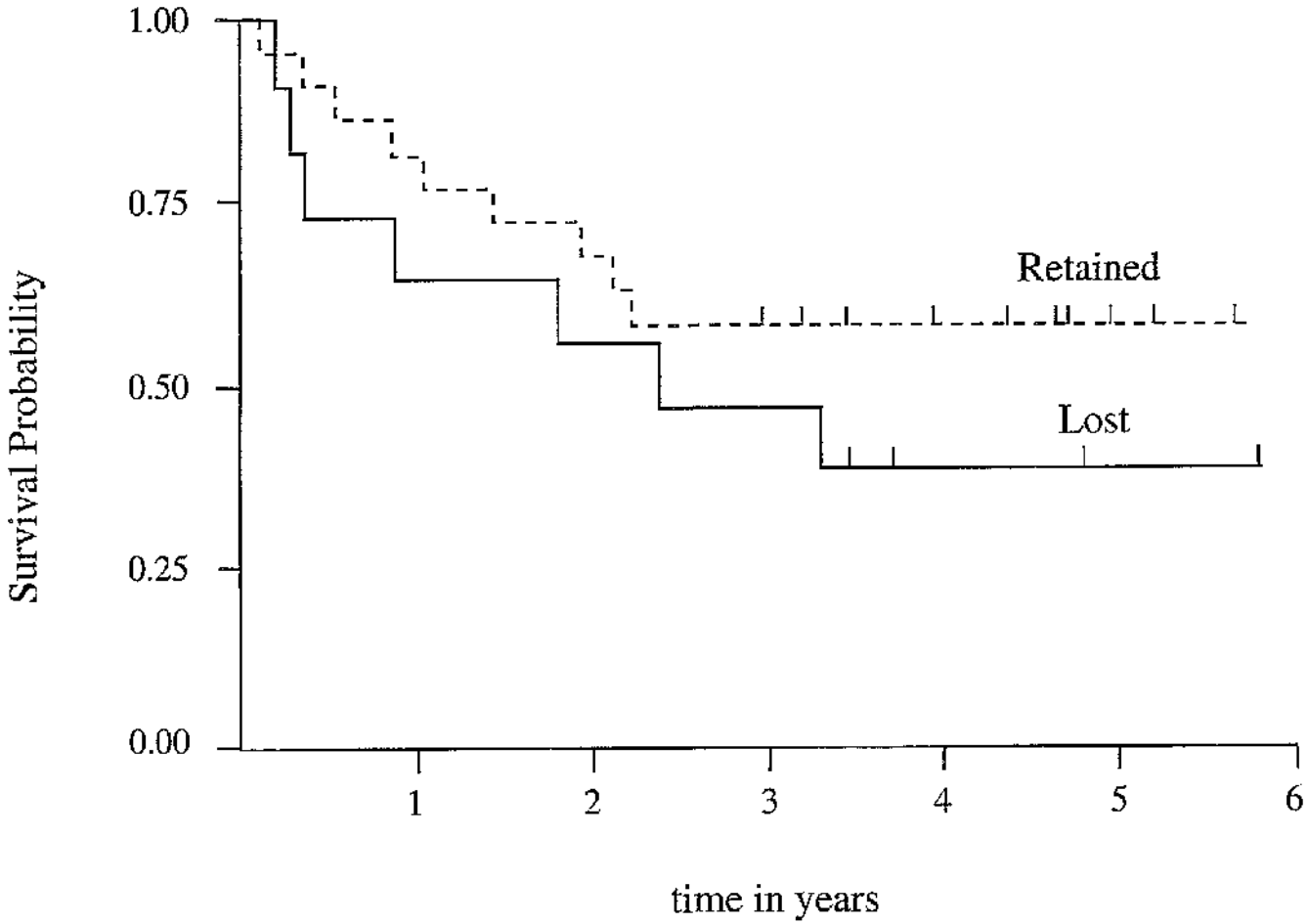

Fig 10.4 Kaplan-Meier Survival Curve for Patients whose Tumours Retained and Lost Heterozygosity at the DCC locus

