Genomic Instability In Colorectal Carcinoma

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by

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Abstract

Microsatellite instability (MSI) in colorectal tumours is demonstrated by PCR amplification of several different microsatellite loci. Minisatellites, which are repeats of longer sequences also found throughout the genome, may also be affected by tumorigenesis. Certain minisatellite alleles contain 2 types of similar repeat unit that are randomly interspersed. The interspersion pattern can be analysed by mapping variant repeat units along an amplified allele, minisatellite variant repeat unit mapping PCR (MVR-PCR). We have applied microsatellite analysis with ten markers and MVR-PCR for locus D7S21 to 33 cases of colorectal neoplasia, 27 sporadic and 6 from patients suspected of having hereditary non-polyposis colorectal cancer (HNPCC). Of the 27 sporadic cases, 3 were MSI-high on microsatellite analysis and one MSI-low. Features such as band loss on PCR product amplification suggesting instability were seen with MVR-PCR only for the MSI-high cases. Four of the HNPCC patients had mismatch repair (MMR) gene mutations proven by sequencing (hMLH1 and hMSH2). All 4 had DNA instability by MVR-PCR, but only two of these had MSI (one high, one low). The other two were negative to mutation analysis. One was from an Amsterdam criteria positive kindred but did not demonstrate instability by any technique. The other had features strongly suggestive of HNPCC and was unstable by both microsatellite analysis (MSI-high) and by MVR-PCR. MVR-PCR detects DNA instability in MSI-high sporadic tumours and in those associated with HNPCC where MSI is observed. Further, in some MMR mutation positive cases MSI was not seen but instability was observed by MVR-PCR. MVR-PCR may be a valuable adjunct to the detection of MMR deficiency in colorectal tumours and it may allow new insights into the nature of DNA instability in this situation.

Publications

An alternative method for the detection of genomic instability in colorectal cancers.
Coleman M.G., Gough A.C., Bunyan D.J., Primrose J.N.
The Association of Coloproctology of Great Britain and Ireland Annual Meeting and the ECCP Biennial Meeting, Edinburgh, 16-19th 1997.
Published as an abstract in *The International Journal of Colorectal Diseases* (1997) 12(3): 119.

Detection of replication error in colorectal cancer: a non-radioactive, single marker technique.
Coleman M.G., Gough A.C., Bunyan D.J., Braham D., Eccles D.M., Primrose J.N.
The Surgical Research Society, The University of Nottingham. 9-11th July 1997.
Published as an abstract in *The British Journal of Surgery* (1997) 84: 1572.

An alternative method for the detection of genomic instability in colorectal cancer.
Coleman M.G., Gough A.C., Bunyan D.J., Primrose J.N.
The British Society of Gastroenterology Diamond Jubilee Meeting, Brighton, 18-21st 1997. Published as an abstract in *Gut* 40(1): A78.
The 38th Meeting of The British Association for Cancer Research, 1-4th April 1997.
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The American Association of Cancer Research Annual Meeting, San Diego 12-16th April 1997.
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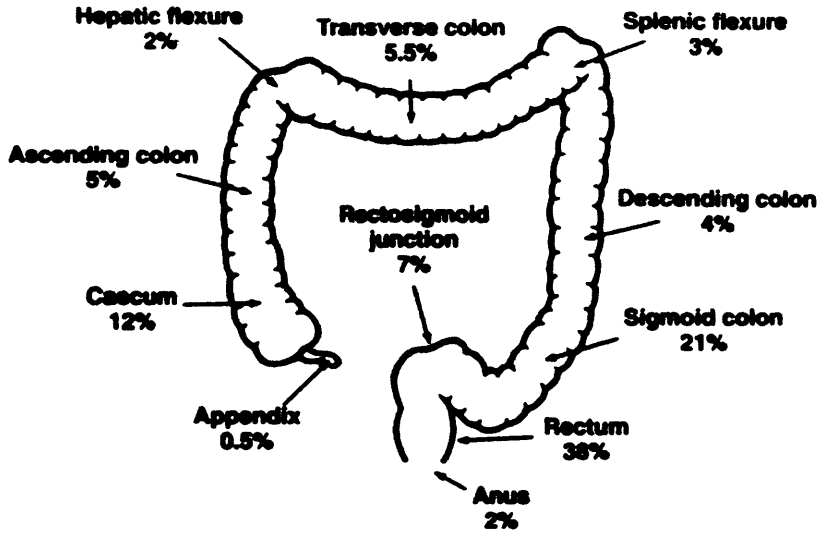
1.1 Epidemiology of colorectal cancer

Cancer of the colon and rectum is the commonest cause of death due to malignancy in non-smokers. Of 305,730 cancers diagnosed in the United Kingdom in 1988, 6% were in the colon and 4% in the rectum (C.R.C. Factsheet 1.1, 1994). It accounts each year for 19250 deaths in the United Kingdom, 85 000 in the European Community 1991 and 61 000 in the United States (CRC fact sheet 18.1, 1993). The estimated lifetime risk of developing cancer of the colon or rectum is 4.6% or 1 in 22 (C.R.C. Factsheet 1.1, 1994). The incidence of colon cancer among newly diagnosed cancers is 6% for males and 7% for females. The incidence of cancer of the rectum is 5% of males and 3% of females newly diagnosed with cancer. In 1987 in the United Kingdom 28 000 new cases of large bowel cancer were diagnosed, with 18 020 in the colon and 10 570 in the rectum (CRC fact sheet 18.1, 1993). 60 - 70% of large bowel cancer affects the colon (Figure 1.1).

Figure 1.1 % Distribution of cases by site within the large bowel (CRC Factsheet 18.1, 1993)



The incidence increases with age from approximately 5 cases per 100 000 between 35 and 40 years old to approximately 400 cases per 100 000 in the population over 85 years old (CRC fact sheet 18.1, 1993). The highest rates of colon cancer occur in social classes I and II, when compared to classes IV and V. No such difference is found with rectal cancer (CRC fact sheet 18.1, 1993). There are worldwide variations in the incidence of cancer of the colon and rectum (CRC fact sheet 18.2, 1993). The highest standardised incidence rates of colon cancer are found in the United States with 34 and 29 per 100 000 population for males and females respectively. The lowest incidence of colon cancer is found in India with 1.8 per 100 000 for both sexes. The highest rates of rectal cancer for males are found in The Federal German Republic with 22 per 100 000 population and for females, in Israel, with 13 per 100 000. The lowest rates of rectal cancer are in Kuwait, with 3 per 100 000 for males and 1.3 per 100 000 for females (CRC fact sheet 18.2, 1993). Although death rates from large bowel cancer have fallen since the 1920s in the

United Kingdom, there has been no significant change over the last 30 to 40 years (CRC fact sheet 18.2, 1993).

1.2 Staging of colorectal cancer

Survival following diagnosis of colorectal cancer is highly dependant on disease stage at presentation. The staging system devised by Dukes in 1929 remains in use today although many modifications of the original classification have taken place Dukes, 1930). The original system was based upon observations of the depth of invasion of the tumour through the bowel wall and regional lymph node involvement in operable rectal cancers. Dukes' A cases were those in which the carcinoma is limited to the wall of the rectum, there being no extension into the extra-rectal tissues and no metastases in lymph nodes. Dukes' B cases were those in which the carcinoma has spread by direct continuity to the extra-rectal tissues but has not yet invaded the regional nodes. Dukes' C cases were those in which metastases are present in the regional nodes (Dukes, 1930 and 1932). The approximate percentage of cases that fall into each stage are: Dukes' A 10%, Dukes' B 50% and Dukes' C 40% (CRC fact sheet 18.2, 1993). By 1935 the importance of proximal node involvement was recognised (Gabriel, Dukes and Bussey, 1935) and the classification was modified to Dukes' C1 in which only regional lymph nodes were involved or in which the proximal spread had not reached the point of ligation of blood vessels; Dukes' C2 was used to describe lymph node spread which had reached the point of the proximal vascular ligature. Dukes' classification was extended to include the colon in 1939 (Simpson and Mayo, 1939). The study of depth of tumour invasion has also resulted in further modifications of Dukes' classification. In 1949, Kirklin reclassified Dukes' A as

those tumours not penetrated through the muscularis mucosa, originally thought by Dukes not to be true invasive cancers (Kirklin, Dockerty and Waugh, 1949). Kirklin further subdivided Dukes' B into B1and B2 according to the depth of invasion of the tumour in the bowel wall and excluded tumours that had reached the peri-rectal tissues in this group. Thus, to Kirklin *et al.,* stage B was Dukes' stage A. Another American modification of Dukes by Astler and Coller sub-classified Dukes' B into B1 and B2 according to whether penetration of the muscularis mucosa (or serosa, where present) had occurred (Astler and Coller, 1954). Despite these modifications, the relationship between extent of spread and prognosis remains the depth of tumour penetration and the presence of lymph node metastases (Phillips, Hittinger, Blesovsky, *et al.*, 1984a). Neither Dukes system nor its modifications took into account the presence of more extensive disease and more recently Dukes' stage D has been added to signify the presence of distant metastases irrespective of the extent of local disease (Turnbull, Kyle, Watson & Spratt, 1967). Such information is accounted for by the universal TNM (tumour, node, and metastasis) system, with criteria set by the Union Internationale Contre le Cancer (UICC) and applied to colorectal cancer by the American Joint Committee Task Force on Colon and Rectum after analysis of 1826 cases of cases (Beart, Moertel, Wieand, *et al.*, 1978) (Table 1.1).

The distribution of Dukes' stages in an Australian population-based study of 1105 cases of histologically confirmed colorectal adenocarcinoma discovered that 15% were Dukes' A lesions, 32% were Dukes' B, 25% were Dukes' C and 29% were Dukes' D using Dukes' original classification (Kune, Kune, Field, *et al.*, 1990). The five year age adjusted survival for each Dukes' stage for colon and rectal cancer

respectively were: 87% and 78% for Dukes' A lesions, 70% and 55% for Dukes' B lesions, 40% and 31% for Dukes' C lesions, and 7% and 0% for Dukes' D (Kune *et al.*, 1990).

Table 1.1 The TNM classification as applied to colorectal cancer.

Clinical-surgical evaluation	Definition	Post-surgical assessment	Definition
T0	No tumour demonstrable	pTis	Carcinoma in situ
T1	Clinically benign or confined to mucosa or sub mucosa	pT1	Confined to mucosa or submucosa
T2	Confined to muscular wall or serosa	pT2	Same
T3	Involvement of all layers of bowel wall with extension to adjacent structures or organs, no fistulas	pT3	Same
T4	Fistula present.	pT4	Same
T5	T3 or T4 present with extension to other organs or structures.	pT5	Same
TX	Depth of penetration not specified	pTX	Same
N0	Nodes not involved	pN0	Same
N1	Nodes involved	pN1	Same
NX	Status of nodes unknown	pNX	Same
M0	No distant metastases or nodal metastases beyond the base of the meso-colon	pM0	Same
M1	Evidence of distant metastases	pM1	Same

Hence Dukes' stage A, according to the TNM classification corresponds to T0-2 N0 M0-1, Dukes' B, T3-4 N0 M0-1, and Dukes' C, T0-4 N1 M0-1.

1.3 Aetiology

1.3.1 Normal colonic cell turnover

Normal colonic epithelium, although polyclonal, is composed of multiple monoclonal expansions from the stem cells in the crypts of Lieberkühn. The daughter cells of any stem cell do not extend beyond the region of confluence at the mucosal surface with the cells from neighbouring crypts. (Fearon, Hamilton & Vogelstein, 1987).

The turnover of cells in normal mucosa formed by division occurring in the lower third of the crypt to their eventual exfoliation from the mucosal surface takes about 4-8 days. This phenomenon was demonstrated by studies in the early 1960s using tritiated thymidine (Cole and McKalen, 1961). The rate of cell division in the crypt and the rate of migration onto the mucosal surface balance the rate of exfoliation in normal mucosa.

1.3.2 Adenomas

Adenomas are neoplastic polyps that are pre-malignant and harbour the ability to develop in to adenocarcinoma of the colon or rectum (Fearon and Vogelstein, 1990a). They are a precursor in the development of sporadic CRC and the phenotypic feature of FAP and certain other inherited neoplastic polyposes.

Accurate information on adenomas is based on large colonoscopic studies (Shinya and Wolff, 1979; Gillespie, Chambers, Chan, Doronzo, Morson and Willams, 1979). In all zones of the colon and rectum, the tubular variety was most common, followed by tubulovillous and then villous adenomas. Broadly, two thirds are found colon distal to the splenic flexure, one third proximal to this. They were most common in the sigmoid colon, followed by the descending colon. All studies show a

relatively low incidence of rectal polyps probably reflecting their ease of removal at rigid sigmoidoscopy. For cases with single adenomas, a disproportionate number are found in the proximal colon (Williams, Balasooriya & Day, 1982). This finding is diluted in cases with multiple adenomas. There is also a shift to the right colon seen for older age groups (Eide and Stalsberg, 1978), which is reflected in the higher proportion of right-sided carcinomas in that group (Snyder, Heston, Meigs, and Flannery, 1977). Therefore there is undoubtedly a relationship between the anatomical distribution of colorectal carcinomas and adenomas within the colon and rectum, which supports the link between the two conditions. Most of these are situated distal to the splenic flexure.

The incidence of polyps increases with advancing age and is greater in males. Autopsy studies both show that 52.4% of men and 32.8% of women over 75 have adenomas (Williams, Balasooriya & Day 1982). These figures contrast with those under 54 years of age (20 and 14.8% respectively) and are confirmed by colonoscopic studies (Gillespie, Chambers, Chan, Doronzo, Morson and Willams, 1979).

The development of a colorectal adenocarcinoma is usually the result of a progressive set of morphological changes that begin as a monoclonal hyper-proliferation of epithelial cells leading to the development of a small adenoma. The transition from adenoma to carcinoma is a continuum and is the result of further clonal expansion during which distinct changes in morphology, apart from its increase in size, are seen. An early adenoma is typically small, spherical and pedunculated, although they may be sessile or grow up to 5 cms. They consist of

closely packed tubules, separated from the lamina propria, which grow and branch horizontally into the muscularis mucosae (Morson, 1978). The acquisition of villous morphology results in a polyp that is larger and with a less well-defined edge. It consists of a central core of connective tissue covered by epithelial cells growing towards the bowel lumen. An intermediate from also may exist, a tubulovillous adenoma that contains elements of both tubular and villous characteristics but in whom the villous elements tend to be broad and stunted (Morson and Dawson, 1990). Normal differentiation in colonic epithelium involves the formation of mucus secreting goblet cells and absorptive cells. The cells forming an adenoma may initially microscopically resemble those of normal colonic epthilelial cells. Differentiation fails to occur and, during carcinogenesis, immature cells are found on the surface, with crowding of cells representing their hyperproliferative state, and hyperchromasia and pleomorphism of nuclei. In addition, there may be an increase in mitotic figures, and the cells may form several layers projecting into the lumen in the case of tubular adenomas or into the connective tissue core in villous adenomas. These changes are known as atypia or dysplasia. The grading of dysplasia is based on the degree and extent of these features (Kozuka, 1975). Severe dysplasia, generally seen in large villous polyps, is essentially synonymous with carcinoma-in-situ.

1.3.3 Neoplastic Polyposes

i. Familial Adenomatous Polyposis

Familial Adenomatous Polyposis (FAP), a condition that is inherited in an autosomal dominant fashion is characterized primarily by numerous adenomatous polyps of the colon, which arise during adolescence. The first description of multiple colonic polyps occurred in 1881 (Lynch, Smyrk, Watson, *et al.,* 1991). It is distinguished clinically from other polyposes by the number of polyps and the presence of characteristic extracolonic manifestations such as upper gastrointestinal polyps, desmoid tumours, osteomas, epidermoid cysts and Congenital Hypertrophy of the Retinal Pigment Epithelium (CHRPE). It has long been recognised that there is a strong association between FAP and the development of cancer but Lockhart-Mummery first described the presence of multiple adenomas within a kindred with a tendency to undergo malignant change (Lockhart-Mummery, 1925). Although defined by the presence of at least 100 colonic polyps, there is a vast range, as discovered by Bussey who found an average of 981 polyps with a range of 157 to 3673 polyps (Bussey, 1975).

The first clue towards finding the molecular pathogenesis of FAP came when an interstitial deletion in chromosome 5q was discovered in a man with polyposis due to Gardner syndrome (Herrera, Kakati, Gibas, Pietrzak and Sandberg, 1986). This observation stimulated molecular studies that demonstrated tight linkage of the disease to markers on chromosome 5q21 (Bronner, Baker, Morrison, *et al.*, 1994; Leppert, Dobbs, Scambler, *et al.*, 1987). The gene (Adenomatous polyposis coli gene, APC) could then be cloned, sequenced and mutations identified in FAP

kindreds and in sporadic colorectal neoplasia (Nishisho, Nakamura, Miyoshi, *et al.*, 1991; Groden, Thliveris, Samowitz, *et al.*, 1991; Joslyn, Carlson, Thliveris, *et al.*, 1991, Kinzler, Nilbert, Su, *et al.*, 1991). In sporadic non-polyposis colorectal neoplasms inactivation of the APC gene has been demonstrated in the majority of carcinomas and early adenomas indicating a pivotal role in colorectal carcinogenesis (Powell, Zilz, Beazer-Barclay, *et al.*, 1992).

The APC gene is large and consists of 15 exons encoding a protein containing 2843 amino acid residues. This is found in the cytoplasm of epithelial cells and is known to be associated with cadherins, which are cell surface adhesion molecules (Su, Vogelstein and Kinzler, 1993). The APC gene product is also associated with microtubules, which are vital to cell division (Smith, Levy, Maupin, Pollard, Vogelstein and Kinzler, 1994). There is a spectrum of mutations seen in the APC gene in both FAP and sporadic colorectal neoplasia, which result in the creation of premature stop codons (Nagase and Nakamura, 1993). This leads to the generation of truncated protein product (Nagase and Nakamura, 1993). Most of the mutations are found in a region of 600 codons within exon 15 at the 5' end of the gene (Nagase and Nakamura, 1993). Mutation analysis is successful in detection of mutation in only a proportion of cases leading to the development of functional assays that determine the presence of truncated protein product by electrophoresis (Powell, Zilz, Beazer-Barclay, *et al.*, 1992). Using well-characterized mutations as controls, the causative mutation can be identified and the relevant gene sequenced. The location of mutations within the APC gene in FAP determine the phenotypic manifestations of the condition in terms of the number and aggression of the colonic polyps and extra-colonic features such as CHRPE and desmoid tumours (Nagase,

Miyoshi, Horii, *et al.*, 1992; Olschwang, Tiret, Laurent-Puig, Muleris, Parc and Thomas, 1993; Caspari, Olschwang, Friedl, *et al.*, 1995). Direct mutation analysis in a FAP kindred once an APC gene mutation is found enables the exclusion of unaffected individuals for genetic counselling and screening thus reducing distress and cost while detecting carriers and helping in their management in terms of the optimal type and timing of prophylactic surgery taking into account the predicted phenotype (Cunningham and Dunlop, 1996).

ii. Other neoplastic polyposes

Attenuated polyposis syndrome

This syndrome is also linked to chromosome 5q, like FAP and it is inherited in an autosomal dominant fashion. There is however a phenotypic distinction from FAP with fewer adenomas (5-100) and, often, rectal sparing. The mutations for attenuated polyposis syndrome are located to the 5' end of the APC gene and are located in close proximity to each other. The mutations, like those in APC also consist of deletions and frameshift mutations that produce truncated protein product (Spirio, Olschwang, Groden, *et al.*, 1993).

1 to 50 but not more than 100 polyps usually appear 10 to 15 years later than FAP. They tend to be flat rather than polypoid and predominate proximal to the splenic flexure. There is also an increased risk of colorectal cancer with a later average age of diagnosis of 55 years. Gastric fundal polyps and duodenal adenomas are also found but desmoids and CHRPE have not been described.

Turcot's syndrome

Colorectal polyposis associated with malignant tumours was first described in 1959 (Turcot, Depres and St Pierre, 1959). Either CNS or colorectal neoplasms may develop first, in the first or second decade of life. Skin lesions such as café-au-lait spots may also be present. Most CNS lesions are gliomas and MMR gene mutations have been identified in most families with the condition.

Hamartomatous polyposis

Focal malformations resulting from disordered differentiation (hamartomas) characterize several different rare polyposis syndromes. These include juvenile polyposis, Peutz-Jeghers syndrome (Peutz, 1921) Cowden's disease (Lloyd and Dennis, 1963) Cronkhite-Canada syndrome (Cronkhite and Canada, 1955) and ganglioneuromatous polyposis (Haggitt and Reid, 1986).

Hereditary mixed polyposis syndrome

This syndrome is inherited in an autosomal dominant fashion and is associated with the presence of hamatomatous, adenomatous or hyperplastic polyps and a predisposition to early onset CRC. (Whitelaw, Murday, Tomlinson, *et al.,* 1997). There are usually fewer than 15 polyps of mixed features scattered throughout the colon.

1.3.4 Inflammatory Bowel Disease And Colorectal Cancer

i. Ulcerative colitis

The relationship between ulcerative colitis and colorectal cancer has long been recognized (Bargen, 1927). The risk is greatest in patients with pan-proctocolitis and increases with time (Devroede, Taylor, Sauer, Jackman and Stickler, 1971).

After 10 years of symptomatic disease the incidence becomes apparent, at around 3% (Kewenter, Ahlman and Hultér, 1978) in those with extensive colitis. Dysplasia or carcinoma-in-situ may develop even during the first 5 years of the disease (Myrvold, Kock and Åhrén, 1974). The incidence of cancer rises to a cumulative incidence of 34% at 25 years but is higher in those with disease onset before the age of 25 years at 43% in this series (Kewenter, Ahlman and Hultér, 1978), and estimates of incidence in other series agree with these figures (Devroede, Taylor, Sauer, Jackman and Stickler, 1971; Greenstein, Sachar, Smith, *et al.*, 1979).

Cancer, although less common, may also develop in patients with left-sided colitis (Greenstein, Sachar, Smith, *et al.*, 1979) or even proctitis (Devroede, Taylor, Sauer, Jackman and Stickler, 1971). The risk of cancer in patients with left sided disease occurs 10 years later than those with pancolitis (Greenstein, Sachar, Smith, *et al.*, 1979) and 20 years later in those with proctitis (Devroede, 1980). In this series the age of onset did not appear to be of significance (Greenstein, Sachar, Smith, *et al.*, 1979).

ii. Crohn's Disease

Crohn's disease has long been thought not to carry a risk of malignancy but recent evidence has shown a small risk of developing adenocarcinoma of both the small and large intestine when affected by the inflammatory process (Lightdale and

Sherlock, 1980). A significant proportion of the cases occur before the age of 40 years and they tend to be more proximal than the normal distribution of colorectal cancers. As in ulcerative colitis, there is an association between duration of symptoms (over 20 years in 87%) and onset of cancer (Ribeiro, Greenstein, Sachar, *et al.*, 1996).

1.4 *Molecular Genetics Of Colorectal Cancer*

1.4.1 Introduction

This clonal expansion of one or a small number of cells to form a tumour is accompanied by a series of somatic genetic alterations. These alterations are seen in all or virtually all neoplastic cells studied within a particular tumour suggesting that they confer a selective growth advantage on the cell that has undergone the change (Fearon and Vogelstein, 1990a).

Evidence for the molecular genetic events that underlie the adenoma-to-carcinoma sequence are based on studies of tumours at all stages of development from hyperproliferative colonic epithelium to advanced carcinomas. Regardless of aetiology, colorectal carcinogenesis follows a common pathway involving the mutational activation and inactivation of oncogenes and tumour suppressor genes. Four to five changes are necessary for transformation from normal epithelium to invasive carcinoma and it is their accumulation rather than order that determines the eventual nature of the tumour (Fearon and Vogelstein, 1990a).

1.4.2 Oncogenes

Oncogenes are dominant genes whose protein products promote abnormal cell proliferation and tumour formation when inappropriately expressed (Bishop, 1987). They were first discovered in detailed studies of the mechanism of action of tumour-producing animal viruses, which reflects their nomenclature (e.g. ras: rat sarcoma, myc: avian myelocytomatosis) (Moore, Jones, Schofield and Harnden, 1989). Thus, they are designated viral oncogenes (v-onc), the oncogenes present in

retroviruses and cellular oncogenes or proto-oncogenes, which are their normal cellular counterparts. (Rigas, 1990). The function of proto-oncogenes in humans is thought to be in the control of normal cellular growth via the synthesis of proteins involved in signal transduction from cell surface growth factor receptors to the cell nucleus (Rigas, 1990). Mutation of proto-oncogenes to oncogenes leads to the production of defective proteins and results in deregulation of normal cell growth. A prolonged growth stimulus then results in inappropriate proliferation and possible malignant transformation. Thus, activated proto-oncogenes have been shown to play a major role in many human cancers (Vogelstein, Fearon & Hamilton, *et al.,* 1988). In colorectal cancers, activating mutations in codons 12 and 13 of Kirsten *ras* (K-*ras*) occur in 50% of carcinomas and adenomas greater than 1 cm in diameter cancers (Vogelstein, Fearon & Hamilton, *et al.,* 1988). The frequency of K-*ras* mutations is much lower (less than 10%) in adenomas less than 1cm. This was found to be regardless of whether the adenoma arose sporadically or as a result of an inherited mutation (Farr, Marshall, Easty, Wright, Powell & Paraskeva, 1988) and suggests that the K-*ras* oncogene is involved in the progression from adenoma to carcinoma (Shibata, Schaeffer, Li, Capella and Perucho, 1993). Furthermore, K-*ras* expression although high in primary cancers is lower in more advanced cases suggesting that after a tumour progresses to a certain stage, *ras* activation is no longer required. Most K *ras* activation is by point mutation, rather than amplification or rearrangement. (Fearon and Vogelstein, 1990a).

Ras has been demonstrated in colorectal adenomas and cancers. Inheritance of the rare Harvey *ras* appears to play a role in the aetiology of 1 in 11 colorectal cancers (Krontiris, Devlin, Karp, Robert and Risch, 1993).

1.4.3 Tumour Suppressor Genes

Tumour suppressor genes are genes that function normally to suppress malignant transformation (Klein, 1987). In contrast to oncogenes, it is their inactivation or loss that leads to malignant transformation. It is not known how they normally exert their anti-tumour effect. Evidence supporting the role of tumour suppressor genes in colorectal carcinogenesis is based on the knowledge that deletions in specific chromosome regions are found in most tumours. This promotes the hypothesis that these regions encompass genes that code those products that normally affect growth and regulation in a negative fashion. It is assumed that loss of the allele results in an insufficient level of expression of these tumour suppressor genes (Knudson, 1985). Evidence that inactivation of such genes was a common event in colorectal tumour development was first observed in the Adenomatous Polyposis Coli (APC) Gene. Germline mutations of the APC gene are associated with FAP and other polyposes whereas somatic mutations appear to play a critical role in the development of the majority of colorectal adenomas and carcinomas (Vogelstein, Fearon, Hamilton *et al.*, 1988). The APC gene located in chromosome 5q is frequently affected by chromosome loss events in sporadic adenomas and carcinomas; these losses involve one of the two parental chromosome sets and are therefore defined as allelic losses or loss of heterozygosity (LOH). Similar LOH events have been found to affect other chromosomes in a large number of tumour types (Weinberg, 1991). LOH results in inactivation of the affected tumour suppressor gene (Weinberg, 1991) and inactivation of one of the two copies is sufficient to alter the normal growth regulation of colonic epithelial cells (Ichii Horii, Nakatsuru *et al.*, 1992). In sporadic colorectal neoplasia, the frequency of mutations of the APC gene appears to be the

same regardless of their histopathological status. They are found in approximately 60% of adenomas and carcinomas including adenomas less than 0.5 cms in diameter (Powell, Zilz, Beazer-Barclay *et al.*, 1992). Therefore it seems likely that APC gene mutations occur very early in the adenoma-to-carcinoma sequence and could be the initiating event in the development of adenomas in patients without polyposis. Probably all CRCs have either APC or β-Catenin mutations (see section 1.4.5). LOH has also been observed in other chromosome regions in colorectal tumours. One of these is the gene p53 found on the chromosome 17p. p53 is mutated in 75-83% of colorectal carcinomas, but infrequently in adenomas (Baker, Fearon, Nigro *et al..*, 1989). Thus, p53 mutation may be associated with the progression from adenoma to carcinoma. The function of the p53 gene product is thought to be in the regulation of normal growth via the control of the expression of a number of cell genes (Kern, Pietenpol, Thiagalingam, Seymour, Kinzler and Vogelstein, 1992). Loss of normal function in p53 may promote genetic instability in cells and it therefore may be selected for during the later stages of tumour development (Livingstone, White, Sprouse, Livanos, Jacks and Tolstoy, 1992).

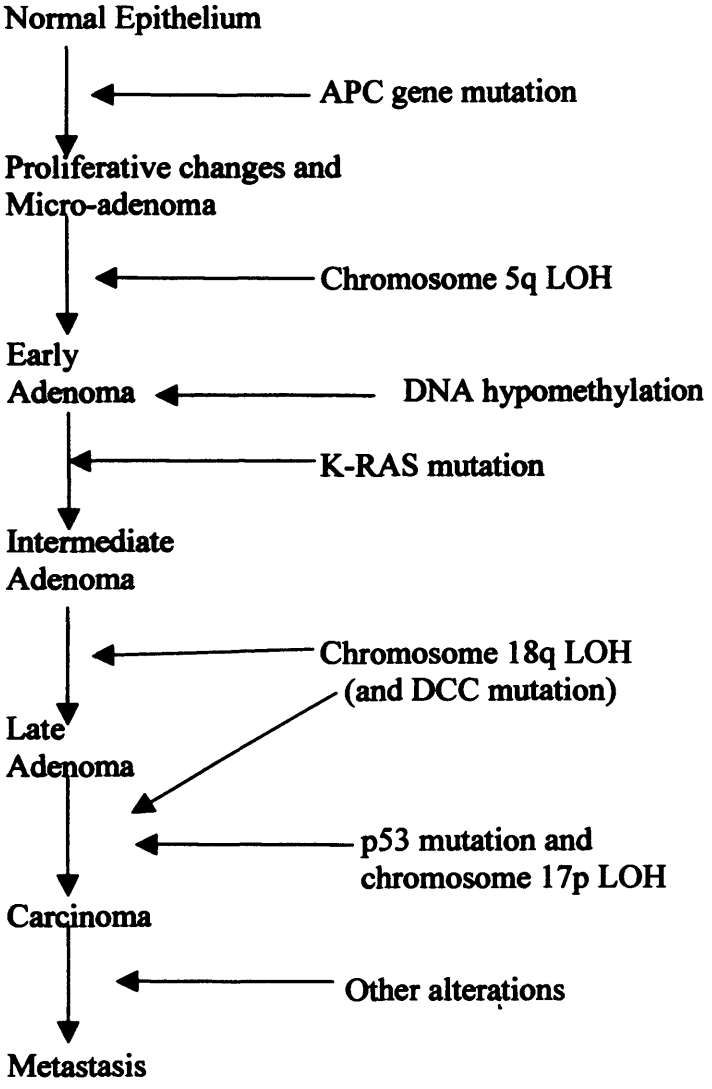
Another tumour suppressor gene, termed *Deleted in Colorectal Cancer* (DCC) has been identified on chromosome 18q (Fearon, Cho, Nigro. *et al.*, 1990b). LOH can be detected in about 70% of colorectal carcinomas, 50% of advanced adenomas and is uncommon in early adenomas (Vogelstein, Fearon, Hamilton *et al.*, 1988). Little is known of the function of the DCC gene product but alterations at the DCC locus does not seem to mediate a transition into any morphologically identifiable stage in the adenoma-to-carcinoma sequence (Boland, Sato, Appelman, Bresalier, and Feinberg, 1995).

1.4.4 A genetic model for the adenoma-to-carcinoma sequence

The knowledge that most, if not all, colorectal carcinomas arise from pre-existing benign adenomas and the observations of genetic alterations in oncogenes and tumour suppressor genes led to the description of the genetic basis for the adenoma-to-carcinoma sequence by Fearon and Vogelstein (1990a). The following are the salient features of the model:

1. Colorectal tumours appear to arise as the result of the mutational activation of oncogenes, such as K-RAS, and inactivation of tumour suppressor genes, such as APC, DCC and p53.
2. Mutation in at least one of these genes is necessary for colorectal carcinogenesis. A few (one to two) may be necessary for adenoma formation, while multiple (four to five) are needed for carcinoma development. The particular genetic alterations involved demonstrate a relative preference for different stages of the adenoma-to-carcinoma sequence (Figure 1.2).
3. The accumulated changes rather than their order determines the biological properties of the tumour.
4. Mutated tumour suppressor genes appear to exert a phenotypic effect at the cellular level even the heterozygous state.

Figure 1.2 A genetic model for colorectal tumourigenesis (Fearon and Vogelstein, 1990a)



1.4.5 E-cadherin expression in CRC

The majority of CRC probably develops in accordance with the Fearon and Vogelstein model of the adenoma-to carcinoma sequence. Significant differences, however, are noted in HNPCC associated CRC and a proportion of sporadic CRC which are also RER positive. For example, the type II transforming growth factor (TGF) β receptor gene is prone to mutation in RER + tumours. So called ulcerative colitis associated CRC also has different clinico-pathological characteristics from sporadic CRC such as a low frequency of APC and K-*ras* mutations, suggesting an alternative molecular pathogenesis. One of the genes associated with tumour growth and differentiation (and therefore, metastasis) is E-cadherin, whose protein is a trans-membrane molecule involved in cell adhesion. It is normally expressed along intercellular borders and functions by way of calcium dependant homophilic adhesion and is linked to actin in the cytoskeleton via α-, β- or γ-catenin. Its expression, although reduced in a proportion of CRC does not differ between RER positive or negative tumours, or in ulcerative colitis associated CRC (Ilyas, Tomlinson, Hanby, Talbot and Bodmer, 1997).

The APC protein product also binds β̃Catenin and activating mutations in the gene is thought to have similar consequences to inactivation of the APC tumour suppressor gene (Kitaeva, Grogan, Williams *et al.*, 1997). Although some researchers have found that β̃Catenin gene expression is down regulated in colorectal carcinoma, others have not found this. Perhaps there is transient down-regulation resulting in metastasis (Van der Wurff, Vermeulen, Van der Linden, Mareel, Bosman and Arends, 1997).

1.4.6 Clinical applications of molecular genetic studies

i. Non-invasive detection and early diagnosis

DNA containing K-ras mutations has been detected in the circulating plasma of patients with colorectal cancer though these are the results of small studies and such mutations are only found in cases whose tumours have K-ras mutations (Anker, Lefort, Vasioukhin *et al.*, 1997). Antibodies to p53 have also been found in the serum of patients with colorectal cancer by immunohistochemistry (Hammel, Boissier, Chaumette *et al.*, 1997).

Viable exfoliated colonic epithelial cells can be isolated from faecal samples and these can be analysed for specific biomarkers of malignant transformation such as K-ras mutation, abnormal expression of the cell surface glycoprotein CD44, and tumour associated antigens such as carcinoma embryonic antigen and carcinoma antigen 19-9. Such assays may detect small adenomas and cancers (Kim, Yang, Rosada, Hamilton and August, 1994).

ii. Prognostic markers

Deletions of p53 are associated with advanced metastatic disease and its detection may be associated with a survival disadvantage (Goh, Yao and Smith, 1995). There are divergent opinions as to the prognostic value of LOH in chromosome 18q. An initial study (Jen, Kim, Piantadosi *et al.*, 1994) demonstrated poor prognosis for

colorectal carcinomas with 18q LOH but subsequent studies have failed to confirm this finding (Cohn, Ornstein, Wang *et al.*, 1997). Gene markers may also be useful in determining response to chemotherapy or radiotherapy (Lowe, Bodis, MacClatchey *et al.*, 1994).

1.5 Minisatellites

1.5.1 Introduction

DNA polymorphisms have revolutionised human genetic analysis. The advent of DNA analysis developed by Southern by restriction endonuclease digestion, electrophoretic separation and hybridisation allowed the structure of DNA to be studied in detail (Southern, 1975). Variable DNA loci were first recognised in humans in 1978 by the use of single strand DNA copies to detect restriction fragment length polymorphisms (RFPLs) adjacent to the human β-globin structural gene. (Kan and Dozy, 1978). They studied DNA from Africans, some of whom had haemoglobin S and its associated mutation in the β-globin gene and found alteration in a restriction endonuclease site (*Hpa* I) such that instead of the normal 7.6 kilobase fragment, fragments of either 7.0 or 13.0 kilobases were produced. This polymorphism was related to, and therefore a linkage marker for, the sickle cell disease genotype. It could be used for genetic diagnosis of sickle cell. In such restriction fragment length polymorphisms (RFPLs), the restriction enzyme cleavage site is either present or absent, the chance of a parent having two different alleles is never more than 50%. The informativeness of such a locus as a discriminative marker in pedigree analysis is therefore limited to a maximum heterozygosity of 50%. Highly variable RFPLs were first discovered by an arbitrary probe in 1980 which found at least 16 different allele length combinations in a small sample of unrelated individuals by digestion of hybridised cloned human DNA (Wyman and White, 1980). Subsequently similar systems have been discovered in other loci including the insulin gene (Bell, Selby and Rutter, 1982), the α globin

gene (Higgs, Goodbourn, Wainscoat, Clegg, and Wetherall, 1981), the Harvey-*ras* oncogene (Capon, Chen, Levinson, Seeburg and Goeddel, 1983), the □-globulin pseudogene (Proudfoot, Gil and Maniatis, 1982) and the myoglobin gene (Jeffreys, Wilson and Thein, 1985a). These regions all contain a tandem array of short-sequence DNA repeats known as minisatellites or variable number tandem repeats (VNTRs). By detailed analysis of the polymorphic region upstream of the Insulin gene (see above), Owerbach and Aagaard (1984) found 139 repeating sequences with a consensus structure related to the ACAGGGTGTGGGG nucleotide sequence. The polymorphism of this region was found to be related to differences in the number of repeats. This variable number of repeats and hence length variation presumably arises by DNA slippage during replication or by unequal exchange during mitosis or meiosis. The length variation in minisatellite alleles may be detected by the use of any restriction endonuclease (hybridisation probe), which does not cleave the repeat unit. Variable number tandem repeat or minisatellite loci are abundant in the human genome and may be simultaneously detected by the use of multiple repeated probes resulting in large numbers of polymorphic loci. This provides a powerful tool for genetic analysis and, because the resulting profile is individual specific, it is called a DNA or genetic fingerprinting. Multilocus DNA fingerprint probes have a wide variety of applications including the establishment of family relationships (Jeffreys, Turner and Debenham, 1991a), immigration disputes (Jeffreys, Wilson and Thein, 1985b), and monitoring bone marrow transplants (Thein, and Jeffreys and Blacklock, 1986). Although VNTR loci have a high germline mutation rate, these can almost always be distinguished from incorrect parentage.

Single minisatellite loci may also be studied individually using cloned locus-specific probes. These have a wide variety of applications as highly informative genetic markers, especially in segregation analysis as linkage markers to genetic disease. They are also used in the forensic identification of individual identity where results can be obtained from small amounts of partially degraded DNA (Wong, Wilson, Patel, Povey and Jeffreys, 1987). Techniques utilising hybridisation probes were limited in sensitivity, especially in forensic work because they required at least 50ng of undegraded DNA for multilocus (Jeffreys, Wilson and Thein, 1985b) and 0.1-1ng for locus specific analysis (Wong, Wilson, Patel, Povey, and Jeffreys, 1987).

The polymerase chain reaction (PCR), developed in 1986 by Mullis *et al.* (Mullis, Faloona, Scharf, Saiki, Horn, and Erlich, 1986) allowed the specific enzymatic amplification of DNA in vitro. This technique was refined in 1988 with the discovery of a thermostable DNA polymerase that simplified it and greatly increased the specificity, yield, sensitivity and the length of targets that can be amplified (Saiki, Gelfand, Stoffel, *et al.*, 1988). The accurate analysis of minisatellite allele length variation by PCR amplification of DNA enables much smaller amounts of DNA to be analysed but suffers from the disadvantage that many such highly polymorphic and therefore informative loci are too large to amplify efficiently (Jeffreys, Wilson, Neumann, and Keyte, 1988b). Only a few minisatellite alleles combine a high level of informativeness with restricted size such that complete PCR profiles can be obtained. Microsatellite alleles whose repeat length is no more than 3 bps and smaller tandem repeats (4bps) may in theory be defined precisely as their allele size is smaller (100-200bps), which may also be of

advantage in that they will be more resistant to degradation. However the informativeness of individual microsatellite loci is limited such that multiple loci in the same case need to be examined to give sufficient data (Hagelberg, Gray and Jeffreys, 1991) and this is further limited by artefactual slippage of products that occurs during amplification (Weber and May, 1989).