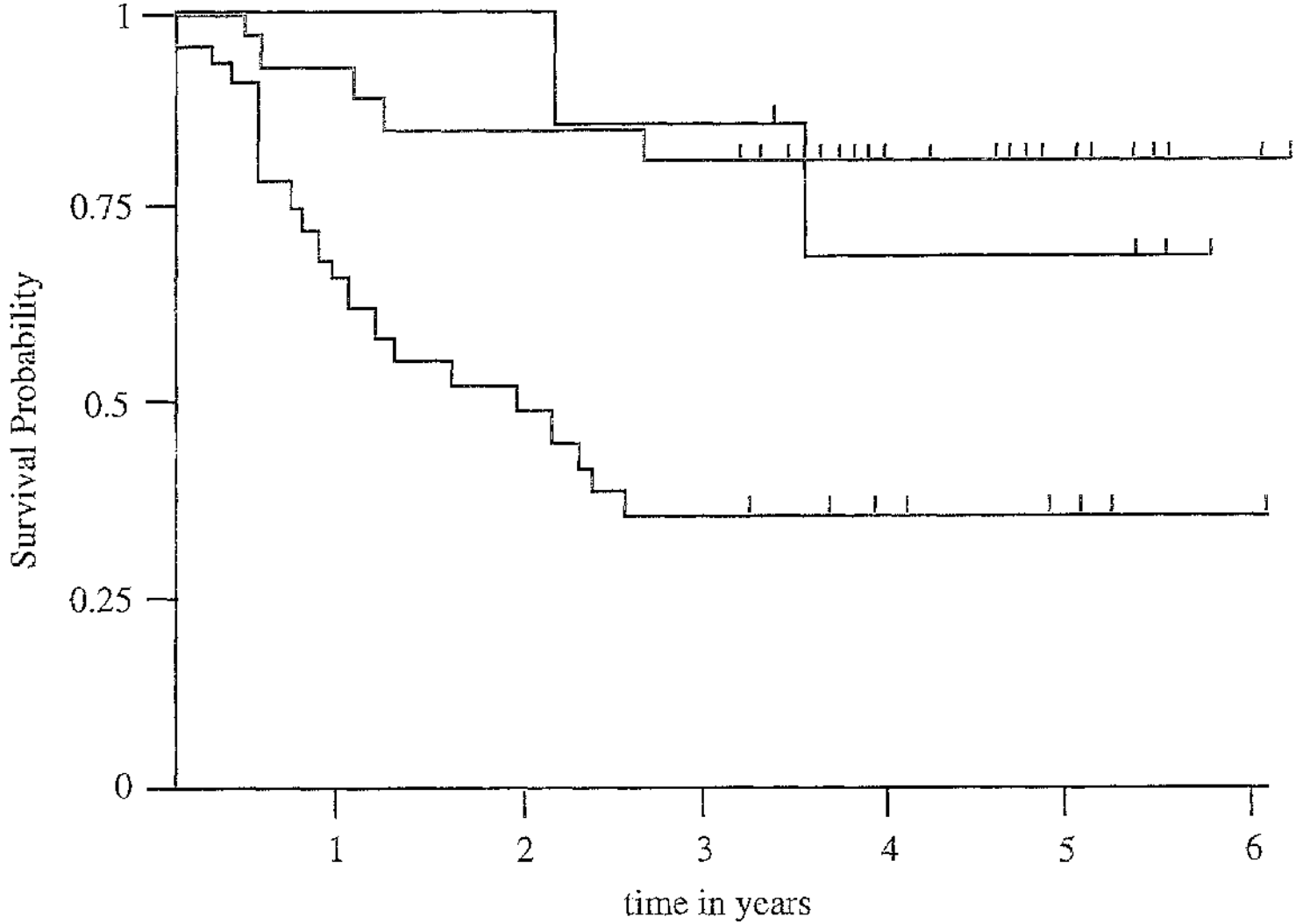

Fig. 10.5 Kaplan-Meier Survival Curve showing
Relationship between Dukes' stage and Survival