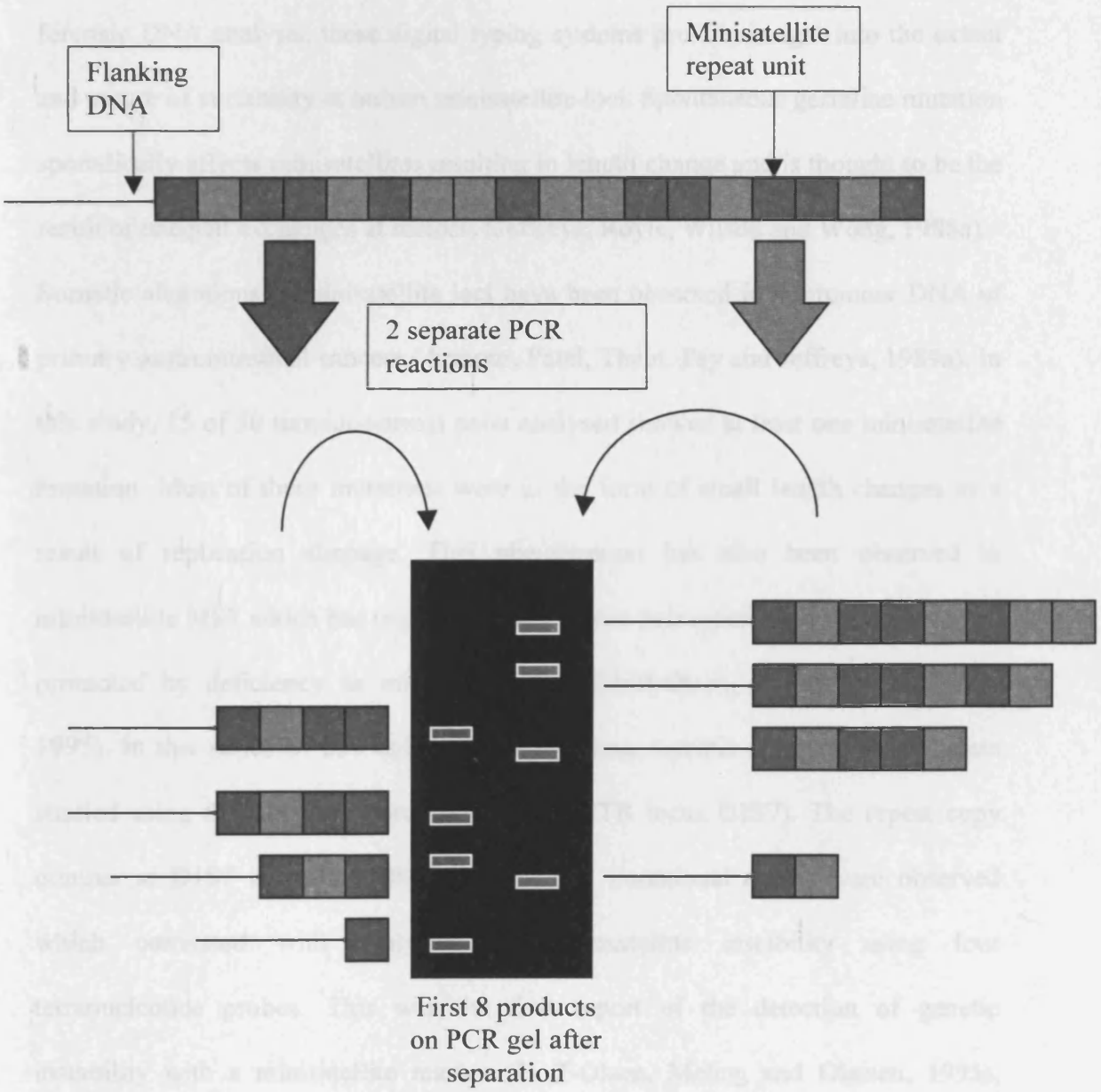
Early work characterising the inter-allelic variability at highly polymorphic minisatellite loci relied on Southern blot estimation of allele length from PCR amplified DNA (Nakamura, Julier, Wolff *et al.*, 1987b). Polymorphism seen in minisatellite loci is the result of a high rate of germline and somatic mutations (Armour, Patel, Thein, Fey and Jeffreys, 1989a). In addition to variation, minisatellites show a tendency towards clustering, and the more variable minisatellites cluster in atypical regions of the genome that tend to be near the ends of human autosomes (Armour, Wong, Wilson, Royle and Jeffreys, 1989b). Repeat units within a minisatellite are seldom all identical but display variation in sequence between repeat units (Jeffreys, Neumann and Wilson, 1990). Minisatellite alleles, which have the greatest level of allelic variability in repeat copy number, in contrast show low levels of inter-repeat unit sequence variability. In such minisatellite loci the inter-repeat unit variability takes the form of base substitutions (Smith, 1976) and in even the most variable minisatellites maintain some degree of inter-repeat unit variation (Wong, Wilson, Patel, Povey, and Jeffreys, (1987). Initial work on the variation in internal structure of minisatellite alleles revealed several different minisatellite repeat units (MVRs) per allele (Owerbach and Aagaard, 1984) but little information on the allelic variation in the distribution of MVRs along a minisatellite allele was known until 1990.

1.5.2 Minisatellite variant repeat unit mapping

In 1990, Jeffreys Neumann and Wilson (1990) mapped the internal sequence of repeat units along a minisatellite allele. By mapping the hypervariable locus D1S8 located on chromosome 1 (at 1q42-q43) using the human minisatellite clone MS32, using PCR they discovered that roughly half the 29bp repeats contained an A to G transposition which made that site cleavable by the enzyme *HaeIII*. In contrast the enzyme *HinfI* cleaved once in all repeat units. Therefore by using two MVR specific primers which prime off either type of repeat unit together with a primer directed at a fixed site in the MVR flanking DNA, a set of products can be generated which extend from the flanking site to each repeat unit (Jeffreys, MacLeod, Tamaki, Neil and Monckton, 1991b). Internal priming of PCR products by MVR specific products, however, favoured the generation of the shorter PCR products with each cycle (Jeffreys, MacLeod, Tamaki, Neil and Monckton, 1991b). This is prevented by providing each MVR specific product with a 20 nucleotide 5' extension (TAG) and carrying out amplifications with a low concentration of one or other tagged repeat unit specific primer and high concentrations of the flanking and TAG primers. This process generates complimentary ladders of PCR products of different lengths extending from the flanking DNA to each specific repeat unit (Figure 1.3). The result is the generation of a binary map of the interspersion pattern of repeat units along a minisatellite allele. This revealed a substantial intermingling of the two types of repeat unit along the allele. They were able to reproduce MVR mapping patterns that could be encoded as a binary string. In this locus (MS32), alleles up to 24 repeats could be mapped, allowing in theory the discrimination of 10^{72} different allelic states. The generation of a digital binary code provides an

objective method for DNA fingerprinting. Further MVR loci suitable for digital typing were later identified at MS205 (D16S309, Armour, Harris and Jeffreys, 1993), and MS31A and B (D7S21, Neil and Jeffreys, 1993). For a digital MVR-PCR locus to be sufficiently informative it must conform to certain criteria (Neil and Jeffreys, 1993). It must have allele length heterozygosity greater than 95% to ensure that most alleles are rare. Repeat unit heterogeneity must not be too extensive and must be suitably positioned to allow design of repeat unit specific primers. All primers must work at the discriminatory annealing temperature of the repeat unit specific primers. These rare loci (MS32, MS205 and MS31) conform to the above requirements and may be analysed by MVR-PCR. Most minisatellite loci show marked polarity of the variation in internal structure (Armour, Harris and Jeffreys, 1993). At one end of the tandem array (the 3' end) there is a far greater allelic diversity and this may be the result of there being a recombination 'hotspot' at this site.

Figure 1.3 Schematic representation of MVR-PCR. The 2 different repeat units are differentiated by colour. PCR amplification results in two sets of products whose sequences both initiate in the flanking DNA. The two sets differ by the terminal repeat subunit (red or blue in this diagram)



1.5.3 Minisatellite instability

The advent of MVR-PCR, by charting the interspersion pattern of variant repeat units along minisatellite arrays, provides detailed information on allele structure (Jeffreys, MacLeod, Tamaki, Neil and Monckton, 1991b). In addition to their use in forensic DNA analysis, these digital typing systems provide insight into the extent and nature of variability at human minisatellite loci. Spontaneous germline mutation sporadically affects minisatellites resulting in length change and is thought to be the result of unequal exchanges at meiosis (Jeffreys, Royle, Wilson and Wong, 1988a). Somatic alterations in minisatellite loci have been observed in the tumour DNA of primary gastrointestinal cancers (Armour, Patel, Thein, Fey and Jeffreys, 1989a). In this study, 15 of 50 tumour-normal pairs analysed showed at least one minisatellite mutation. Most of these mutations were in the form of small length changes as a result of replication slippage. This phenomenon has also been observed in minisatellite MS1 which has unusually short 9 base pair repeat, and is thought to be promoted by deficiency in mismatch repair (Hoff-Olsen, Meling and Olaisen, 1995). In this series of 224 colorectal carcinomas, somatic mutation events were studied using the MS1 minisatellite probe (VNTR locus D1S7). The repeat copy number at D1S7 is 100 to 2000 and frequent mutational events were observed which correlated with analysis for microsatellite instability using four tetranucleotide probes. This was the first report of the detection of genetic instability with a minisatellite marker (Hoff-Olsen, Meling and Olaisen, 1995), although in this study, PCR amplification of the D1S7 locus length was analysed rather than the interspersion pattern of the repeat units by MVR-PCR. Additionally, only four tetranucleotide probes were used and none of these were from the panel of

microsatellite markers recommended for use by many workers in this field (Bocker, Diermann, Friedl *et al.*, 1997, Boland, Thibodeau, Hamilton *et al.*, 1998).

In contrast to the mutation processes that affect simple tandem repeats or microsatellites which arise through replication slippage (Aaltonen, Peltomäki, Leach *et al.*, 1993; Thibodeau, Bren, and Schaid 1993; Parsons, Li, Longley *et al.*, 1993), the mutation processes that arise in minisatellites appear to involve mitotic recombination, either intra-molecular or by unequal exchange between sister chromatids during meiosis (Jeffreys, Bios, Buard *et al.*, 1997). The evidence provided by Hoff-Olsen, Meling and Olaisen (1995) suggests that defective mismatch repair processes may also affect longer repeats which have not been as extensively investigated as microsatellite loci in the context of CRC.

1.6 Microsatellites

1.6.1 Microsatellite instability

Sequences of tandem DNA repeats are known as satellites (Smith, 1976), and are widespread in the human genome and in other organisms such as eukaryotes where they where first discovered (Britten and Kohne, 1968). These are tracts in which a single base or small number of bases are repeated. Repeats of 6 base pairs or fewer are termed microsatellites and generally the length of the stretch is 100 bps or fewer. They appear to change length at frequencies much higher than expected for 'standard' point mutations (Strand, Prolla, Liskay and Petes, 1993). These phenomena were first observed in simple organisms such as yeast and bacteria (Levinson and Gutman, 1987; Strand, Prolla, Liskay and Petes, 1993) The tandem nature of these repeat units is thought to render them prone to slipped-strand mis-pairing (SSM), and hence particularly susceptible to insertion or deletion mutagenesis during replication (Levison and Gutman 1987). Slipped-strand mismatches result in length alteration (deletion or duplication of an integral number of repeat units) of a satellite sequence known as DNA or genomic instability. The integral characteristics of SSM are their occurrence in repetitive regions; that they involve the deletion or duplication of an integral number of repeat units; a bias toward frameshift of a single rather than multiple repeat units, and a proportionate relationship to the length of a satellite (Levison and Gutman 1987). Methyl directed repair mechanisms that could detect and repair single unpaired bases in *Escherichia coli* were discovered in 1986 (Dohet, Wagner and Radman, 1986). These repair mechanisms could recognise and repair over 90% of DNA mismatches in newly

synthesised DNA. When absent in mutator strains of *E. Coli* (*mutL* and *mutS)* increases of frameshift mutation frequency by over 13-fold are observed. Ionov *et al.*. observed such spontaneous errors in colorectal carcinomas in 1993 by a PCR technique using primers whose nucleotide sequence is chosen arbitrarily (Ionov, Peinado, Malkhosyan, Shibata, and Perucho, 1993). They discovered ubiquitous somatic mutations in 12% of colorectal cancers examined. These tumours displayed distinctive genotypic and phenotypic features and they proposed that this subgroup might be caused by mutation in a gene coding for a factor essential for replication fidelity of simple repeat sequences. They noted the propensity for early onset of the tumours that manifested widespread alterations, as well as a high prevalence of tumours in the proximal colon. This led them to believe that these changes were the molecular manifestation of HNPCC. Once genome-wide alterations had been demonstrated in colon cancer, the detection of alterations in a few microsatellite loci could be extrapolated to a general alteration in all such repetitive areas or a mutator phenotype. This phenomenon became dubbed the replication error (RER+) phenotype (Aaltonen, Peltomäki, Leach *et al.*, 1993), and its detection formed the basis for many papers describing 'microsatellite instability' in a variety of tumours (reviewed in Marra and Boland, 1995). In humans the commonest microsatellites are mononucleotide (A_n/T_n) or dinucleotides repeats (CA_n/GT_n) and they are characteristically located in non-coding sequences. The function of these sequences is presently unknown. The length differences in microsatellites are inherited in a stable fashion and that have a high degree of polymorphism. These features make such loci useful in linkage studies, which are done by examining restriction fragment length polymorphisms (RFPLs). Over 2000 such loci have been identified

in the human genome (Gyapay, Morissette, Vignal, *et al.*, 1994) and this number continues to increase.

1.6.2 Replication error

Confirmation of the pivotal role of replication error in HNPCC and a proportion of sporadic colorectal cancers became evident through the observation that nearly 86% of HNPCC associated tumours exhibited RER (Aaltonen, Peltomäki, Mecklin *et al.*, 1994). These alterations were also observed in 16% of sporadic tumours that shared certain features with HNPCC tumours (Thibodeau, Bren and Schaid, 1993). Genetic mapping using linkage analysis with highly polymorphic microsatellite DNA markers first localised a HNPCC susceptibility gene to 2p15-16 (Peltomäki, Aaltonen, Sistonen *et al.*, 1993a). The description of the discovery of mismatch repair genes and their role in HNPCC is given elsewhere. Defects in microsatellites stability have also been studied in other familial colon cancer syndromes (e.g. Muir-Torre syndrome) and extra-colonic tumours associated with HNPCC. The Muir-Torre syndrome is a rare variant of HNPCC in which there is an additional propensity to the development of sebaceous and skin tumours (Cohen, Kohn, and Kurzock, 1991). Widespread microsatellite instability has been observed in about 50% of tumours studied (Honchel, Halling, Schaid, Pittelkow, and Thibodeau, 1994). Sites where extra-colonic cancers develop in HNPCC kindreds include the female reproductive tract, the stomach and urinary tract (Lynch, Smyrk, Watson *et al.*, 1993). Studies of microsatellite instability in these cases have shown a particular association with HNPCC compared with sporadic cancers of the same sites.

RER+ sporadic tumours do not have detectable germline mutations in MMR genes (Liu, Nicolaides, Markowitz, *et al.*, 1995a). This suggests that they either acquire a somatic MMR gene mutation or they possess a hitherto undiscovered MMR gene mutation, both of which have important implications for family members of an individual whose tumour showed this phenotype. Opinions differ as to the usefulness of MSI detection as an adjunct to clinical data in the diagnosis of HNPCC. (Samowitz, Slattery and Kerber, 1995; Jass, Cottier, Jeevaratnam *et al.*, 1995b). Regardless of association with HNPCC, the majority of young patients (35 years or younger) with colorectal cancer exhibit genetic instability with microsatellite marker analysis (Liu, Farrington, Petersen, *et al.*, 1995b). This suggests that the mechanism for carcinogenesis in younger patients differs from that in older patients.

Microsatellite instability has been studied in relation to the adenoma to carcinoma sequence (Jacoby, Marshall, Kailas, Schlack, Harms, and Love, 1995). The acquisition of genetic instability occurs at an early stage of adenoma development in HNPCC and is associated with a greater likelihood of progression to carcinoma. The RER phenotype in adenomas causes a large number of mutations to accumulate rapidly and these mutations continue to accumulate during neoplastic progression. The specific targets that the RER+ phenotype acts upon have not been demonstrated. Rare allelic variants of the H-ras gene are associated with an increased risk of cancer (Krontiris, Devlin, Karp, Robert, & Risch, 1993). It contains a simple minisatellite repeat sequence that might be expected to be vulnerable to mutation in HNPCC patients as a result of DNA mismatch repair. The genetic alterations that are responsible for the RER+ phenotype detected by the study of microsatellite loci may also be responsible for widespread mutations in

almost any site, not necessarily restricted to simple repeats. Thus regions upstream of genes responsible for colorectal cancer progression such as APC, p53, K-ras, and DCC could be affected by defects in mismatch repair resulting in an increase in the mutation rate of these genes and consequent acceleration of carcinogenesis.

There are several problems with the detection of RER+ phenotype by the use of microsatellite markers: first the absence, until recently, of a general consensus as to the number of microsatellite loci that need to be examined to determine RER status and the proportion that are altered such that a RER+ phenotype may be inferred, and, second, that some of the loci examined are not in genes involved in carcinogenesis and therefore instability in these areas is of no consequence. Since microsatellite alleles are short in sequence length (up to 100bps), individual loci may not necessarily manifest DNA instability even though the genome as a whole may be affected. Therefore several microsatellite loci are amplified and analysed per case and if a proportion manifest differences between tumour and normal DNA, then instability or replication error (RER) is inferred. The likelihood of alteration in a microsatellites varies from 55 to 91% in different loci in familial colon cancer, and between 12 and 28% in sporadic colorectal tumours (Aaltonen *et al.*, 1993, Thibodeau, Bren and Schaid, 1993). Therefore the choice of microsatellite loci affects the sensitivity of the RER assay. All leading workers in this field use at least 4 microsatellite markers and designate the RER+ phenotype with abnormality in at least 2 markers (Table 1.2).

Table 1.2 Microsatellite markers and RER phenotype

AUTHOR	Number of microsatellite markers	Minimum number altered markers for RER+	Comment
Liu, B. *et al.*, (1995a)	4	2	HNPCC, 92% RER+
Patel, U. *et al.*, (1994)	9	?	Sporadic, 40% RER+
Ishimari, G. *et al.*, (1995)	8	2	Sporadic, 20% RER+
Thibodeau, S. *et al.*, (1993)	4	?	
Lothe, R. *et al.*, (1993)	7	3	HNPCC, 31% RER+, sporadic 17% RER+
Liu, B. *et al.*, (1996)	4-5	2	<45yrs 58% RER+, >45yrs 17% RER+
Peltomaki, P. *et al.*, (1993b)	7	2	HNPCC 79% RER+, sporadic 13%
Jacoby, R. *et al.*, (1995)	3-17	24%	Different tumours
Parsons, R. *et al.*, (1995)	4	?	RER associated with TGFβ(II) receptor

More recently, international criteria have been developed by the National Cancer Institute for the determination of microsatellite instability (MSI)(Boland, Thibodeau, Hamilton *et al.*, 1998). They considered hitherto published research in the field of microsatellite instability and made several conclusions, now generally adopted. The first was to rationalise nomenclature. Several terms including RER, MIN (microsatellite instability), MMP (mismatch repair phenotype), USM (ubiquitous somatic mutations) were discarded in favour of the term MSI (microsatellite instability). Tumours could then be divided into several groups.

Microsatellite Stable (MSS), where no instability is apparent; MSI-L (low), where only a few markers exhibit MSI and MSI-H (high), where the majority of markers exhibit MSI.

In order to provide uniformity in testing for MSI in CRC, they recommended a panel of 5 microsatellite markers based on reviewed evidence. MSI-H could be designated if 2 or more markers (out of 5) were altered. The markers selected were those known to be accurate in detecting MSI in tumours with an underlying defect in mismatch repair due to mutation in *hMLH1* or *hMSH2*. MSI-L could be interpreted where one marker was altered and MSS where none were altered (Table 1.3). This 'working reference panel' was not intended to replace existing validated markers but to act as a method of comparing new markers in the field. A further nineteen markers were also recommended where problems are encountered or additional information is required (Table 1.3). The alternative loci are recommended for use where differentiation between MSI-L and MSS is important such as where an attenuated phenotype may be present.

Table 1.3

Reference panel and alternative loci for MSI testing recommended by the National Cancer Institute (Boland, Thibodeau, Hamilton *et al.*, 1998).

Reference panel		Alternative loci	
Marker	Repeating unit	BAT40	BAT34C4
BAT25	Mononucleotide	TGF-β-RII	ACTC (635/636)
BAT26	Mononucleotide	D18S55	D18S58
		D18S64	D3S1029
D5S346	Dinucleotide	D10S197	D13S175
D7S123	Dinucleotide	D17S588	D5S107
D17S250	Dinucleotide	D8S87	D18S69
		D13S153	D17S787
		D7S519	D20S100

Evidence for the indications for testing for MSI were reviewed by the National Cancer Institute in 1997 to form the 'Bethesda guidelines' (Rodriguez-Bigas, Boland, Hamilton *et al.*, 1997). Apart from individuals from families that meet the Amsterdam criteria, testing is also recommended in the following situations:

a) Individuals with 2 HNPCC related cancers or metachronous and synchronous CRC.

b) Individuals with CRC who have a first-degree relative diagnosed with HNPCC related neoplasia.

c) Individuals with CRC or endometrial cancer diagnosed at less than 45 years of age.

d) Individuals with CRC in the right colon with an undifferentiated histopathological pattern diagnosed less than 45 years of age.

e) Individuals with CRC with a signet-ring cell type diagnosed at age less than 45 years.

f) Individuals with adenomas diagnosed at age less than 40 years.

Table 1.4 Frequency of the replication error phenotype in human cancers

Tumour Type	RER %, (n)	Study
Familial (HNPCC)		
Colon (adenocarcinoma)	79 (14)	Aaltonen *et al.*, 1993
	86 (29)	Aaltonen *et al.*, 1994
Colon (adenoma)	57 (14)	Aaltonen *et al.*, 1994
Endometrial	75 (4)	Risinger *et al.*, 1993
Sporadic		
Colon (adenocarcinoma)	13 (46)	Aaltonen *et al.*, 1993
	16 (49)	Aaltonen *et al.*, 1994
	12 (137)	Ionov *et al.*, 1993
	28 (90)	Thibodeau *et al.*, 1993
	16 (241)	Lothe *et al.*, 1993
	13 (137)	Kim *et al.*, 1994
Colon (adenoma)	3 (33)	Aaltonen *et al.*, 1994
Endometrial	17 (36)	Risinger *et al.*, 1993
	23 (30)	Burks *et al.*, 1994
	20 (45)	Duggan *et al.*, 1994
Blast crisis CML	74 (19)	Wada *et al.*, 1994
Pancreatic	67 (9)	Han *et al.*, 1993
Skin (squamous cell)	50 (10)	Zaphiropolous *et al.*, 1994
Skin (sebaceous tumours)*	46 (13)	Honchel *et al.*, 1994
Lung (small cell)	45 (33)	Merlo *et al.*, 1994
(non-small cell)	34 (38)	Shridhar *et al.*, 1994
	2 (87)	Peltomaki *et al.*, 1993
Stomach	39 (57)	Han *et al.*, 1993
	18 (33)	Peltomaki *et al.*, 1993
	31 (52)	Rhyu *et al.*, 1994
Renal cell	25 (36)	Uchida *et al.*, 1994
Oesophageal (adenoma)	22 (36)	Meltzer *et al.*, 1994
Oesophageal (squamous)	2 (42)	Meltzer *et al.*, 1994
Ovary	16 (19)	Han *et al.*, 1993
Cervix	15 (13)	Han *et al.*, 1993
Bladder	3 (200)	Gonzalez-Zulueta *et al.*, 1993
	21 (61)	Orlow *et al.*, 1994
Breast	0 (84)	Lothe *et al.*, 1993
	4 (26)	Han *et al.*, 1993
	11 (104)	Wooster *et al.*, 1994
	20 (20)	Yee *et al.*, 1994
Testis	0 (86)	Lothe *et al.*, 1993
		Murty *et al.*, 1994
Prostate	3 (40)	Schoenberg *et al.*, 1994
Liver	3 (29)	Han *et al.*, 1993

% = percentage of tumours that exhibit at least one shifted focus
n = number of cases analysed CML = chronic myeloid leukaemia *Muir-Torre syndrome

1.7 Chromosome Alterations In Colorectal Cancer

The majority of colorectal cancers do not exhibit defects in DNA replication error leading to an increase in the rate of spontaneous mutation (Orr-Weaver and Weinberg, 1998). Therefore the large number of mutations seen in sporadic cancers cannot be explained by the predicted rate of spontaneous mutations seen in non-germline cells (Loeb, 1991). Loeb (1991) proposed that an early step in tumour progression must be the development of a mutator phenotype in which the genomes of pre-malignant cells are more hypermutable than normal cells. This process hastens each rate-limiting step in tumour progression. In contrast to MSI + cancers that tend to be euploid (normal chromosome complement), most colorectal cancers have abnormal chromosome number (aneuploidy) and loss of heterozygosity (LOH) at many genetic loci suggesting an alternative mechanism in driving tumour progression than defective mismatch repair (Orr-Weaver and Weinberg, 1998). Aneuploidy is associated with poor prognosis compared with diploid or near diploid tumours (Rognum, Thorud and Lund, 1987) but there are no differences in tumour differentiation or pathological stage (Armitage, Robins, Evans, Turner, Baldwin and Hardcastle, 1985). Aneuploidy is the result of instability of the chromosome (Lengauer, Kinzler and Vogelstein, 1997). During mitotic cell division chromosomes are allocated to one of either mitotic spindles. During this process the chromosomes become attached to the spindles and this process is monitored by a mitotic checkpoint (Orr-Weaver and Weinberg, 1998). Failure of the checkpoint results in unequal allocation of chromosomes during cell division and leads to aneuploidy. The mutations that result in the disruption of the cell-division checkpoint have recently been identified in colorectal cancer cells (Cahill,

Lengauer, Yu *et al.*, 1998). Their normal protein products bind to the kinetochore, a specialised region of the chromosome and cause metaphase arrest if stable binding of the microtubular part of the mitotic spindle has not occurred (Orr-Weaver and Weinberg, 1998).

1.8 DNA mismatch repair

DNA replication is a highly accurate process following which there are less than one error per million replicated nucleotides (Lindahl, 1994). Post-replication mechanisms exist in human cells that recognise and repair these errors or 'mismatches', and defects in them result in widespread genetic instability. Mismatch repair refers to the cellular capability of recognizing abnormal base pairs and correcting the sequence on one to retrieve a normal A-T or G-C pairing. The activity was also found to correct stretches of unpaired bases that result from insertion or deletion of nucleotides on one of the two DNA strands (Modrich, 1991). HNPCC tumours and a proportion of sporadic colorectal cancers contain numerous insertions and deletions in microsatellite sequences, a phenomenon known as microsatellite instability (Aaltonen, Peltomäki, Leach *et al.*, 1993). This increase frequency of acquired mutations is characterised as the replication error phenotype (RER+) and is thought to develop early in tumour progression (Parsons, Li, Longley *et al.*, 1993). Similar instability had already been found in association with several inherited neurological and neuromuscular disorders such as the fragile X syndrome and Huntingdon's Chorea (Martin, 1993) where a genetic instability produces an expansion in trinucleotide repeats.

However, in colorectal cancer, a different mechanism was found to exist. DNA repair mechanisms have previously been well recognised in bacteria such as *Escherichia coli* (Modrich, 1991) and the yeast *Saccharomyces cerevisiae* (Reenan and Kolodner, 1992). During DNA replication, DNA polymerases often misalign newly synthesised DNA templates creating small 'loop-outs' of unpaired bases that are recognised and repaired by mismatch repair enzymes. The enzymes are the

specific gene products of the mismatch repair genes mutS, mutL and mutH that are thought to act as a complex (Modrich, 1991). Mutation in any of these genes results in an increased rate of frameshifts, base substitutions and recombinations (Radman, Matic, Halliday, and Taddei, 1995). Similar observations of such widespread genetic phenomena in mismatch repair deficient organisms and the tumours of HNPCC patients led to the prediction that a defect in the integrity of DNA mismatch repair mechanisms was responsible for carcinogenesis in HNPCC.

In *Escherichia coli*, the mutS protein recognizes newly created DNA strands by differences in methylation between the template and the new strand and binds directly to mismatched DNA. It then recruits mutL protein to form a complex that aligns the mismatched DNA with appropriate DNA sequences for excision by an endonuclease coded for by the mutH gene. This endonuclease incises specifically at hemimethylated GATC sequences in DNA removing an extensive section of DNA between the mutH incision site and the mismatch. (Modrich, 1991). Gap filling by DNA polymerase III and joining by DNA ligase complete the repair process (Lindahl, 1994).

1.9 Mismatch Repair Gene Mutations

Patients with a genetic predisposition to colorectal cancer were noted to have a reduced capacity for DNA repair synthesis in peripheral blood leukocytes, in 1983 (Pero, Miller, Lipkin *et al.*, 1983). DNA repair deficiencies had already been implicated in other inherited predispositions to cancer such as Xeroderma Pigmentosum, Bloom's syndrome, ataxia telangectasia and Fanconi's anaemia (Setlow, 1978). Until 1993 the search for molecular pathogenesis of HNPCC focussed mostly on proposed mutations in oncogenes and tumour suppressor genes (Peltomäki, Sistonen, Mecklin et *al.*, 1991), already integral to the adenoma-to-carcinoma as described by Fearon and Vogelstein (1990a). Several significant events occurred in 1993; linkage analysis established a locus for HNPCC in two large kindreds to chromosome two (2p15-16), (Peltomäki, Aaltonen, Sistonen *et al.*, 1993a) by using a panel of microsatellite markers covering the entire genome and confirmed the autosomal dominant mode of inheritance of the condition. The same group compared tumour DNA from individuals with HNPCC to those with sporadic tumours and found widespread alterations in short repeat DNA sequences (microsatellite instability) suggesting an alternative mechanism to that previously envisaged and coined the term 'replication error (RER+) phenotype (Aaltonen, Peltomäki, Leach *et al.*, 1993). They reinforced this theory when they found that none of the HNPCC tumours showed no loss of heterozygosity and the incidence of mutations in k-ras, p53 and APC were the same in both groups. This coincided with Thibodeau (Thibodeau, Bren and Schaid, 1993) and Ionov (Ionov, Peinado, Malkhosyan, Shibata, and Perucho, 1993) who found that a significant proportion of tumours of the proximal colon exhibited microsatellite instability, and they

correlated this with survival and, inversely, with loss of heterozygosity. A second locus predisposing to inherited colorectal cancer, this time, to chromosome three (3p) was also found by linkage analysis using restriction fragment length polymorphisms (RFPLs) and microsatellite markers the same year (Lindblom, Tannergård, Werelius, and Nordenskjöld, 1993). Defects in the *Escherichia coli* MutHLS repair pathways were known to result in destabilisation of tracts of simple repetitive DNA (Strand, Prolla, Liskay and Petes, 1993) similar to microsatellite instability in HNPCC tumours and this led to the discovery of genes in humans homologous to those in yeast and bacteria. The human homologue of the hMSH2 (mutS homolog) gene was discovered by PCR amplification using oligonucleotide primers designed to target the amino acid sequences in the most conserved sequences of the known MutS homologs (Fishel, Lescoe, Rao *et al.*, 1993). The amplified sequences were then cloned and used to read a cDNA library that localised the gene to 2p22-21, close to the area previously discovered by linkage (Peltomäki, Aaltonen, Sistonen, *et al..*, 1993a). This work was almost simultaneously duplicated by Leach *et al..*, (Leach, Nicolaides, Papadopoulos *et al.*, 1993b), and the discovery of a second gene soon followed, this time, hMLH1, localised to 3p21-23 and homologous to the MutL MMR gene in *Escherichia coli* (Bronner, Baker, Morrison *et al.*, 1994; Papodopoulos, Nicolaides, Wei *et al.*, 1994). Eight other MutL homologues (hPMS1-8) and a mutS homologue (*MSH6*, GTBP) have since been identified, though not all have been directly implicated in disease (Palombo, Gallinari, Iaccarino, *et al..*, 1995; Nicolaides, Papodopoulos, Liu, *et al..*, 1994). Three somatic mutations of hMSH6 have been described in sporadic colorectal carcinomas and in one case of HNPCC. (Miyake, Konishi, Tanaka *et al.*, 1997).

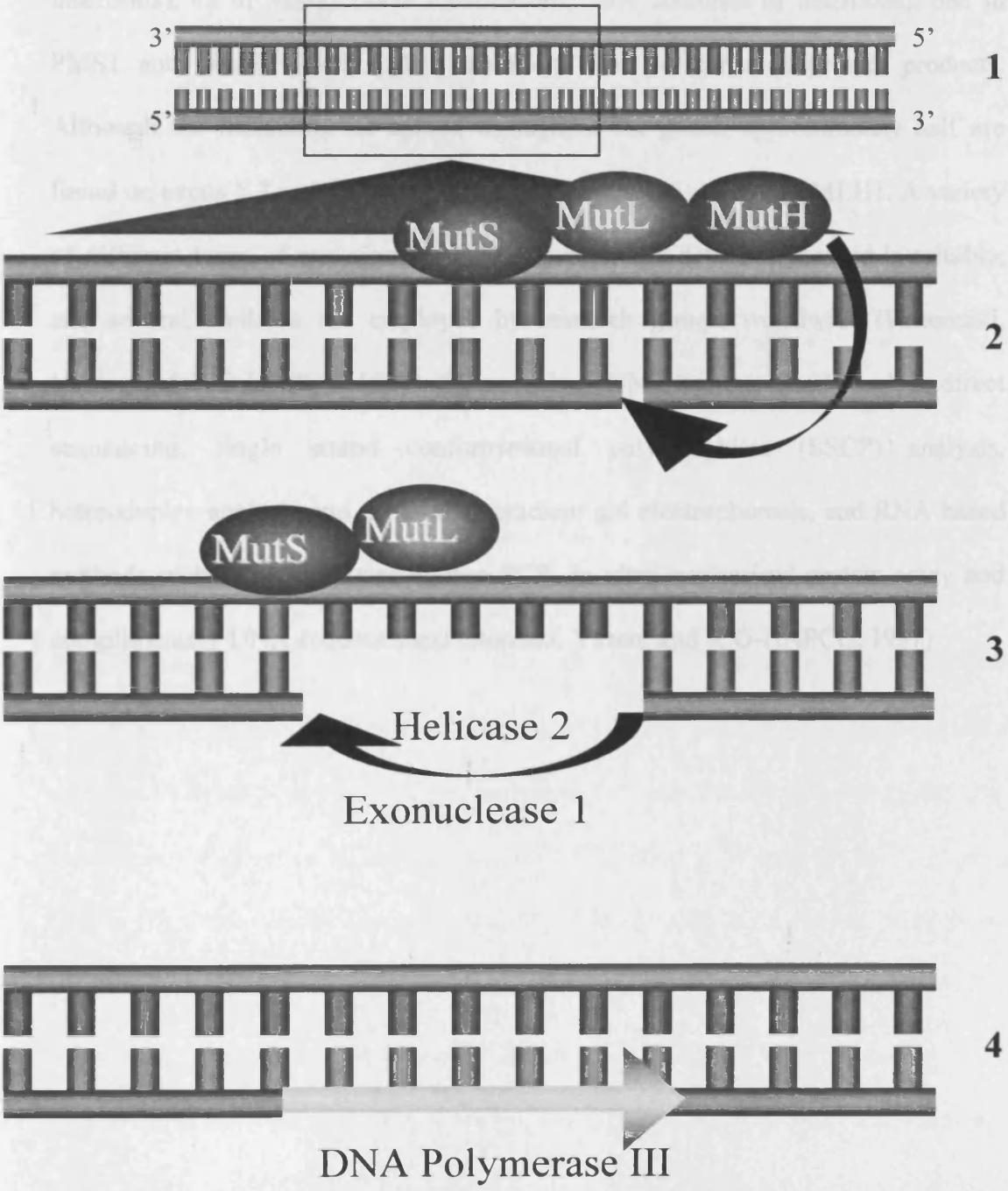
Table 1.5

MMR genes, their locations and the proportions of detectable mutations in HNPCC cohorts.

Gene	Chromosome	Apparent contribution to HNPCC (Liu *et al.*, 1996)
HMSH2	2p21-22	31%
hMLH1	3p21	33%
HPMS1	2q31-33	2%
HPMS2	7p22	4%

In humans, mismatch repair the MutS homologues hMSH2 and GTBP form a heterodimeric complex that binds to mismatches, and MutL homologues hMLH1 and hPMS2 form a similar complex that participates in MMR (Li and Modrich, 1995). There is no MutH homologue in humans and repair of the correct strand appears to be directed by nicks in the newly created strand (Figure 1.4)(Li and Modrich, 1995).

Figure 1.4 Schematic diagram of mismatch repair. The enzymes involved in the recognition, excision and repair of DNA mismatches. Repair begins with the recognition of the mismatch (1, green base, 3'-5' strand) by Mut S followed by the formation of a complex with MutL (2). The binding of the MutS-MutL complex close to the mismatch activates an endonuclease (MutH), which creates a nick close to the mismatch (3). Subsequently the mismatch is excised and replaced by the appropriate portion of DNA created by a DNA polymerase (4).



The International Collaborative Group on HNPCC has established a database of hitherto discovered DNA mismatch repair gene mutations, which has accumulated 126 predisposing mutations (Peltomäki, Vasen, and ICG-HNPCC, 1997). 74 mutations were in MLH1 (67% single base substitutions, 33% deletions or insertions), 48 in MSH2 (44% substitutions, 56% deletions or insertions), one in PMS1 and two in PMS2. Most mutations lead to truncated protein products. Although the mutations are spread throughout the genes, approximately half are found on exons 5,7 and 12 of MSH2 and exons 9,13,16, and 19 of MLH1. A variety of different types of mutation are found so no single detection method is suitable, and several methods are employed by research groups worldwide (Peltomäki, Vasen, and ICG-HNPCC, 1997). These include DNA based methods such as direct sequencing, single strand conformational polymorphism (SSCP) analysis, heteroduplex analysis and denaturing gradient gel electrophoresis, and RNA based methods such as reverse transcriptase PCR, in vitro synthesised protein assay and complimentary DNA sequencing (Peltomäki, Vasen, and ICG-HNPCC, 1997).

1.10 Mismatch Repair and Cancer

Defects in mismatch repair alone are not sufficient to cause cancer. Study of the non-neoplastic lymphoblast cells in HNPCC patients reveal deficiencies in MMR yet no increase in their malignant transformation (Parsons, Li, Longley, *et al.*., 1995a) and the incidence of tumours in some non-colonic sites is not increased above the expected considering the elevated rate of mutation at these sites. The overall increase in DNA damage resulting from MMR defects could enhance the likelihood of inducing mutations in tumour suppressor genes and oncogenes thereby increasing the rate of tumourigenesis. This hypothesis is reinforced by the finding that mutS and mutL may be important in restoring G-T mispairs that result from 5-methylcytosine deamination (Lieb, 1987). In 5 of the 6 mutational hotspots in the p53 gene contain G-C sites where the cytosine is methylated to 5-methylcytosine. Deamination of 5-methylcytosine changes this modified base to thymine forming the G-T mispair (Rideout, Coetzee, Olumi and Jones, 1990). It is also possible that certain genes such as the Transforming Growth Factor β receptor gene (TGFβRII) which is involved in cell growth and replication are particularly prone to replication defects in the absence of mismatch repair (Markowitz, Wang, Myeroff, *et al.*., 1995). This study found that the majority of RER+ colon cancer cell lines have mutations in the TGFβRII gene that were localized to mononucleotide (A_{10}) microsatellite sequences. It was also found that TGFβRII gene expression was normal in RER- cell lines. These findings have been subsequently confirmed by others (Parsons, Myeroff, Liu *et al.*, 1995b; Lu, Akiyama, Nagasaki, Saitoh and Yuasa, 1995). TGFβRII gene mutation results in failure of the receptor to bind TGFβ which has potent tumour suppressor activity and the affected cells lose their

responsiveness to growth suppression. HNPCC patients with CRC have a high rate of mutations in the TGFβRII gene. In one study comparing tumours from HNPCC patients to those from sporadic cases, 63% of the HNPCC tumours compared to 10% of the sporadic cases had frameshift mutations in TGFβRII. APC and K-RAS mutation rates were the same in both groups (Tannergård, Liu, Weger Nordenskjöld, and Lindblom, 1997).

1.11 Hereditary Non-Polyposis Colorectal Cancer

1.11.1 Historical background

The possibility of a link between cancer and genetics was noted as early as 100 AD in the Roman literature with a familial clustering of breast cancer (Lynch, Lynch & Albano 1988a). In the 1860s Broca wrote about a link between breast cancer and gastrointestinal cancer, and its transmission through his wife's family in several generations (Lynch, Lynch & Albano 1988a). Aldred Warthin, a physician and pathologist in 1895 in Michigan performed a meticulous documentation of his seamstress's extended family in which cancer was widespread (Warthin, 1913). This study on what became known as "Warthin's Family G" was followed subsequently for more than 75 years by other researchers (Lynch and Krush, 1971). They found, in a family of descendants now numbering nearly 650 that cancer, predominantly adenocarcinoma of the colon and endometrium, was transmitted in an autosomal dominant fashion, that individuals affected had an unusually early age of onset, and there was an increased incidence of other primary malignant neoplasms. They found no physical, biochemical or cytogenetic phenotypes comparable to other inherited cancer predisposition syndromes such as Gardner's syndrome. This work together with observations also made by Lynch in 1966 (Lynch, Shaw, Magnuson *et al.*, 1966) on two large Midwestern kindreds led to the term "cancer family syndrome" to describe the condition and formed the basis for the study of countless other cancer families world-wide (Lynch, Lynch, Albano *et al.*, 1988a).