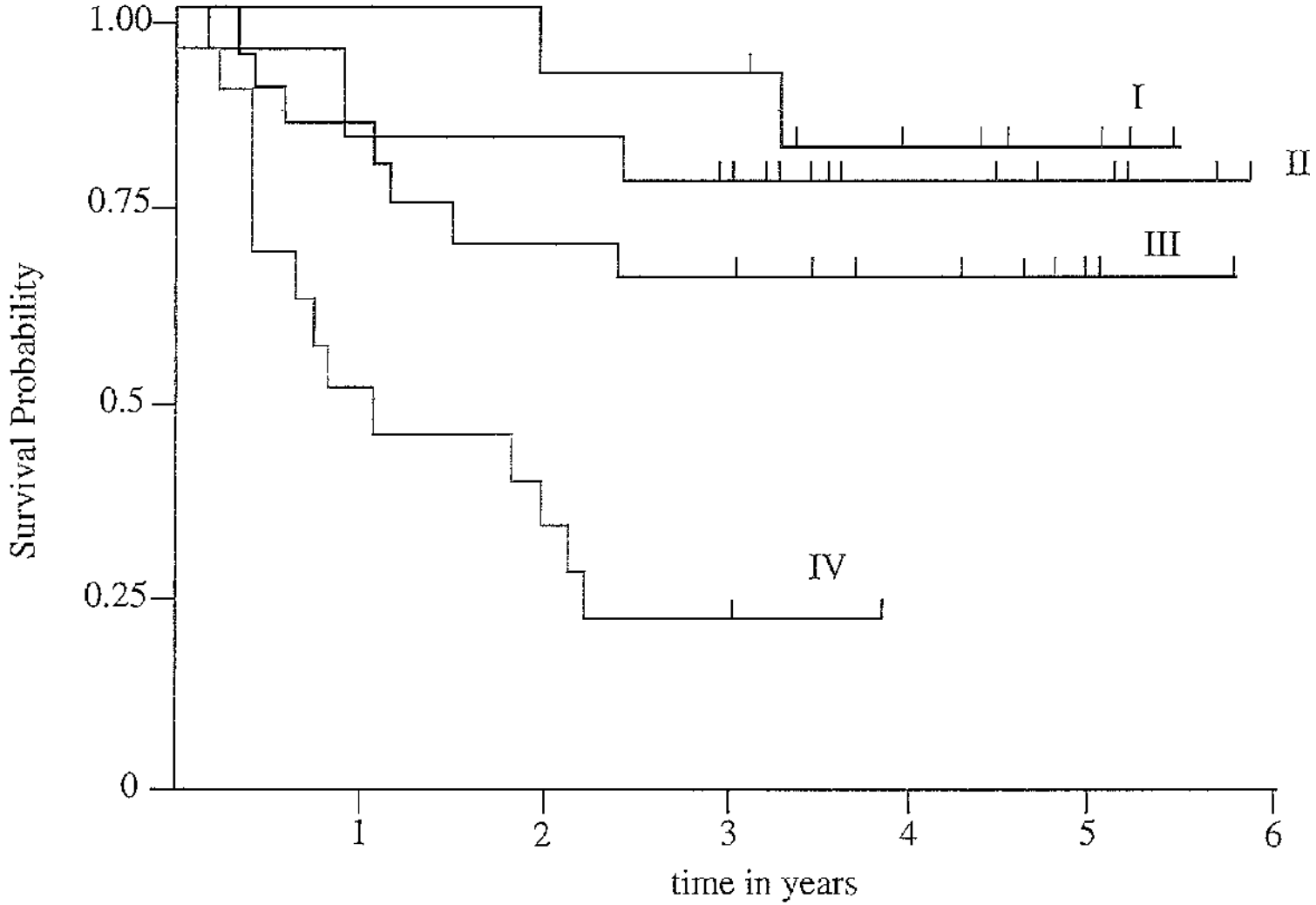

Fig 10.6 Kaplan-Meier Survival Curve showing Relationship between Jass Scoring System and Survival

Specimen	Genetic Marker					
	D5S135	D17S34	D17S28	D17S5	D17S308	D18S8
324	0.97	1.00	0.98	0.99	H	1.03
338	0.95	0.92	0.89	0.32	H	1.00
344	0.41	0.33	0.96	0.88	1.01	0.39
406	H	0.58	0.86	1.01	H	
456	1.03	0.23	H	0.97	H	0.44
500	H	H	H	0.96	H	H
506	H	0.46	0.40	0.39	0.87	H
509	0.76	0.82	1.01	H	1.00	0.91
522	1.00	0.95	H	H	H	H
529	0.18	0.23	0.16	0.26	H	0.14
533	0.14	0.21	0.15	0.12	H	0.22
540	H	0.92	0.97	0.97	0.97	1.02
556	0.22	0.40	0.99	0.96	H	H
562	H	1.00	0.98	0.99	0.88	0.34
563	H	0.33	0.25	0.31	H	H

Table 10.1 Scanning Densitometry Measurements of matched Tumour/Normal DNA pairs . H = Homozygous.