1.11.2 Lynch syndromes

In 1985 Lynch refined the criteria for the diagnosis of affected families according to clinical and pathological criteria (Lynch, Rozen and Schuelke, 1985). He delineated two clinical variants known as the Lynch syndromes. Lynch syndrome I, characterised by an autosomal dominant predisposition to colonic cancer with early age of onset, a predominance to right sided and multiple colonic cancers, and Lynch syndrome II, with the same features, but in addition, an excess of adenocarcinomas in other sites, particularly the endometrium and ovary. By 1985 the term hereditary non-polyposis colorectal cancer (HNPCC) had come into use (Lynch, Kimberling, Albano *et al.*, 1985). At this time there existed little information on the incidence, prevalence, natural history and clinical expression of the condition due to the lack of a distinguishable phenotype and a paucity of well-documented HNPCC families (Vasen Mecklin, Meera-Khan and Lynch, 1991a). These problems led to the formation of the International Collaborative Group on HNPCC (ICG-HNPCC).

1.11.3 The Amsterdam Criteria

30 Leading experts in the condition met in Amsterdam in August 1990 to agree on a structure and workings of the group. In three subsequent sessions they discussed the possibility of collaboration for future studies, developed a uniform minimum data sheet and data collection form. They established genetic diagnostic criteria, agreed on terminology and gave recommendations for screening (Vasen, Mecklin, Meera-Khan and Lynch 1991b). The term 'cancer family syndrome' was discarded in

favour of 'HNPCC' and the diagnosis Lynch syndrome I or II reserved for families with pedigrees of sufficient size such that they could be accurately be conferred. The group agreed on minimum criteria for the diagnosis of HNPCC that became known as the Amsterdam Criteria:

1. At least three relatives should have histologically verified colorectal cancer; one of them should be a first degree relative to the other two. Familial adenomatous polyposis should be excluded.
2. At least two successive generations should be affected.
3. In one of the relatives colorectal cancer should be diagnosed less than 50 years of age.

For studies on linkage, natural history and tumour spectrum, more extended pedigrees were needed. The diagnosis of HNPCC, like other common genetic conditions is confounded by environmental factors (particularly diet), chance clusterings and heterogeneity in penetrance (Kinzler and Vogelstein, 1996).

1.11.4 Incidence of HNPCC

Estimates of the frequency of HNPCC vary since investigations into incidence until recently have been hindered by the lack of an accepted definition until the Amsterdam criteria and the absence of an easily recognizable phenotype. The Amsterdam criteria exclude extracolonic malignancies and small families are not likely to meet the criteria. Therefore estimates of the frequency of HNPCC using the Amsterdam criteria are likely to be low and in such studies range from 3.4% of CRC (Ponz de Leon, Sassatelli, Benatti and Roncucci, 1993) in Italy, to 1-2.6% in

Northern Ireland (Kee and Collins, 1991), 4% in England and 0.7% in Finland with adherence to Amsterdam criteria with 2.4% in putative HNPCC families (Mecklin, Järvinen, Hakkiluoto, Hallikas, Hiltunen, Härkönen, *et al.*, 1995). Variation also occurs within countries. In Italy, Modica *et al.* (Modica, Roncucci, Benatti, Gafa, Tamassia and Dardanoni, 1995) found HNPCC accounted for 3-5% of CRC in Northern Italy and less than 1% in Southern Italy, though this may be partly explained by interactions between the environment and genetic susceptibility factors.

1.11.5 Gene penetrance

In studies of large HNPCC kindreds between 71 and 79% of gene carriers are affected at a mean age of 47 years (standard deviation 10 years) (Bailey-Wilson, Elston, Schuelke, Kimberling, Albano, Lynch *et al.*, 1986). HNPCC kindreds additionally have the expected frequency of sporadic CRC cases with 4% affected by 80 years.

1.11.6 HNPCC phenotype

The colorectal and extra-colonic manifestations of HNPCC vary according to the type of mismatch repair gene mutation in a kindred (Lin, Shashidharan, Ternent, Thorson, Blatchford and Christensen *et al.*, 1998). Apart from the expected differences observed between cases of CRC in HNPCC kindreds and sporadic CRC

of younger mean age of onset and a higher proportion of proximal cancers, there were also differences according to genotype. Kindreds with HMSH2 mutations had proportionately more rectal cancers and extracolonic cancers than those with HMLH1 mutations. (Lin *et al.*, 1998).

1.11.7 Adenomas in HNPCC

Several colonoscopic studies of HNPCC families have shown that adenomas form with the same frequency as the general population, but at an earlier age (Table 1.6). Adenomas are the precursors of cancer in HNPCC as in sporadic cases (Love, 1986). The majority of cancers diagnosed in HNPCC patients have associated adenomas (Lynch, Smyrk and Jass, 1995). In addition to an earlier age of onset there is evidence that HNPCC associated adenomas were more likely to have high grade dysplasia and undergo malignant transformation than sporadic adenomas (Jass and Stewart, 1992). There is good evidence that removal of adenomas from HNPCC patients is effective in reducing the incidence of CRC in this group (Sankila, Aaltonen, Jarvinen and Mecklin, 1996) when compared to a control group with HNPCC who refused screening.

Table 1.6 Adenomas in HNPCC: Colonoscopic screening of first degree relatives.

Study	Number screened	Number with adenomas	Comments
Love, 1984	42	7 (17%)	Includes 8 with previous CRC
Lanspa *et al.*, 1992	55	8 (14.5%)	
Green *et al.*, 1995	61	7 (11.5%)	Younger age of adenoma diagnosis
Vasen *et al.*, 1995	388	33 (8.5%)	Excess villosity and high-grade dysplasia
Jarvinen *et al.*, 1995	133	22 (16.5%)	

1.11.8 Age of Cancer Diagnosis in HNPCC

The average age of onset of CRC in HNPCC is 44 years but within such kindreds there is a wide range (Van der Water, Jeervaratnam, Browett, Stewart, Lane, and Jass, 1994), the youngest reported case being 13 years old (Faragher, Cox and Stevenson, 1993). Therefore not all cases of CRC in HNPCC families will develop at a young age. There may also be cases of sporadic cancer in HNPCC kindreds. CRC in young patients, regardless of HNPCC status appears to be the result of mechanisms different to that in older cases (Liu, Nicolaides, Markowitz *et al.*, 1995a). The majority exhibit the RER+ phenotype implying defective replication fidelity with only a few (5 out of 12) possessing mutations in known MMR genes.

1.11.9 The Proximal colon

Sporadic proximal colonic cancers exhibit biological properties that distinguish them from distal colon and rectal cancers (Bufill, 1990). Sporadic RER+ and HNPCC CRCs share a predilection for the proximal colon and share overlapping characteristics (Table 1.7)(Kim, Jen, Vogelstein and Hamilton, 1994; Järvinen, Mecklin and Sistonen, 1995).

Table 1.7 Features of CRC subgroups compared with all CRC

	All CRC	Right colon	HNPCC	RER+
Age	65-70	70	40-50	60
Sex	M>F	F>M	M=F	M=F
Proximal location	30%	-	70%	94%
RER+	10-15%	29%	86-92%	-
Poorly differentiated	10%	10%	39%	53%
Mucinous	10-20%	20-30%	15-40%	35%
Crohn's-like reaction	20-28%	10-36%	42%	47%

Crohn's like reaction: lymphoid aggregates in the vicinity of the tumour.

1.11.10 Histology of HNPCC

In the normal mucosa of patients within HNPCC kindreds, there appears to be an increase in the gradient of the normal regional proliferative pattern from proximal to distal (Patchett, Alstead, Saunders, Hodgson and Farthing, 1997). There is a higher rate of colonic mucosal proliferation as measured by whole crypt mitotic count in the proximal colon as compared to the distal colon and this gradient is doubled in HNPCC suggesting a possible factor to explain the development of right-sided cancer in these patients (Patchett *et al.*, 1997). There do not appear to be any

histological or mucin histochemical changes in the normal non-neoplastic mucosa of HNPCC patients (Love, Gilchrist and Morrissey, 1985). Similarly, analysis of the background rate of mutational activity in the normal colorectal mucosa of HNPCC subjects shows no abnormalities (Jass and Edgar, 1994).

There is an excess of poorly differentiated, mucinous signet ring cancers in HNPCC however since these features are commonly found in sporadic CRC, they are not pathognomonic (Mecklin and Järvinen, 1986) (Table 1.7).

1.11.11 Extracolonic tumours in HNPCC

The commonest affected site after the colon and rectum in HNPCC families is the endometrium (Watson and Lynch, 1993). The other sites with significantly more than expected cancers are the stomach, small bowel, hepatobiliary system, kidney/ureter and ovary. The distribution among HNPCC kindreds of extracolonic cancers appears to be heterogeneous for cancers of the endometrium and upper renal tract and homogenous for the remainder (Watson and Lynch, 1993). The extent of heterogeneity however is no longer thought to be sufficient to sub-divide HNPCC, as Lynch originally postulated in to two syndromes according to the sites affected by cancer in a HNPCC kindred (Watson and Lynch, 1993).

1.11.12 Diagnosis and Screening

A family history that fits with The Amsterdam Criteria is the best predictor of the presence of a mutation (Nystrom-Lahti, Wu, Moisio, Hofstra, Osinga, Mecklin *et al.*, 1996). Families whose history does not fit the criteria have an extremely low frequency of mutations in mismatch repair genes (Wijnen, Meera Khan, Vasen *et al.*, 1997). Mismatch repair gene mutations are not responsible for those cases which present with multiple colonic adenomas who do not have APC gene mutations or a family history suggestive of HNPCC (Beck, Tomlinson, Homfray, Frayling, Hodgson and Bodmer, 1997). For isolated cases of CRC that present at a young age, mutation analysis yields conflicting results. Dunlop Farrington, Carothers *et al.* (1997) found a relatively high rate of mutations (22%) in a group of patients diagnosed before 36 years of age whilst Yuan *et al.* (1998) found only 2% in a cohort under 45 years of age with CRC. Although only molecular diagnosis by direct causative mutation analysis is of clinical value, useful indicators may be provided by testing for replication error phenotype (Jass *et al.*, 1995, Dunlop *et al.*, 1997), or by testing for the absence of hMLH1 or hMSH2 protein by immunohistochemistry (Kim Piao, Kim *et al.*, 1998). DNA mismatch repair deficient tumours may be reliable and practibly detected by using HMLH1 and hMSH2 immunohistochemistry (Marcus, Madlensky, and Gryfe *et al.*, 1999).

Currently colonoscopy is used to screen at-risk individuals with possible HNPCC (Järvinen, Mecklin and Sistonen, 1995). In this controlled study over 10 years there was a reduction in the rate of CRC by 62% in the group observed by colonoscopy with polypectomy when polyps were detected. There were no deaths from CRC in the treated group. Their recommendation was that screening should begin at 20 to

25 years of age and continue at three yearly intervals. Others groups take the view that known gene carriers should undergo colonoscopy every 1 to 2 years until 35 and then annually thereafter (Lynch and Smyrk, 1996). This is based on evidence that there is an unexpectedly high level of interval cancers and distal adenomas on conventional screening programs suggesting that HNPCC accelerates the adenoma-to-carcinoma sequence (Vasen, Nagengast and Meera-Khan, 1995). When a cancer or large polyp is diagnosed, sub-total colectomy is recommended. Screening by colonoscopy asymptomatic first-degree relatives of affected individuals with HNPCC provides a significant yield of 15% neoplasms with 3% carcinomas (Green, Chapman, Burn, Bishop and Varma, 1995) (Table 1.8).

Table 1.8 Current recommendations for HNPCC screening by the ICG-HNPCC (http://www.nfdht.hl/guidelines.htm)

Site	Procedure	Lower age limit (yrs)	Interval (years)
Colon	Colonoscopy	20-25	2
Endometrium (+ ovaries)	• Gyn. Examination • Transvaginal sonography • Ca-125	30-35	1-2
Stomach*	Gastroscopy	30-35	1-2
Urinary tract*	Sonography Urine analysis	30-35	1-2

*Only if it runs in the family

1.11.13 Muir-Torre Syndrome

Originally categorised separately from HNPCC (Muir, Bell and Barlow, 1967, Torre, 1968), this condition is now known to be caused by the same mutations as HNPCC (Hall, Murday, Chapman *et al.*, 1994). It is characterised by neoplasms of sebaceous glands and internal cancers, mainly affecting the colon and rectum, as with HNPCC (Cohen, Kohn and Kurzrock, 1991).

1.11.14 Prophylactic surgery in HNPCC

Carriers of germline HNPCC mutations are currently recommended to conisder prophylactic sub-total colectomy when colorectal adenomas or cancer are found (DeCosse, 1995). They should be counselled as to the natural history of HNPCC, particularly the penetrance of the mutation and potentially rapid rate of cancer progression, the limitations and risks of surveillance colonoscopy, and the possible

sequelae of surgery. They must be told that they will require life-long evaluation of their rectal segment following subtotal colectomy. Rectal cancer developed in approximately 11% of 71 patients a median of 158 months following subtotal colectomy in one study (Rodriguez-Bigas, Vasen, Pekka-Mecklin, Myrhøj, Rozen, Bertario *et al.*, 1997). The significant risk factors after multivariate analysis were age of first procedure and whether or not the patient was under surveillance. This reinforced the view that life-long yearly endoscopic follow up of the rectal stump should take place after colectomy. The 10 year cumulative risk of metachronous cancer is 40% if the first cancer is not treated by sub-total colectomy (Lynch, Lanspa, Boman *et al.*, 1988c). There is no clear evidence to recommend prophlactic colectomy in mutation carriers who have no evidence of adenomas but given the 80% lifetime risk of developing CRC, it should be considered as an option, especially if there are technical difficulties in performing a complete colonoscopy. Female patients should be also counselled with regard to prophylactic total hysterectomy and bilateral salpingo-oophorectomy as an alternative to lifelong gynaecological surveillance, particularly where colonic resection is being planned (Burke, Petersen, Lynch *et al.*, 1997).

1.12 *Treatment Of Colorectal Cancer*

1.12.1 Introduction

Despite more recent advances in other modalities such as chemotherapy and radiotherapy, the mortality following treatment of colorectal cancer has not changed over the last 30 years and surgical resection remains central to the curative treatment (CRC factsheet 18.3, 1993, Abcarian, 1992). The mortality of newly diagnosed CRC is approximately 50% and 85% of local recurrences are clinically evident within 2 years (Abcarian, 1992). Phillips *et al.,* (1984), in The British Large Bowel Cancer Project analysed the surgical pathology and its relationship to outcome in CRC. Of 4583 cases of adenocarcinoma of the large bowel, 82% underwent resection of the primary of which two-thirds (2518) were deemed curative. The factors influencing survival were:

i. Depth of tumour invasion. Dukes' stage A patients had better survival than those with Dukes' stage B.
ii. Cell differentiation. Those with poorly differentiated tumours had a worse prognosis compared to those with well or moderately well differentiated tumours.
iii. Vascular invasion. This feature was also an independent prognostic variable.
iv. Lymph node metastases. The outcome in this group, as with Dukes' A and B also varied according to whether the primary tumour invaded the full thickness of the bowel wall.

v. Location of positive lymph nodes. Involvement of the apical lymph node (Dukes' stage C2) was associated with poorer prognosis than those with no apical node involvement (Dukes' stage C1).

vi. Number of lymph nodes involved. Four or less involved lymph nodes were also an independent prognostic variable.

The British Large Bowel Cancer Project also followed 4200 cases following resection for two years and determined the factors associated with local recurrence (Phillips *et al.*, 1984b and c):

i. Younger patients had a higher rate of local recurrence than older ones.

ii. Patients with rectal cancer treated by anterior resection had a higher rate of local recurrence than those treated by abdomino-perineal resection. The greater the length of the distal tumour margin in anterior resections the lower the local recurrence rate.

iii. Obstruction, perforation and tumour fixation were all associated with higher incidence of local recurrence.

iv. There appeared to be a wide variation in local recurrence between different surgeons from 5 to 30%.

1.12.2 Colonic resection for cancer

The importance of surgical technique has long been recognized in the surgical resection of CRC (Turnbull, Kyle, Watson & Spratt, 1967). The recognition of cancer cell dissemination in the portal venous blood by tumour manipulation during

surgery led to the formulation of the no-touch isolation technique. The lympho-vascular pedicle is first isolated and divided; the colon is then divided at the elected sites of resection and the cancer-bearing segment is removed last (Turnbull *et al.*, 1967). The vascular pedicle is ligated at a level to allow wide excision of the lymph node bearing mesocolon (Abcarian, 1992). The location of the tumour in the colon determines the ligation of the major vascular pedicle, the extent of the resection and the type of anastomosis (Abcarian, 1992). Opinion differs as to the extent of resection required to achieve a curative lymphadenectomy. In a prospective randomized French study no differences in either immediate outcome or long-term survival were found for patients who had either a segmental resection or a left hemicolectomy for left colonic carcinoma (Rouffet, Haye and Vacher *et al.*, 1994).

1.12.3 Rectal cancer

The anatomical situation of the rectum within the pelvis and its relations create a surgical challenge that separates the curative excision of rectal cancer from that of cancer of the colon. The optimum form of surgical treatment for rectal cancer remains a topic of debate around two areas:

1. The role of sphincter preservation versus the need for permanent colostomy in low rectal cancer.
2. The extent of pelvic dissection in order to enhance survival and minimise local recurrence against the need to preserve autonomic (bladder and sexual) function.

The first reliably curative operation for rectal cancer was described by Ernest Miles, a surgeon from St Mark's Hospital, London (Miles, 1908). Based upon studies which found that rectal cancer could spread upwards, downwards and laterally, he described an operation with a combined abdominal and perineal approach where the entire rectum, anal canal and sphincters, most of the levators and ischiorectal fat, the sigmoid colon and mesocolon, and the pelvic peritoneum, were all removed. It was not until the 1930s that Miles' view of the spread of rectal cancer was challenged (Dukes, 1930) and the technique of anterior resection of the rectum with anastomosis via the abdominal route was described from the Mayo clinic (Dixon, 1939). Numerous studies have since established that anterior resection is as effective as abdomino-perineal for cancers in the upper rectum (Lockhart-Mummery, Ritchie and Hawley, 1976) and it is favoured by patients (Williams and Johnston, 1982). This has led, together with the widespread availability of modern stapling instruments and the recognition that a shorter distal margin is adequate

(Williams, Dixon and Johnston, 1983), to the increasing adoption of sphincter preserving procedures for cancers in the middle and lower thirds of the rectum. Sphincter saving procedures can now be accomplished without detriment to local recurrence for more than three-quarters of cancers less than 5 centimetres from the anal verge (Heald, Smedh, Kald, Sexton and Moran, 1997). The major problem following curative excision of the rectum for cancer is that of local recurrence.

The recognition that tumour deposits occur in the mesorectum distal to the distal palpable edge of the tumour in the rectum (Heald, Husband and Ryall, 1982), and the role of circumferential margin involvement in the local recurrence of rectal cancer (Quirke, Durdey, Dixon and Williams, 1986) has led to total mesorectal excision (TME) being widely adopted after reports of lower local recurrence rates (Heald and Karanjia, 1992) for this technique. The goal of TME is the complete excision of the rectum and mesorectum by careful, sharp dissection, as an intact unit thereby removing the primary tumour and its loco-regional field of spread enveloped by the visceral layer of the pelvic fascia. The disadvantage of TME compared with conventional anterior resection with mesorectal transection is a higher rate of anastomotic failure and the need, therefore, for a temporary defunctioning stoma (Karanjia, Corder, Holdsworth and Heald, 1991).

1.12.4 Hepatic Resection for metastases from colorectal cancer

Over half the patients with colorectal cancer will develop liver metastases (Taylor, 1996). Untreated, the prognosis is poor with only the occasional survivor (Stangl, Altendorf-Hofmann, Charnley and Scheele, 1994). Surgery has become increasingly accepted in the last decade with 5-year survival figures of 22-44% in those in whom resection is feasible (Logan, Meier, Ramming, Morton & Longmire,1982, Scheele, Stangl and Altendorf-Hofmann, 1990, Sugihara, Hojo, Moriya, Yamasaki, Kosuge & Takayama,1993). Since only 5-20% of patients with liver metastases are resectable (Hartley and Poston, 1994), accurate preoperative imaging is vital to exclude those with unresectable disease from surgery, and to plan radical surgery in the remainder (Moran, O'Rourke and Rees, 1995). Providing that extra hepatic disease has been excluded and a radical resection can be achieved preserving sufficient remaining liver to avoid hepatic failure, there are no absolute contraindications to resection (Rees, Plant and Bygrave, 1997). Tumour size and differentiation, resection margin and the proportion of liver volume replaced by tumour all significantly affect prognosis following curative liver resection (Rees, Plant and Bygrave, 1997, Scheele and Altendorf-Hofmann, 1996).

1.12.5 Adjuvant systemic chemotherapy

Given that a high proportion of cases of CRC are classified as curatively resectable at presentation, yet only 50% survive to 5 years, much effort has been directed toward the identification of those at high risk of recurrent disease, and the determination of the most appropriate form and mode of administration of chemotherapeutic agents, together with agents designed to enhance their potency (Buyse, Zelenuich-Jacquotte, Chalmers, 1988). Early large randomized controlled trials failed to demonstrate survival benefits in patients treated with chemotherapy following curative surgery but meta-analysis of such trials revealed a marginal 5-year survival advantage in groups treated with 5-fluorouracil (5-FU) 3.4% better than patients treated by surgery alone with a reduction in the odds of death ratio of 17% (P=0.03) (Buyse, Zelenuich-Jacquotte, Chalmers, 1988). Lack of sufficient detail in individual trials resulted in failure to define possible advantages to certain subgroups such as colon versus rectal tumours and with different disease stage (Buyse, Zelenuich-Jacquotte, Chalmers, 1988). These early trials encouraged the use of combination chemotherapy and in 1984, Windle *et al.* reported a benefit from the use of 5-FU and levamisole (Windle, Bell and Shaw, 1984). In 1988 Wolmark *et al.* (1988) reported the results of the National Surgical Adjuvant Breast and Bowel Project (NSABP) on 1166 patients with Dukes' B or C carcinoma of the colon who had undergone curative resection. The were randomized into three groups: surgery alone; surgery followed by chemotherapy using 5-fluorouracil (5-FU), semustine (methyl-CCNU, a nitrosurea) and vincristine, and inoculation of live *Mycobacteria* (autologous tumour Bacille Calmette Guérin vaccine, BCG). The rationale for incorporating semustine, vincristine and BCG in addition 5-FU had been established

by previous randomized studies of their use in the adjuvant setting, though not always in humans (Corbett, Griswold, Roberts, Peckham & Schabel 1977; Malvigit, Gutterman, Malahy *et al.*, 1977). These appeared to demonstrate superior results in terms of survival and disease free interval. Wolmark *et al.* Demonstrated a significant prolongation of disease free interval and increase in 5-year survival from 59% in the control group to 67% in the chemotherapy group (Wolmark, Fisher, Rockette *et al.*, 1988). There was failure to complete chemotherapy in 29% due to adverse symptoms and three cases of semustine-related leukaemia. The results in those treated with BCG were inconclusive.

Levamisole, an antihelminthic drug has been in use for some time but it became of interest in the treatment of cancer when it was found to have immunostimulatory properties (Laurie, Moertel, Fleming *et al.*, 1989). In combination with 5-FU, levamisole has been found to be effective in delaying recurrence in Dukes'B_2 and C cancers, and in increasing survival in Dukes' C cancers. This work was confirmed by another trial in patients with Dukes' B_2 and C cancers. 5-FU and levamisole decreased the recurrence rate in Dukes' B_2 from 20% to 14% at three years although this did not reach significance and the recurrence rate by 41% and the death rate by 33% in Dukes' C cancers after a median follow up of 3 years (Moertel, Fleming, Macdonald *et al.*, 1990). This study led the National Cancer Institute (NCI) in the United States to recommend that 5-FU and levamisole be standard adjuvant treatment for all patients who had undergone curative resection for Dukes' stage C colorectal cancer. The interim and final follow up data from this study in 1992 continued to show a maintenance of the benefit to those randomized to 5-FU and

levamisole (Moertel, Fleming, MacDonald, Haller and Laurie, 1992; Moertel, Fleming, MacDonald *et al.*, 1995).

Leucovorin (Folinic acid) potentiates the effect of Fluorouracil by stabilisation of its metabolite. Combination therapy with the two agents has been shown to be of benefit in advanced colorectal cancer in terms of tumour response rates (The Advanced Colorectal Cancer Meta-Analysis Group, 1992). A large Canadian and European trial of nearly 1500 patients has shown a 22% reduction in mortality in those treated with high dose 5-FU and folinic acid and this effect predominated in Dukes' C cancers (IMPACT trial, 1995). The survival advantage of 5-FU and folinic acid also appears to be superior to previous effective regimes (Wolmark, Rockette, Fisher *et al..*, 1993). After three years the mortality in a control group treated with semustine, vincristine and fluorouracil was 32% compared with 23% in those given fluorouracil and leucovorin. There appears to be no benefit to 12 month *versus* 6-month regimes of 5-FU and folinic acid (O'Connell, Laurie, Shepard *et al..*, 1996).

The efficacy of leucovorin *versus* levamisole as potentiators of 5-FU has also been studied and leucovorin appears to be marginally better than levamisole in terms of survival and disease free survival (Wolmark, Rockette, Mamounas *et al.*, 1996). Provisional results appear to show that the addition of levamisole to 5-FU and folinic acid does not appear to confer additional benefit (Haller, Catalano, Donald and Macer, 1996). Overall, adjuvant chemotherapy regimes with 5-FU and levamisole or leucovorin appear to be of benefit in terms of survival in Dukes' stage C colon cancer but this has not been demonstrated universally and the optimum

dose and combination remains uncertain. Their role in Dukes' stage B is also in some doubt.

An ongoing trial (QUick And Simple And Reliable, QUASAR) in the United Kingdom under the aegis of the UKCCR is comparing treatment with 5-FU plus high or low dose folinic acid, with or without levamisole (UKCCR QUASAR Protocol, 1994).

1.12.6 Adjuvant Chemotherapy by Portal Vein Infusion

Lack of evidence that systemic chemotherapy was of conclusive benefit led for the search for more direct means of administration. Early reports of the use of cytotoxics infused directly into the portal vein were based on the knowledge that the majority of recurrent CRC is found in the liver. In a randomized controlled trial, Taylor *et al.* (Taylor, Machin, Mullee, Trotter, Cooke and West, 1985) commenced a one week regime of portal vein 5-FU infusion with heparin at the time of resection of the primary and found that the treated group had fewer liver metastases and prolonged survival. This benefit appeared to be confined to Dukes' B colon cancers, and was not seen in Dukes' C or rectal cancers. Results in subsequent trials, however, have not universally repeated this success. The Swiss Group for Clinical Cancer Research found a 21% reduction in disease recurrence using 5-FU, mitomycin C and heparin (Swiss Group for Clinical Cancer Research, 1995). Another group found a 16% survival increase in Dukes' C carcinomas treated by portal vein 5-FU infusion following resection when compared to those treated by surgery alone, but no benefit in other groups (Fielding, Hittinger, Grace and Fry, 1992). Other groups have failed to demonstrate a survival benefit in similar studies of portal vein infusion of 5-FU (Beart, Moertel, Wieand *et al.,* 1990 and Wolmark, Rockette, Wickerham *et al.,* 1990). The recent long-term follow up data from the NSABP (Wolmark, Rockette, Petrelli *et al.,* 1994) does, however show a reduction in death at five years and incidence of liver metastases.

More recently a meta-analysis of nine randomised trials of portal-vein infusion showed a 13% reduction in risk of death in patients with Dukes' B or C cancers (Slevin, 1996).

The current AXIS (Adjuvant X-ray and 5-FU infusion study) is the largest colorectal cancer trial ever conducted in Europe. It was launched in 1989 by the UK Co-ordinating Committee on Cancer Research (UKCCR) to investigate the possible benefits of adjuvant therapy with intra-portal 5-FU and preoperative or postoperative radiotherapy on colon or rectal cancers (AXIS Steering Group, 1994).

1.12.7 Adjuvant Therapy for Rectal Cancer

The anatomical characteristics of the rectum and pelvis distinguish the rectum from the colon in terms of the more limited scope for curative resection and its greater suitability for radiotherapy. The use of radiotherapy as an adjunct to curative surgery has long been advocated but by no means is generally accepted as obligatory (Baslev, Pedersen, Teglbjaerg *et al.*, 1986). The Gastrointestinal Tumour Study Group in 1985 showed that neither postoperative radiotherapy nor 5-FU alone improved survival (GITSIG, 1985), but that a combination of the two did appear to be of benefit in patients who have had curative resection of rectal cancer with involvement of the rectal fat (Dukes' B2) or regional nodes (Dukes' C), or both. The combination of two modalities however resulted in considerable toxicity with a high dropout rate.

More recently a trial that randomized patients to post-operative radiotherapy alone or with 5-FU and methyl-CCNU (Krook, Moertel, Gunderson *et al.*, 1991). Both local recurrence and survival rates were improved in the group with combination treatment but there was no control group of surgery alone and the local recurrence rates as in other studies was high (13.5%, combination treatment group, 25%, radiotherapy alone). Radiotherapy alone has been evaluated in pre-operative and post-operative settings with short and long courses. In 1986 a group from Copenhagen, Denmark presented the results of a randomized trial of a seven week course of radiotherapy commencing thirty days after surgery for Dukes' B and C carcinomas of the rectum and recto-sigmoid (Baslev *et al.*, 1986). They found no benefit to survival or distant metastases in either Dukes' B or C cancers but a

reduction in the local recurrence rate in Dukes' C cancers. The local recurrence rates were high (22% post radiotherapy, 28% no radiotherapy) for Dukes C cancers suggesting inadequate local clearance at the time of surgery. In the same year an American group presented the results of a randomized trial of pre-operative radiotherapy for cancer of the rectum (Higgins, Humphrey, Dwight, Roswit, Lee, & Keehn *et al.*, 1986). Males with rectal cancer requiring abdomino-perineal resection were randomized to receive radiotherapy and surgery or surgery alone. Again, no differences were seen in survival but the local recurrence rate appeared to be lower for the irradiated group.

Meta-analysis of trials of radiotherapy for rectal cancer show a small but non-statistically significant survival benefit to those receiving radiotherapy in addition to surgery, especially in cases resected with the hope of cure (Buyse, Zeleniuch-Jacquotte and Chalmers, 1988).

Wolmark NSABP R-02 The same group conducted a study of Dukes' B and C rectal cancers that were randomized to surgery alone, postoperative chemotherapy by the same regimen as for the colon study, or radiotherapy alone.

1.12.8 Chemotherapy for Advanced Disease

The outcome for patients with surgically incurable disease is poor with median survivals of between 8 and 12 months (Machover, 1997). 5-FU was first introduced in 1958 (Curreri, Ansfield, McIver, Waisman and Heidelberger, 1958) and remains the most commonly used single agent though the optimal route and combination for administration remains uncertain (Machover, 1997). The most impressive biomodulatory drug used with 5-FU is leucovorin. A meta-analysis of 9 trials comparing the use of 5-FU alone and 5-FU in combination with leucovorin revealed a significant benefit in terms of tumour response rate from 11% to 23%, though there was no improvement in survival (Advanced Colorectal Cancer Meta-Analysis Project, 1992). The use of high dose methotrexate with leucovorin appears to result in a marginal, but significant increase in survival of up to 2.8 months (Poon, O'Connell, Wieand *et al.*, 1991) when compared with 5-FU alone, with comparable side effects.

1.13 Statement Of Aims

The aim of this study was to re-evaluate existing methods for analysis of the replication error phenotype in the primary colorectal tumours from patients with sporadic colorectal cancer and those related to hereditary non-polyposis colorectal cancer. Those suspected of having HNPCC were to be tested for the presence of mutations in DNA mismatch repair genes. Multivariate repeat unit mapping by the polymerase chain reaction (MVR-PCR) was to be applied to the tumour-normal tissue pairs of such cases to determine whether this could be an alternative method for the detection of DNA instability. Non-radioactive MVR-PCR was to be evaluated as a simpler, cheaper and safer method than those described before.

2.1 Tissue Collection and DNA Extraction

2.1.1 Tissue collection and storage

i. *Sporadic colorectal adenocarcinoma tissue specimen collection.*

(Appendix 1)

At the time of laparotomy for histologically verified colorectal adenocarcinoma, the excised tissue was taken immediately from the theatre to the sluice where it was opened along the wall of the specimen opposite the tumour where possible. The specimen was washed briefly in saline solution. A small piece of tumour was removed from the luminal aspect taking care to avoid important tumour margins. A strip of macroscopically normal mucosa was dissected from the sub-mucosa at least 5 cms away from the tumour from the same specimen. Both tissue samples were placed in separate labelled cryo-tubes and snap-frozen immediately in liquid nitrogen. The specimens were then transported in liquid nitrogen and placed in a –80°C container freezer for storage.

ii. *Wax blocks* (Appendix 1)

Since 1978, in The North Hampshire Hospital, Basingstoke, UK, all patients with a diagnosis of colorectal adenocarcinoma have been prospectively entered on to a database. This has collected the following information:

- Patient demographics
- Type of operation
- Dates of death and/or recurrent disease (including site)
- Pathology

- Post-operative complications and mortality

Several wax blocks from the tumours and normal tissues of each case were made available for analysis. They were examined macroscopically by a consultant pathologist (C. DuB.) to select blocks that obviously contained exclusively tumour tissue or normal tissue in order to evaluate DNA extraction methods.

In order to facilitate macroscopic visual segregation of tumour and normal tissues to determine the effectiveness of extraction techniques on an initial cohort, Dukes' stage C and 'D' stage tumours were excluded. By this means, lymph node blocks could be used to extract 'normal' DNA because they by definition contained no tumour tissue. Out of 25 Dukes stage A and B specimens, only one could not be effectively separated into tumour and normal blocks by inspection and was therefore excluded. It was intended that, once DNA extraction had been successfully carried out, for the remaining blocks to be sectioned and stained with Haematoxylin and Eosin to identify tumour and normal tissue with a microscope and allow dissection of each block to remove tumour and normal tissue in separate sections.
For the initial cohort of Dukes stage A and B cases, five 30 micron sections were taken from each block of normal and tumour tissue for DNA extraction and placed in a cryo-tube.

2.1.2 DNA Extraction

i. *DNA extraction from frozen tissue specimens*

Approximately 2-3mm^3 of tissue sample was digested overnight in 500µl of phosphate buffered saline (PBS, Sigma, UK) with 100µl of Proteinase K (200 µl/ml, ICN, UK) and 0.5% N-Lauroyl Sarcosine (BDH, UK) at 55 °C. An equal volume of water saturated phenol (Lancaster synthesis, UK) was added, then mixed and centrifuged at 13 000 rpm for 3 minutes in a bench top centrifuge (MSE Microcentaur). The aqueous phase was transferred to a new tube and this step repeated once with phenol, and once with chloroform (BDH, UK). The aqueous phase was aliquoted and transferred to a new tube and twice the volume of ice cold 100% ethanol with Sodium acetate (1%, 3.5M) added. The tubes were refrigerated at -80°C for greater than one hour and centrifuged at 13 000 rpm for 15 minutes. The supernatant was discarded. The pellets were gently washed with 100µl of 70% ethanol, left to air dry and finally re-suspended in 150µl of TE Buffer (Tris and EDTA, appendix 2).

ii. *DNA extraction from paraffin wax embedded tissue sections*

1. 10 Wax embedded 10µm thick sections of tissue from a microtome were placed in a labelled Eppendorf tube.
2. 400µl of xylene was added, the mixture was agitated in a vortex, then incubated at room temperature for 10 minutes and centrifuged (Microcentaur) for 5 minutes at 13000 rpm.

3. The waste supernatant was then discarded to a xylene waste bottle, another 400µl of xylene was added, and the mixture was agitated and spun as in step 2 (above).
4. 400µl of 70% ethanol was then added, the mixture was shaken and spun in the centrifuge for 5 minutes at 13000 rpm. The waste supernatant was then discarded.
5. Step 4 was repeated.
6. The washed pellet in the bottom of the Eppendorf was then left to air dry in a incubator at 37 °C for 10 minutes.
7. 250µl of Proteinase K (200µl/ml) with N Lauroyl Sarcosine (0.5%) was added to the tube containing the dried pellet.
8. The mixture was then incubated at 55 °C in a heated water bath for 3 hours and then agitated in a vortex to dissolve. If the pellet was still not dissolved at this stage, further Proteinase K was added and the mixture was incubated again for one hour.
9. 250µl of phenol at 65 °C was added, the mixture was agitated in a vortex and then centrifuged for 10 minutes at 13000 rpm.
10. The top aqueous phase was carefully poured out into a clean Eppendorf tube.
11. An equal volume of a mixture of Phenol, Chloroform and Indole Acetic acid (IAA, ratio 25:24:1) (Sigma, UK) was added to the new tube.
12. The contents of the tube were mixed by hand and spun in the centrifuge for 5 minutes at 13000 rpm.
13. Step 10 was repeated.
14. An equal volume of Chloroform was added.
15. Step 12 was repeated.

16. Step 10 was repeated.
17. A volume twice that in the tube of 100% ethanol and 1% Sodium Acetate was added and the mixture placed in a -80 °C freezer for 30 minutes, then spun in the centrifuge for 20 minutes at 13000 rpm.
18. The ethanol/sodium acetate supernatant was removed and the pellet was gently washed with 50µl of 70% ethanol (stored at -20 °C).
19. The ethanol supernatant was removed and the pellet air-dried at room temperature for 20 minutes.
20. The pellet was finally re-suspended in 100µl of TE (appendix 2).

iii. *DNA extraction from peripheral blood.*

Ten millilitres of a sample of peripheral venous blood were pipetted into a 50 ml centrifuge tube, to which 10 ml of phosphate buffered saline (PBS, Sigma, UK) was added to wash the blood. The mixture was inverted several times and spun at 3000rpm for 10 minutes in a centrifuge (Jouan GR422). The supernatant was discarded and the residue re-suspended in 2ml of PBS. 200µg per ml of Proteinase K (ICN, UK) with 0.5% Lauroyl Sarcosine (BDH, UK), and the mixture was vortexed, then incubated at 55°C for 3 hours until lysis of the pellet occurred. An equal volume of water-saturated phenol (Lancaster synthesis, UK) was added, the mixture was vortexed, then spun in the centrifuge for 10 minutes. The supernatant was removed to a fresh tube and the previous step repeated if the supernatant was not clear at this stage. An equal volume of chloroform (BDH, UK) was added to the

supernatant and the mixture was vigorously vortexed and then spun in the centrifuge at 300rpm for 10 minutes. The supernatant was removed and discarded, and twice the volume of 100% ethanol with 0.35M Sodium Acetate was added. The mixture was inverted several times and placed in a -80°C freezer for 30 minutes, and then spun at 3000rpm for 30 minutes in a centrifuge. The supernatant was removed and the pellet was carefully washed with 1ml of 70% ethanol, which was then removed. The pellet was air-dried at 37°C for 10 minutes and re-suspended in 1ml of TE (appendix 2)

2.2 *DNA Validation*

2.2.1 DNA yield

DNA yield was checked on a 0.6% agarose gel (appendix 3) by horizontal electrophoresis at 80 mAmp (Scotlab, UK), with a Lambda Hind marker (Promega, UK). The markers, in all cases are provided with blue/orange loading dye (Promega, UK) which is used for loading DNA samples into gel electrophoresis wells and tracking migration during electrophoresis. The dye contains xylene cyanol FF which migrates at approximately 4kb, bromophenol blue at approximately 300bp and Orange G at approximately 50bp in a 0.5% to 1.4% agarose gels in 0.5X TBE.

2.2.2 DNA quality

i. *Glutaraldehyde-phosphate dehydrogenase (GAPDH) PCR*

Quality was confirmed by control PCR for a fragment of the Glutaraldehyde-phosphate dehydrogenase (GAPDH) gene. This is a ubiquitously expressed gene whose detection by PCR provides a useful method for the qualitative assessment of DNA extraction (Ercolani, Florence, Denaro & Alexander, 1988). The PCR reagent and conditions are as follows:

0.2 mM dNTPs (Dinucleoside triphosphates, Pharmacia Biotech, UK, see appendix 3), 1 x PCR buffer (Biogene Ltd), 1.5 mM $MgCl_2$, 2% DMSO (Dimethyl sulfoxide, Sigma, UK), 450mM of forward and reverse primer specific for the GAPDH gene (appendix 4), 0.1units Bio/Polythermase^{TM} and 0.5µl genomic DNA. Sterile water was added to each mix in a 0.5ml microfuge tube to make up a final volume of 25µl. Tubes were placed in an automatic thermal cycler (Techne) and denatured for 3 minutes at 94°C followed by 35 repeated cycles of 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 30 seconds. This was followed by a final elongation step at 72°C for 2 minutes. A negative PCR reaction control (using sterile water in place of genomic DNA) was used in all reactions.

10µl of each product was separated with 3µl of bromophenol loading dye by 8% polyacrylamide gel electrophoresis (PAGE, see appendix 3) vertically at 40 mAmp (Sigma, UK).

ii *CYP2D6 PCR*

CYP2D6 is another locus whose amplification by PCR provides another means of assessing DNA extraction (Gough, Miles, Spur & Moss, 1990).