pairs

Specimen	Genetic Marker					
	D5S135	D17S34	D17S28	D17S5	D17S308	D18S8
571	0.88	H	0.91	H	H	H
587	0.32	H	H	0.36	0.99	H?
596	H	H	H	H	1.03	1.04
638	H	0.96	0.32	0.23	H	0.96
708	0.85	0.90	H	0.24	0.95	0.29
715	H	0.98	H	0.31	H	0.92
717	H	0.90	H	0.88	0.94	0.94
734	H	0.33	H	0.28	0.94	H
762	0.41	H	1.01	H	H	H
769	0.34	0.38	0.24	0.31	H	H
776	H	0.86	H	0.79	H	H
778	H	1.00	1.01	H	H	0.93
838	0.90	H	H	0.96	1.02	H
860	0.45	1.01	H	0.99	H	0.79
864	H	H	0.83	H	H	H

Table 10.1 Scanning Densitometry Measurements of matched Tumour/Normal DNA pairs

Specimen	Genetic Marker					
	D5S135	D17S34	D17S28	D17S5	D17S308	D18S8
880	0.37	1.17	H	H	H	H
890	0.95	0.93	H	H	H	H
904	H	0.22	H	H	0.93	H
906	H	0.98	H	H	H	H
927	0.88	0.91	H	H	0.90	H
930	1.00	0.96	0.98	1.02	0.99	0.98
937	0.25	0.97	0.89	0.21	H	0.31
960	H	1.03	1.01	0.97	H	0.91
961	H	0.13	H	0.17	H	H
966	0.99	0.49	0.86	0.88	H	H
968	0.98	H	H	0.34	0.96	H
973	0.91	H	1.02	0.40	H	H
980	0.96	0.93	H	0.96	1.00	H
982	0.35	1.04	0.99	1.00	H	0.84
1000	H	0.98	H	0.93	H	0.28
1011	0.38	0.42	0.36	0.40	H	H

Table 10.1 Scanning Densitometry Measurements of matched Tumour/Normal DNApairs

locus	probe	location	No. of tumours informative	No.(%)with LOH
D17S5	YNZ22	17p13.3	48 (76%)	23 (47.9%)
D17S34	P144D6	17p13	52 (82.5%)	21 (40.3%)
D17S28	YNH37.3	17p13	30 (47.6%)	10 (33.3%)
D17S308	Nm23-H1	17q21	24 (34.7%)	0
D5S135(APC)	54D	5q21	36(57%)	14 (38.8%)
D18S8 (DCC)	OLVIIE10	18q21.3	31 (49.2%)	12 (38.7%)

Table 10.2 Loss of heterozygosity in colorectal cancer specimens.

clinico-pathological features	17p	18q	5q
	p value from exact test		
sex	0.2	0.3	0.07
site	0.8	0.7	0.8
Jass	0.002	0.4	0.6
Dukes	0.004	0.1	0.7
distant metastasis	0.003	0.06	1
metastasis site	0.03	1.0	0.6

Table 10.3 Summary of univariate analysis relating molecular genetic findings to clinical variables.

clinical feature	X^2	df	p
17p	5.7	1	0.02
18q	1.1	1	0.3
5q	0.2	1	0.7
scx	0.0	1	1.0
age	8.2	2	0.02
site	0.9	2	0.6
Jass	21.4	3	< 0.0001
Dukes	14.8	2	0.0006

Table10.4 Summary of univariate analysis relating histo-pathological variables with survival.

variable	coding	hazard ratio	95% CI	p
age	continuous	1.05	(1.01,1.09)	0.01
Jass	I vs. IV	11.7	(2.5,54.2)	< 0.001
	II vs. IV	3.9	(1.5,15.3)	
	III vs. IV	6.5	(1.7, 11.6)	

Table 10.5 Summary of multivariate analysis investigating relative prognostic value of 17p, age and Jass scoring system. The p value given for Jass is that for linear trend across all Jass scores.

Study	Age	Site of tumour		Mean Follow Up (months)	Significant genetic abnormality
		Right	Left		
Kern	66	48%	52%	40.5	17p,18q loss
Laurent-Puig	63.7	19%	81%	45	17p loss
O'Connell	71	43	57%	26	18q loss
Cohn	65.2	Not Specified		25	17q loss
Present	65.4	14%	86%	36.3	17p

Table10.6 A summary of studies investigating prognostic value of LOH in colorectal cancer

Discussion

The molecular genetics of colorectal cancer is possibly the most well characterised of any common adult malignancy, yet the precise biological mechanisms which underlie metastasis of these tumours, remain poorly understood. It is hypothesised that certain alterations in DNA sequence in a sub-population of cells within the tumour, may confer an increased capacity for metastasis (451). Identification of such alterations may lead to the development of more accurate prognostic indicators.

Loss of heterozygosity (LOH) can be detected either by Southern analysis (199) or by the polymerase chain reaction by examining DNA from matched tumour/constitutional samples (135). This study is one of 6, collectively involving over 400 patients, that have addressed the possibility of using LOH at candidate loci as prognostic indicators of survival in colorectal cancer (179,448-450,453,). A further study has looked at the relationship between LOH and the development of metastasis, but contains no information about patient survival (454). Two studies have observed that LOH on chromosome 17p13 is independently associated with shortened survival, three have found a similar association with LOH at the DCC locus on chromosome18q, and one study has reported an association between survival and LOH at the NM23 locus on chromosome 17q (Table 10.6).

The observed frequency of LOH at the17p and 18q loci (Table 10.3) was similar to that in some reports (454,455) , but less than reported in other series. A frequency of LOH at the APC locus of 38% was broadly consistent with other published data (144,454,449,) suggesting that stromal contamination of the tumour specimen with normal tissue, which might mask LOH, was not the main reason for the lower than expected LOH observed at the 17p and 18q loci.

On univariate analysis, 17p LOH was found to be statistically related to age at diagnosis, Dukes' staging and Jass score (Table 10.4), and these factors were also related to shortened survival (Table 10.4). However, the 17p LOH correlation disappeared when entered into the

multivariate regressional analysis model (Table 10.6), supporting the findings of two other reports (448,450).

The lack of relationship between 5q LOH and survival (Fig.10.3) was consistent with all previous reports, and was expected as the APC gene is believed to mutate at an early stage in the adenoma-carcinoma sequence. In contrast to three other studies (448,449,453)no relationship was noted between 18q LOH and survival (Fig. 10.4), although the data set was too small for detailed evaluation.

In one report involving 21 large bowel cancer patients with no evidence of distant metastases at the time of initial operation, 8 of 11 patients whose tumours had LOH at the Nm23 locus, subsequently developed metastatic liver disease (450). Yet in a further study, using a ribonuclease protection assay, no mutations were detected at the Nm23 locus in 26 metastatic colon cancers, 17 non metastatic colon cancers, or 43 matched normal controls (455). Furthermore, Nm23 gene expression was elevated almost as often in metastatic as in non-metastatic cancer. Twenty-four of the patients (34.7%) in our study population were informative for the Nm23-H1 probe, but no LOH was detected. Other investigators, using microsatellite repeat markers for the Nm23 gene, have also been unable to demonstrate LOH at this locus (456).

Although, the use of LOH at specific loci as prognostic indicators is still in its infancy, it is clear, that the results of these studies are far from reproducible between different centres. One explanation may be that the molecular genetics of proximal and distal cancers of the colon differs (178) and there was marked variation in tumour distribution between the studies and/or variable length of follow-up (Table 10.7). Nonetheless, a pattern may be emerging in that 18q LOH had prognostic value in the studies with a high proportion of right sided cancers, while17p LOH had some prognostic value in those studies in which the majority of tumours were left sided (Table 10.7).

In conclusion, this study is suggestive but not confirmatory that LOH on the short arm of chromosome 17 may serve as an independent marker of tumour aggressiveness in colorectal cancer. The data set was of insufficient size to fully evaluate genetic changes at the 5q or 18q loci, but there was no evidence that analysis of the Nm23 gene would provide any useful prognostic information.

Further studies involving a much larger series of patients, which stratify genetic alterations by site, and use standardised methodology, are now needed to clarify whether such data can be interpreted in a meaningful manner for routine use in clinical practice.

Chapter11

Future Prospects

The combined tools of molecular genetic markers and fibre-optic technology now provide us with an unparalleled opportunity to reduce morbidity and mortality from colorectal cancer.