Further validation of the quality of DNA extraction from wax blocks was determined by PCR amplification of the CYP2D6 locus using the E3 and I4 primers (appendix 4).

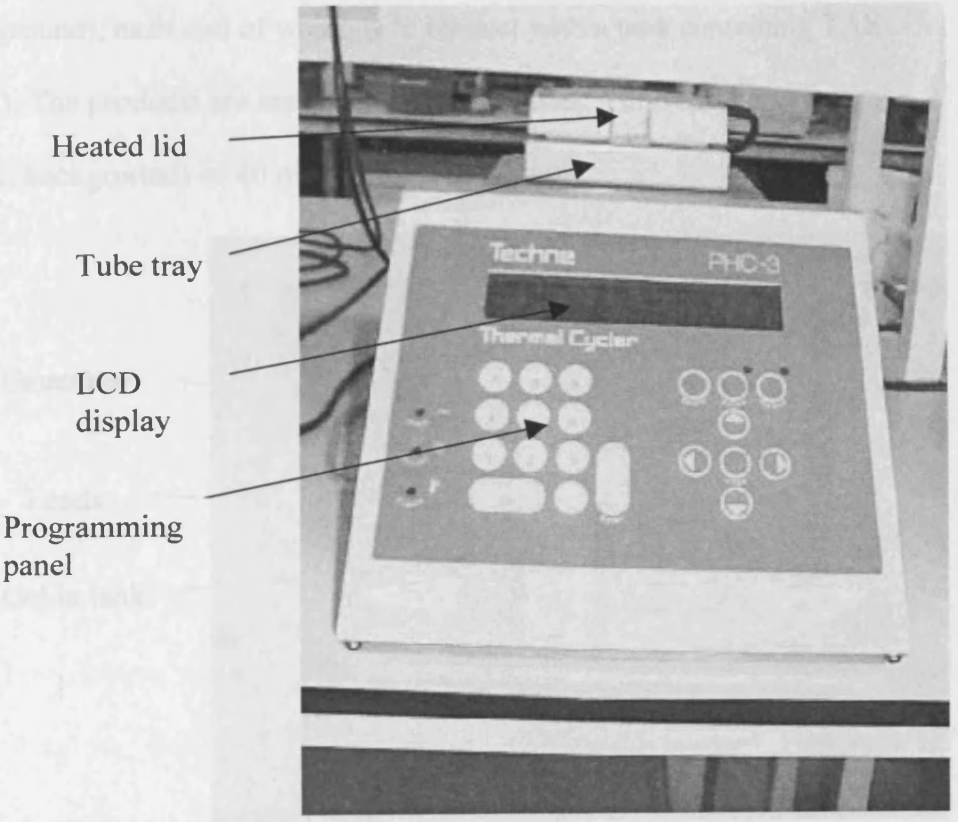
The PCR mixture constituents per reaction were as follows:

DMSO	2%(1µl)
$MgCl_2$	4µl
Buffer A	5µl
Primer (E3 & I4)	1µl x 2
DNTP	1µl x 4
Taq polymerase	0.04µl
dH_20	34µl
Total volume	50µl

The PCR mixture was placed in an automatic thermal cycler (Techne, UK, Figure 2.1) using the following conditions:

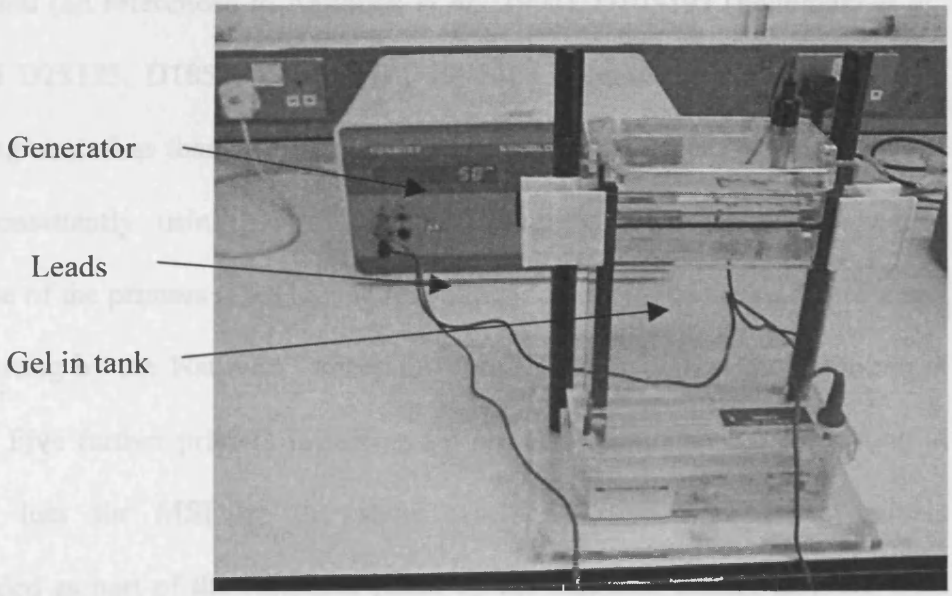
One cycle at 94^0C for 3 minutes, 35 cycles of 94^0C for 30 seconds, 55^0C for 30 seconds and 72^0C for 30 seconds followed by a single cycle at 72^0C for 30 seconds.

Figure 2.1 A Techne Thermal Cycler. Conditions for PCR are programmed in on the front panel and Eppendorf tubes mounted in the heating tray on top. A heated lid is used to compress the tubes and ensure even heating. The lid also prevents evaporation of PCR reagent and obviates the need for oil in the tubes.



10µl of each product was separated with 3µl of bromophenol loading dye by 8% polyacrylamide gel electrophoresis (PAGE) (Figure 2.2).

Figure 2.2 Polyacrylamide Gel Electrophoresis (PAGE) between two glass plates (foreground), each end of which is in contact with a tank containing TAE (appendix 2). The products are separated vertically using a current (generator, Scotlab, UK, background) of 40 mAmp for 2 to 3 hours.



2.3 Microsatellite analysis

2.3.1 Primers and conditions for RER analysis

i. *Primer selection*

Replication analysis was carried out by PCR amplification of 10 di-nucleotide repeat microsatellites: D5S404, D7S519, D8S255, D11S904, D13S175, D15S120, D17S787 and (all referenced in Aaltonen *et al.*, 1994), D10S197 (Peltomaki *et al.*, 1993), and D2S123, D18S58 (Liu *et al.*, 1995a). Primers were chosen so as to amplify fragments less than 180 base pairs in size because larger fragments did not amplify consistently using DNA templates prepared from paraffin-embedded tissues. One of the primers (D2S123) is recommended as part of the reference panel for MSI testing by the National Cancer Institute (Boland, Thibodeau, Hamilton *et al.*, 1998). Five further primers in this group are also recommended for testing at alternative loci for MSI by the same working group. The other primers recommended as part of the reference panel by the National Cancer Institute were not used as this consensus was only achieved after this project had been carried out.

Table 2.1 PCR conditions for microsatellite analysis. See appendix 5 for primer sequences.

MICROSATELLITE MARKER	Primer amount (ng)	[$MgCl_2$] (mM)	[DMSO] (%)	Units bio/poly-thermase	Amount target (µl)	Final volume (µl)	Annealing temp (^{O}C)	Expected product size (bp)
D2S123	75	2.5	1	0.1	0.5	25	60	197-227
D5S404	75	2.75	0	0.1	0.5	25	60	180-198
D7S519	75	2	0	0.1	0.5	25	56	256-268
D8S255	75	2.75	0	0.1	0.5	25	56	107-129
D10S197	75	2	0	0.1	0.5	25	65	161-173
D11S904	75	2	0	0.1	0.5	25	58	185-201
D13S175	75	2	0	0.1	0.5	25	60	101-113
D15S120	75	2	0	0.1	0.5	25	60	121-145
D17S787	75	2.75	0	0.1	0.5	25	65	138-166
D18S58	75	2.5	2	0.1	0.5	25	53	144-160

The PCR conditions were as follows:

As according to Table 2.1 with 0.2 mM dNTPs, 1 x PCR buffer (Biogene), the tubes were placed in an automatic thermal cycler (Techne, Figure 2.1) and denatured for 3 minutes at 94°C followed by 35 repeated cycles of 94°C for 30 seconds, annealing at 58°C for 15 seconds and extension at 72°C for 15 seconds. This was followed by a final elongation step at 72°C for 2 minutes. A negative PCR reaction control using sterile water in place of genomic DNA was used in all reactions.

2.3.2 Separation and visualisation of PCR products

5µl of each product was separated immediately on an 8% polyacrylamide gel by electrophoresis (PAGE) for about 2 hours at 40 mAmp in a vertical axis with 3µl of bromophenol loading dye (appendix 6) and a Hinf I ψX174 marker (Promega, UK, Table 2.2). The gel was then removed and stained with ethidium bromide and photographed under ultraviolet excitation in a dark room with a Polaroid camera.

2.4 Multivariant repeat unit mapping (MVR-PCR)

2.4.1 MVR-PCR 1

i. *The MS31A locus*

Repeat unit specific primers for human minisatellite locus MS31A (D7S21) were used Neil & Jeffreys, 1993). This polymorphic locus contains 20 base pair repeats with 405bp of flanking. MS31A is separated from an adjacent minisatellite, MS31B by a 15bp segment (Armour, Wong, Wilson, Royle & Jeffreys, 1989b). It has an allele length heterozygosity of 98% and all the length variation at D7S21 is due to variation in the repeat copy number at MS31A. The organisation of locus D7S21 is shown in appendix 10.

ii. *Principles of the MVR-PCR I*

The two MS31A repeat units differ only by either a G nt (31-TAG-G) or A nt (31-TAG-A) at their 3' terminus (Neil & Jeffreys, 1993). 31-TAG-A and 31-TAG-G are variant repeat unit specific oligonucleotides (see appendix 7 for sequences, design modified from Neil & Jeffreys, 1993) that terminate at this site. Their 19-nt repeat sequence is preceded by a 20-nt 5' synthetic non-minisatellite extension identical to the TAG amplimer (appendix 7). A third amplimer, 32D, primes from the flanking DNA (appendix 7). Two PCR reactions then proceed with either 31-TAG-A or 31-TAG-G to generate two stable sets of products. The PCR reaction in each case proceeds as follows (Jeffreys *et al.*, 1991b):

1. At low concentration 31-TAG-A will anneal to about one a-type repeat unit per target minisatellite molecule and extend into the flanking DNA.

2. 31A primes from the flanking DNA creating a sequence with an end complimentary to TAG
3. Low concentrations of 31-TAG-A prevents excessive internal priming of these product `which would result in authentic but shorter products.
4. Using high concentrations, 31A and TAG now amplify efficiently to create a stable set of products extending from the flanking DNA to each a-type repeat unit.

(See figure 1.3 for a schematic representation of the process)

iii. *Titration of conditions for MVR-PCR I*

To optimise conditions for PCR, standard concentrations of primers, dinucleotide triphosphates (DNTPs), *Taq* polymerase (Biogene ltd) and genomic DNA were used and the concentrations of $MgCl_2$ and dimethyl sulfoxide (DMSO) were varied (Table 2.2). Each PCR reaction contained:

2.5µl Buffer A (PCR buffer)

100ng genomic DNA

1µM 31 TAG

1µM 31A

20nM 31-TAG-A or G

0.4µl *Taq* polymerase (Biogene ltd)

0.5 µl DNTP x 4 (A,C,G,T)

$MgCl_2$ 0.5µl, 1.0µl, 1.5µl, 2.0µl, or 2.5µl

DMSO none or 2%(0.5µl)

15µl H_2O

Total PCR reaction volume 25µl, 10 x 2 reactions = 20 microcentrifuge tubes.

Table 2.2 Quantities of DMSO and $MgCl_2$ for titration of MVR-PCR I.

The numbers in the table represent the identification number for each different amount of reactant above.

	No DMSO	0.5 % DMSO
0.5 µl $MgCl_2$	1	2
1.0 µl $MgCl_2$	3	4
1.5 µl $MgCl_2$	5	6
2.0 µl $MgCl_2$	7	8
2.5 µl $MgCl_2$	9	10

iv. *Dilutions of Stock Primers*

The desired quantities of primer per reaction were achieved by dilution of stock primers to the desired concentrations (Table 2.3). The volume of primer stock to add per reaction was calculated as follows:

$$\frac{[P_2] \times V_R}{[P_1]} = \text{volume of primer to be added per reaction}$$

$[P_1]$ = concentration of stock primer (µM)

V_R = volume of the PCR reactant (25µl)

$[P_2]$ = desired concentration of primer in the PCR reaction (µM)

Table 2.3 Stock and reaction concentrations for MVR-PCR I

Primers	Stock concentrations (μM)	Desired reaction concentration	Dilution factor
31-TAG	18.5	1μM	1:1.77
31-A	17.7	1μM	1:1.85
31-TAG-A	9.6	20nM	1:48.2
31-TAG-G	7.2	20nM	1:36.15

v. *PCR conditions for MVR-PCR I in an automatic thermal cycler (Techne)*

One cycle of 94 °C for 3 minutes (denaturing), 62 °C for 30 seconds and 72 °C for 1.5 minutes, 35 cycles of 94 °C for 30 seconds, 62 °C for 30 seconds (annealing) and 72 °C for 1 minute (extension). Finally, a single cycle of 3 minutes at 72°C elongation. The first cycle, which includes an annealing stage, allows the flanking primer (31A) and specific primer to operate and generate sequences extending from the flanking site to each repeat unit site. These products are then amplified by the further 35 cycles having expended the repeat unit specific primer at the first stage to generate a stable set of products by amplification using the flanking primer and the TAG amplimer, which are at high concentrations.

vi. *PCR product separation and visualisation*

5□l of each product was separated immediately on an 8% polyacrylamide gel by electrophoresis (PAGE) with 3μl of bromophenol loading dye and a Hinf I ψX174 marker. The gel was then removed and stained with ethidium bromide and photographed under ultraviolet excitation.

vii. *Modulation of conditions for MVR-PCR I*

To increase the yield of products from MVR-PCR I, various alterations were made, in a stepwise fashion. Single parameters were altered in the absence of other change in order to best observe the effects of each alteration.

a) Primer reaction concentrations

The concentration of TAG (amplimer) was doubled to increase the yield of longer PCR products. The higher concentration of TAG was maintained and the concentrations of repeat unit specific primer were halved.

b) Different annealing temperatures

The annealing temperature was increased from 62^{0}C to 64^{0}C and then, on a further reaction, dropped to 60 ^{0}C.

c) Repeat titration

The original titration was performed again, and the primers were first heated to 94 ^{0}C for three minutes, and then put in ice before adding to the PCR reagent.

d) Altered automated thermal cycling

The annealing temperatures were incrementally increased in successive reactions by two degrees centigrade to determine the optimum temperature.

e) The thermal cycling was returned to the conditions specified in the first description of MVR-PCR at this locus (Jeffreys *et al.*, 1991b), with ten cycles of 94 ^{0}C for 45 seconds to denature, 68 ^{0}C for one minute to anneal, then 70^{0}C for 2.5 minutes to extend; this was followed by a further ten cycles, incrementing

the extension time by 20 seconds per cycle and finally with a chase of 68^{0}C for 1 minute and 70^{0}C for 10 minutes.

2.4.2 MVR-PCR II - titration

i. *Design of primers*

Primers were re-designed in order to increase product size and yield (appendix 8).

ii. *Dilutions of Stock Primers*

The desired quantities of primer per reaction were achieved by dilution of stock primers to the desired concentrations (Table 2.4).

Table 2.4 Stock and reaction concentrations for MVR-PCR II

Primers	Stock concentrations (μM)	Desired reaction concentration	Dilution factor
TAG-II	12.2	1μM	1:1.77
31-A	17.7	1μM	1:8
31-TAG-II-AC	6.9	20nM	1:30
31-TAG-II-GT	4.3	20nM	1:20

iii. *Titration of conditions for MVR-PCR II*

To optimise conditions for PCR, standard concentrations of primers, dinucleotide triphosphates (DNTPs), *Taq* polymerase (Biogene ltd) and genomic DNA were used and the concentrations of $MgCl_2$ and dimethyl sulfoxide (DMSO) were varied. Each PCR reaction contained:

2.5μl Buffer A

100ng genomic DNA

1μM 31A

20nM 31-TAG-II-AC or GT

0.4µl *Taq* polymerase (Biogene ltd)

0.5 µl DNTP x 4 (A,C,G,T)

$MgCl_2$ 1.5µl, 2.0µl, 2.5µl, 3.0µl, or 3.5µl

DMSO none or 2%(0.5µl)

15µl H_2O

Total PCR reaction volume 25µl, 10 x 2 reactions = 20 microcentrifuge tubes (Table 2.5).

Table 2.5 Reaction concentrations of $MgCl_2$ and DMSO for titration of MVR-PCR II with each cryo-tube labelled numerically.

	No DMSO	0.5 % DMSO
1.5 µl $MgCl_2$	1	2
2.0 µl $MgCl_2$	3	4
2.5 µl $MgCl_2$	5	6
3.0 µl $MgCl_2$	7	8
3.5 µl $MgCl_2$	9	10

iv. *PCR conditions for MVR-PCR II in an automatic thermal cycler (Techne)*

One cycle of 94 °C for 3 minutes (denaturing), 52 °C for 30 seconds and 72 °C for 30 seconds, 35 cycles of 94 °C for 30 seconds, 52 °C for 30 seconds (annealing) and 72 °C for 30 seconds (extension). Finally, an elongation single cycle of 2 minutes at 72 °C.

v. *PCR product separation and visualisation*

As described for MVR-PCR I (section **2.4.1**).

vi. *Repeat titration of MVR-PCR II* (see results of **2.4.2**)

PCR product from lane 1 for 31-TAG-II-AC and lane 2 for 31-TAG-II-GT were used to repeat the titration, this time, with TAG-II (1 □M per reaction), with the annealing temperature increased from 52 °C to 62 °C, and the extension times increased from 30 seconds to 2 minutes.

2.4.3 MVR-PCR II – final conditions

i. *PCR reaction mixture*

2.5µl Buffer A

100ng genomic DNA

1µM 31 TAG II

1µM 31A

20nM 31-TAG-II-AC or GT

0.4µl *Taq* polymerase (Biogene ltd)

0.5 µl DNTP x 4 (dATP, dCTP, dGTP, and dTTP)

$MgCl_2$ 1.5µl

DMSO 2%(0.5µl)

15µl H_2O

Total PCR reaction volume 25µl, 10 x 2 reactions = 20 microcentrifuge tubes

ii. *Automated thermal cycling*

Based on the results of MVR-PCR II titration (section **2.4.2**), cycling was as follows:

One cycle of 94 °C for 3 minutes (denaturing), 52 °C for 30 seconds and 72 °C for 30 seconds, 10 cycles of 94 °C for 30 seconds, 52 °C for 30 seconds (annealing) and 72 °C for 30 seconds (extension), a further 25 cycles of 94 °C for 30 seconds, 62 °C for 30 seconds (annealing) and 72 °C for 30 seconds (extension). Finally, an elongation single cycle of 2 minutes at 72 °C.

iii. *PCR product separation and visualisation*

As described for MVR-PCR I (section **2.4.1**).

iv. *MVR-PCR II and colorectal tumours*

MVR-PCR II was used on a variety of tumour and normal DNA samples from sporadic and HNPCC associated colorectal cancers and those associated with HNPCC. The RER phenotype of each tumour-normal pair had already been ascertained by microsatellite analysis (See section 2.3). MVR-PCR was repeated several times on both RER negative and positive paired samples on different occasions to determine the reproducibility of the results. Results were interpreted blind to the identity of each DNA pair.

2.4.4 MVR-PCR III

i. *The MS32 locus*

As with MS31A, this hyper variable locus contains two types of repeat unit that differ by a single base substitution that creates or destroys a *Hae*III restriction site. Each repeat unit is 29bps:

MS32 repeat unit:

5' G(G or A)CCAGGGGTGACTCAGAATGGAGCAGGY 3'

ii. *Primers*

The variant repeat unit primers (32-TAG-III-C or T) (Jeffreys *et al.*, 1991b) each consisted of a 20 nucleotide minisatellite repeat sequence terminating at the site of base substitution and preceded by a 25nt 5' synthetic non-minisatellite repeat sequence identical to the TAG III amplimer (appendix 9).

iii. *Dilutions of Stock Primers*

The desired quantities of primer per reaction were achieved by dilution of stock primers to the desired concentrations (Table 2.6).