Presymptomatic diagnosis is now possible for the majority of individuals at risk of developing FAP and for many individuals at risk of developing HNPCC. It seems probable that in the near future, predictive DNA testing will also be possible for the other highly penetrant hereditary colorectal cancer syndromes such as juvenile polyposis and the Peutz-Jeghers syndrome. This will enable gene carriers to be identified early and treated appropriately, while those not carrying a predisposing mutation can be spared many years of unnecessary anxiety about their health, as well as that of their children. Many questions remain unanswered, however, regarding the most appropriate way to apply this technology. Genetic epidemiologists need to determine the exact population frequency and penetrance of the different mutations within families before accurate prediction can be made for gene carriers, and functional assays will have to be developed to distinguish true mutations from harmless polymorphisms. Furthermore, safeguards will need to be put in place to prevent uncontrolled predictive DNA testing leading to stigmatisation in employment, insurance and interpersonal relationships. The issue of genome "ownership" awaits resolution.

The management of the individual who has a "weaker" family history of colorectal cancer also needs to be clarified. The majority of people falling into this category will develop their cancers over the age of 50 years, and there are presently no genetic markers to identify those individuals who may have inherited a common low penetrant gene. Most benefit, in terms of reduced colorectal cancer incidence appears to result from periodic screening accrued at the initial screen. As a result, some authors have advocated that a single 60 cm flexible sigmoidoscopic examination performed at the age of 55 years, followed by a single diagnostic colonoscopy to detect and treat proximal as well as distal neoplasia, may be sufficient

to achieve a cost-effective reduction in cancer mortality (457). In one study, less than 1% of persons whose only lesions at sigmoidoscopy were small tubular adenomas had an advanced lesion proximally (458). The endoscopic workload created by introducing such a programme would be formidable but some argue that this workload could be met by the employment of specialist nurse endoscopists and general practitioners who have been trained to perform flexible sigmoidoscopy (459).

Alternatively, it might be possible to develop a test which can detect genetic mutations which is present in all large adenomas and early cancers in stool DNA. Sidransky et al have reported the identification of ras oncogene mutations in the stool DNA from shed epithelial cells in patients with colorectal cancer , and eventually tests with greater sensitivity and specificity may be developed (181).

Genetic counselling is a time-consuming process, and a designated family cancer clinic is probably a more appropriate setting than a busy surgical out-patient clinic. Presently, most counselling is provided by clinical geneticists, but in future, this role might be filled also by an interested surgeon or gastroenterologist with appropriate training in cancer genetics. Public demand for cancer screening may be greater than is generally appreciated, and may be"news sensitive". When President Ronald Reagan had colorectal cancer diagnosed in 1985, the health insurance company Medicare computers noticed a 300% increase in the demand for colonoscopic services (460).

Gene therapy is perhaps one of the most exciting prospects for the treatment of colorectal cancer (461-463. Many obstacles remain to be overcome, not least of which, is the possibility of insertional mutagenesis and the activation of oncogenes leading to neoplasia.. Nonetheless, several gene therapy trials in oncology have been approved in the U.S.A. and Europe. In FAP, it is possible that gene therapy may be used to reduce the tendency for adenomas to form in the duodenum and in the rectal stump.

Other therapeutic strategies include the insertion of genes which would enhance the expression of cytokines (464) and the suppression of oncogene expression (465). Methods might be discovered to make tumours expressing the Multidrug Resistance Gene, p-glycoprotein, more responsive to chemotherapy, or it may be possible to treat the cancer patient's normal bone marrow with this gene, abrogating marrow suppression, a major side effect of chemotherapy (466).

The search for the ideal prognostic molecular marker will no doubt continue. To date, attempts to identify the specific property of the cancer cell, which makes it more likely to metastasise, have been disappointing. Cellular heterogeneity within tumours remains a problem and studies analysing molecular markers are often qualitative rather than quantitative in nature. Furthermore, it is always possible that metastases may result from the random survival of shed epithelial cells (467). In 1986, Nobel Prize-winning virologist, Renato Dulbecco, stated " We have two options: either to discover the genes involved in cancer by a piecemeal approach, or to sequence the whole genome.....I think it would be far more useful to begin by sequencing the cellular genome. The sequence will make it possible to prepare probes for all genes.....Classification of the genes will facilitate the identification of those involved in cancer progression " (468). The Human Genome Project is targeted for completion by the year 2005. Hopefully, by this time, we will know whether the reductionist approach of Dulbecco was correct.

Medical historians may look back on the last twenty years, as one of the most exciting periods in colorectal cancer research. In the past 8 years, 9 genes directly implicated in the carcinogenic process have been identified, and the part that each of these plays in the cancer jigsaw is presently being unravelled. The assertion of Bailar and Smith that " we are losing the war against cancer" may yet be disproved (300).

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