Table 2.6 Stock and reaction concentrations for MVR-PCR III

Primers	Stock concentrations (μM)	Desired reaction concentration	Dilution factor
TAG-III	11.0	1μM	1:1.77
32-D	8.0	1μM	1:8
32-TAG-III-C	8.3	20nM	1:30
31-TAG-III-T	10.2	20nM	1:20

iv. *MVR-PCR III titration* (see section **2.4.1**)

2.5µl Buffer A (PCR buffer)

100ng genomic DNA

1µM TAG-III

1µM 32D

20nM 32-TAG-III-C or T

0.4µl *Taq* polymerase (Biogene ltd)

0.5 µl DNTP x 4 (A,C,G,T)

$MgCl_2$ 1.5µl, 2.0µl, 2.5µl, 3.0µl, or 3.5µl

DMSO none or 2%(0.5µl)

15µl H_2O

Total PCR reaction volume 25µl, 10 x 2 reactions = 20 microcentrifuge tubes

Table 2.7 Reaction concentrations of $MgCl_2$ and DMSO for titration of MVR-PCR III with each cryo-tube labelled numerically.

	No DMSO	0.5 % DMSO
0.5 µl $MgCl_2$	1	2
1.0 µl $MgCl_2$	3	4
1.5 µl $MgCl_2$	5	6
2.0 µl $MgCl_2$	7	8
2.5 µl $MgCl_2$	9	10

v. *PCR conditions for MVR-PCR III in an automatic thermal cycler (Techne)*

One cycle of 94 °C for 3 minutes (denaturing), 50 °C for 30 seconds and 72 °C for 15 seconds, 10 cycles of 94 °C for 30 seconds, 50 °C for 30 seconds (annealing) and 72 °C for 15 seconds (extension), 30 cycles of 94 °C for 30 seconds, 66 °C for 30 seconds (annealing) and 72 °C for 15 seconds (extension). Finally, a single cycle of 2 minutes at 72 °C elongation.

vi. *PCR product separation and visualisation*

As described for MVR-PCR I (section **2.4.1**).

vii. *Repeat titration of MVR-PCR III*

Repeat titration was carried out in two steps. First, TAG III amplimer was omitted and 35 cycles of PCR were used at an annealing temperature of 53°C. The same ranges of concentrations of DMSO and $MgCl_2$ were used. The best products from the first titration were then used as target for another titration against the flanking primer (32D) and the amplimer (TAG III) at the same annealing temperature.

2.4.6 MVR-PCR IV

i. *The MS31A locus*

A attempt was to made repeat analysis of locus MS31A using redesigned repeat unit specific primers (31-IV-AC and GT) which had no TAG amplimer in order to generate product extending from the flanking site to the first few repeat units. Equivalent concentrations of 31-A, the flanking primer and the specific primers were used and no TAG amplimer.

ii. *Primers*

The variant repeat unit specific primers (31-IV-AC or GT) each consisted of a 20nt minisatellite repeat sequence terminating at the site of base substitution with a sequence complimentary to that of the MS31A repeat units.

iii. *Dilutions of Stock Primers*

The desired quantities of primer per reaction were achieved by dilution of stock primers to the desired concentrations (Table 2.8).

Table 2.8 Stock and reaction concentrations for MVR-PCR IV

Primers	Stock concentrations (μM)	Desired reaction concentration	Dilution factor
31-IV-AC	22.0	1μM	1:30
31-IV-GT	22.8	1μM	1:20

iv. *MVR-PCR IV titration (see section* **2.4.1***)*

2.5µl Buffer A (PCR buffer)

100ng genomic DNA

1µM 31A

1µM 31-IV-AC or GT

0.4µl *Taq* polymerase (Biogene ltd)

0.5 µl DNTP x 4 (A,C,G,T)

$MgCl_2$ 1.5µl, 2.0µl, 2.5µl, 3.0µl, or 3.5µl

DMSO none or 2%(0.5µl)

15µl H_2O

Total PCR reaction volume 25µl, 10 x 2 reactions = 20 microcentrifuge tubes

v. *PCR conditions for MVR-PCR III in an automatic thermal cycler (Techne)*

One cycle of 94 °C for 3 minutes (denaturing), 60 °C for 30 seconds and 72 °C for 45 seconds, 35 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds (annealing) and 72 °C for 45 seconds (extension), and finally, a single cycle of 2 minutes at 72 °C for elongation.

vi. *PCR product separation and visualisation*

As described for MVR-PCR I (section **2.4.1**).

2.5 *The Wessex HNPCC Register*

2.5.1 Guidelines for clinicians for referral to the Wessex Clinical Genetics Service

The criteria for referral to the genetics clinic set out below were modified from the Amsterdam criteria (Vasen, Mecklin, Meera-Khan & Lynch, 1991b) and are therefore based on clinical definitions not genetic testing.

1. Three close relatives on the same side of the family with colorectal cancer, at least one under 55 years of age and occurring in at least two generations.
2. Three close relatives as above with any of the cancers associated with HNPCC but at least one must be colorectal and at least one diagnosed before 55 years of age.
3. An immediate relative (parent, sibling or child) with colorectal cancer under 45 years of age.
4. Dual primary cancers, for example, colorectal and endometrial in a close relative under 55 years of age.

2.5.2 Data collection

Patients referred to the Wessex Clinical Genetics Service for evaluation of a personal or family history of colorectal cancer had their family history carefully documented. Families were categorized as follows:

1) Family history meets the Amsterdam criteria (as above).
2) Family history affects two or more generations with at least two colorectal cancers and one ore more HNPCC associated tumour was present.

3) Family history of CRC with two cases of colorectal with average age less than 55 or three more cases affected at any age.
4) Isolated case less than 45 years of age.
5) Multiple colonic neoplasms in an individual with or without a family history where multiple adenomas amount to fewer then 100 in total and where no mutation in the APC gene had been detected using heteroduplex analysis for the entire gene (Bunyan, Shea-Simmons, Reck, Finnis and Eccles, 1995).

Patients referred to the Wessex Clinical Genetics Service for evaluation of a personal or family history of colorectal cancer had their family history carefully documented. Histological confirmation of cancers from records or death certificates was obtained wherever possible. Information entered onto a database included the reason for referral, the family history (number, age of diagnosis, site, and type of neoplasms) and whether this conformed to the Amsterdam Criteria, whether tumour blocks were available for analysis, and subsequently whether a mismatch repair gene mutation was found. Peripheral blood samples were taken for this purpose

2.5.3 HNPCC PCR

PCR amplification of the hMLH1 and hMSH2 genes was carried out using 35 separate pairs of primers that are specific for the 19 exons of the hMLH1 gene and the 16 exons of the hMSH2 gene. All primers were designed and supplied by Dr Ian Frayling as part of the UK HNPCC consortium. PCR reactions were carried out using a Perkin Elmer 480 PCR machine. The same basic PCR protocol was used for every primer set: -

1. 1 µl of sample DNA was mixed with 9 µl of distilled water in a 0.5 ml PCR tube. One drop of oil on top was placed, and then the tube was heated at 95°C in a Perkin Elmer PCR machine for 6 minutes.

2. Meanwhile, the following pre-mix was prepared for each sample sample: -

10 x PCR buffer (Promega, UK)	2.5 µl
25 mM $MgCl_2$ (Promega, UK)	2.75 µl
2 mM dNTP mix	2.5 µl
10 µM forward primer	1.25µl
10 µM reverse primer	1.25µl
Taq polymerase (Promega, UK)	0.1 µl
Distilled water	4.65 µl

3. 15 µl of pre-mix was added to the hot DNA sample and 35 PCR cycles were performed using the following conditions: -

95°C for 30 seconds, 60°C for 1 min, 72°C for 1 min

This was followed by an elongation step of 10 minutes at 72°C.

2.5.4 Heteroduplex analysis

1. 5 µl of Hydrolink loading buffer (AT Biochem) was added to the PCR sample, heteroduplices were formed by heating the sample to 95°C for 3 minutes then slowly cooling to 37°C over a period of 25 minutes using the ramp cycle of a Perkin Elmer 480 PCR machine.

2. 12 µl of the sample was loaded onto a 1 mm thick Hydrolink gel (AT Biochem), using a 20 cm Sturdier gel apparatus set-up. The gel was run overnight at a constant 235 Watts.

3. The gel was stained for 10 minutes in 800 mls of gel running buffer containing 50 µl of 20 mg/ml ethidium bromide, and viewed on an ultraviolet transmission light box.

2.6 *Mismatch Repair Gene Sequencing*

2.6.1 Isolation of PCR Template

1. PCR amplification of the relevant mismatch repair gene exonic region (as described elsewhere) was performed using a biotin-tagged forward primer to a final volume of 100 µl of PCR product per person (2 x 50 µl reactions).
2. The PCR products were pooled and the samples run on an ethidium bromide stained 1% low melting point agarose gel until separation of the required PCR product from the other PCR reaction components was complete.
3. Place the gel on a UV transilluminator to allow visualisation of the bands, then cut out the required PCR product from the gel using a sterile scalpel, keeping the agarose block to the minimum size required.
4. Transfer the agarose block to a 1.5 ml eppendorf tube and add 400 µl of 1 x TBE buffer. Place the eppendorf tube in a 65°C water bath until the agarose has melted.
5. Add 16 µl of M280 streptavidin beads (Dynabeads, Dynal), mix and replace the eppendorf tube at 65°C for 30 minutes.
6. Separate the beads coated with the biotinylated PCR product by placing the eppendorf tube in the MPC (magnetic particle concentrator which is supplied in the Dynabeads kit). Discard the supernatant.
7. Remove the eppendorf tube from the MPC. Add 200 µl of distilled water and mix. Place the eppendorf tube in the MPC and discard the supernatant.

8. Repeat step 7.

9. Remove the eppendorf tube from the MPC. Add 200 µl of 0.15M NaOH and mix. Leave at room temperature for 5 minutes. Place the eppendorf tube in the MPC and discard the supernatant.

10. Repeat step 7 twice.

11. Remove the eppendorf tube from the MPC. Add 200 µl of distilled water and mix. Transfer to a 0.5 ml PCR tube.

2.6.2 Mismatch repair gene sequencing and sequencing gel electrophoresis

Sequencing was carried out using a Sequenase version 2 sequencing kit from the United States Biochemical Corporation (USB).

1. Place the PCR tube from step 11 (above) in the MPC. Discard supernatant. Remove the tube from the MPC, add 7 µl of distilled water and mix.

2. Add 2 µl of reaction buffer (USB) and 1 µl of the appropriate unbiotinylated reverse primer (10 µM stock), which was used in the original PCR reaction.

3. Place the PCR tube in a Perkin Elmer PCR machine. Using a ramp cycle, heat the tube to 65^{o}C for 2 minutes then cool to 30^{o}C over a period of 30 minutes to anneal the primer to the template strand.

4. Chill the tube on ice for use in step 7.

5. While cooling, label, fill and cap 0.5 ml PCR tubes with 2.5 µl of each Termination Mixture (USB) - one each for A, C, G and T. Dilute the

Labelling Mix (USB) to a working concentration using 1 volume Labelling Mix to 4 volumes of distilled water, and dilute the Sequenase enzyme to a working concentration using 1 volume of Sequenase to 3 volumes of ice-cold Sequenase Dilution Buffer (USB). Place the diluted Labelling Mix and the diluted Sequenase on ice.

6. Pre-warm the 4 termination tubes (A, C, G and T) from step 5 in a 37°C waterbath.

7. To the ice-cold DNA mixture (10 µl) add: -

DTT (Dithiothreitol, USB)	1µl
Diluted Labelling Mix	2µl
^{35}S-dATP (Amersham)	1µl
Diluted Sequenase Polymerase	0.5µl

Mix and incubate at room temperature for 4 minutes.

8. Transfer 3.5 µl of labelling mix to each termination tube (A, C, G and T), mix and continue incubation of the termination reactions at 37°C for 5 minutes.

9. Stop the reactions by adding 4 µl of Stop Solution (USB).

10. Heat the samples to 75°C for 2 minutes immediately before loading onto a 6% non-denaturing polyacrylamide sequencing gel. Run the gel until the xylene cyanol dye of the Stop Solution reaches the end of the gel (approximately 2 - 3 hours at 50 Watts).

11. After electrophoresis, transfer the gel to Whatman 3MM paper and cover with clingfilm. Visualise the bands by standard autoradiography (1 - 3

days, Kodak Xomat AR film) or by using the Fuji phospho-imaging system (4 - 20 hours).

3.1 Development of MVR-PCR I

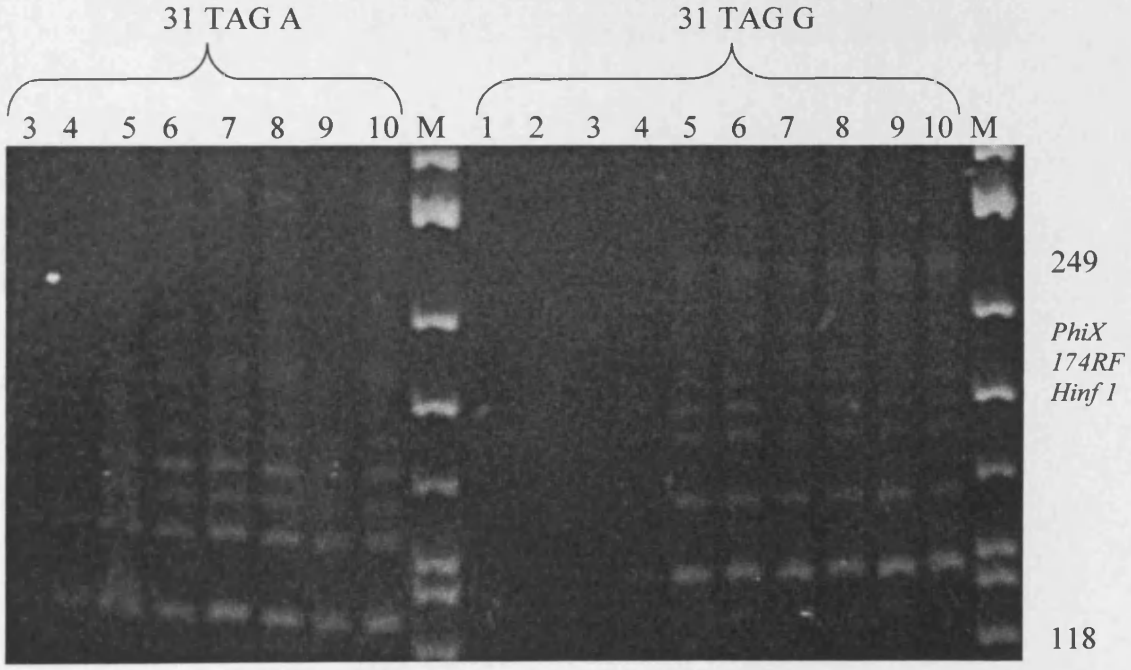
3.1.1 MVR-PCR I titration (Methods 2.4.1)

Initial titration using control DNA revealed several bands for reaction containing both 31-TAG-A and G for most concentrations of DMSO and Magnesium Chloride (Figure 3.1; Table 2.2, Methods). The optimum conditions in terms of concentrations of DMSO and $MgCl_2$ are then used for subsequent titration of primer concentration and thermal cycling.

Figure 3.1

Titration of MVR-PCR using different concentrations of $MgCl_2$ (see table 2.3, Methods).

The numbers represent different concentrations of the 2 reagents. Several DNA bands appear for both sets of reactions (31-TAG A and G). Conditions were optimal for reaction number seven for both markers, which contained 2.0 µl $MgCl_2$. Little or no product was present for reactions containing less than 1.5 µl $MgCl_2$. The band intensities were weaker for reactions containing no DMSO. For all MVR-PCR reactions a ψX174 HinfI (Promega, UK) size marker was used (M).



3.1.2 MVR-PCR I

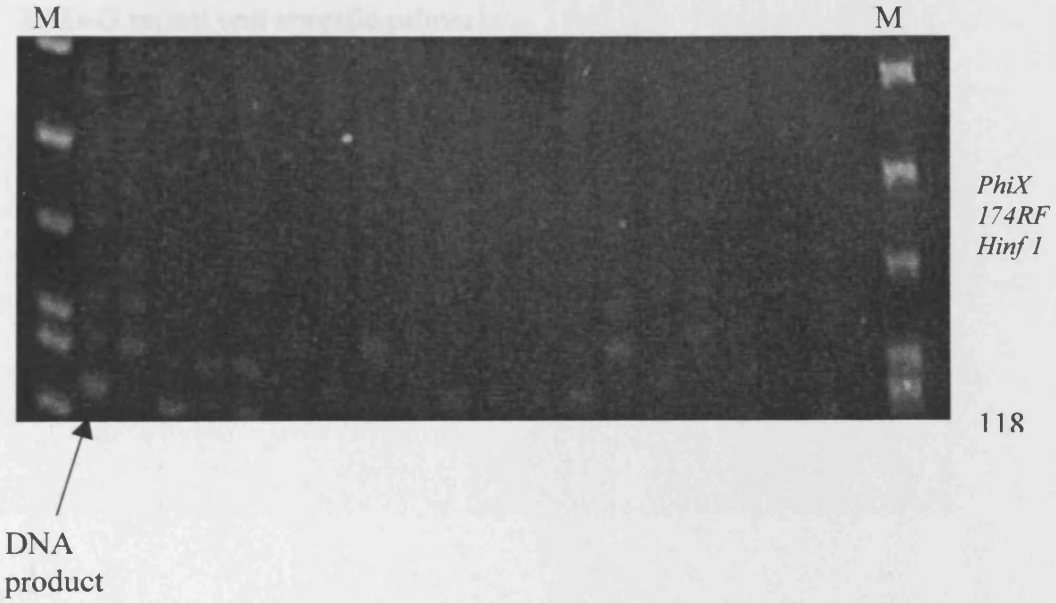
MVR-PCR I was used to analyse five tumour / normal tissue pairs of cases two, three, four, five, and six of the sporadic CRC group using both repeat units. Hence there are two separate reactions for both the normal and tumour DNA and therefore four lanes of PCR product for each patient. The reaction conditions were altered at each successive reaction to improve results:

i. For the first reaction using conditions identical to those in the titration reaction, there was a poor yield generally and the shorter products were favoured (Figure 3.2).

Figure 3.2

MVR-PCR I for 5 tumour (T) / normal (N) tissue pairs

Each of the T/N pair has two reactions, one for each repeat unit specific primer. The band intensity is low and inconsistent and favours shorter products. DNA product is present between 100 and 150 base pairs.

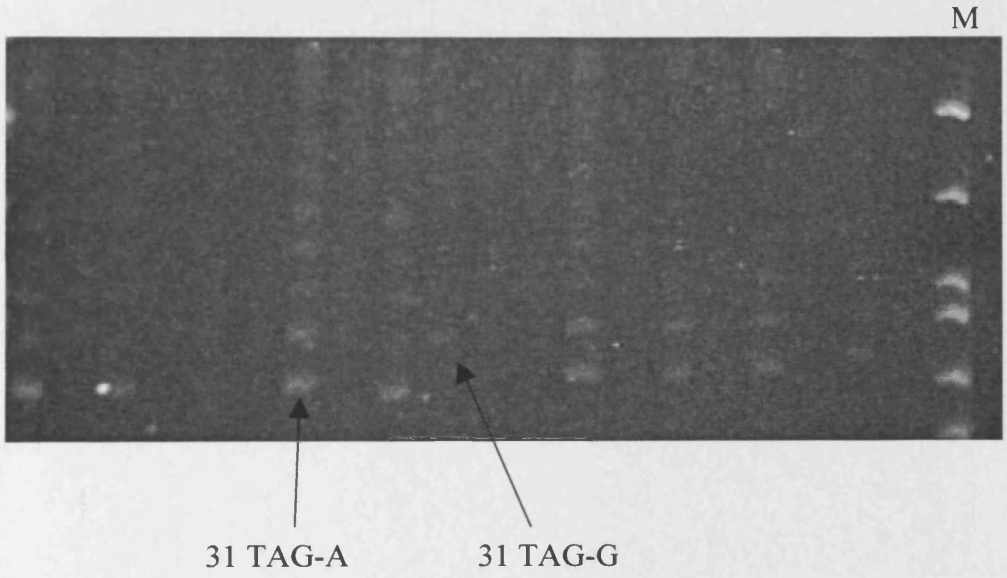


ii. Twice the concentration of TAG amplimer and half the concentration of repeat unit specific primer in order to prevent the generation of shorter PCR product. The result (Figure 3.3) was the generation of a greater spread in terms of sizes, but again, the yield was poor and inconsistent. There was no PCR product for reactions containing the 31 TAG-G repeat unit specific primer.

Figure 3.3

Repeat MVR-PCR I for 5 tumour (T) / normal (N) tissue pairs

MVR-PCR I for five tumour (T) / normal tissue (N) pairs, each having two separate reactions for each repeat unit primer, hence four lanes per patient. The band intensity is poor and the yield inconsistent. There is no product at all for 31 TAG G reactions. For 31 TAG-A, bands are now seen for product fragments up to 200bp.

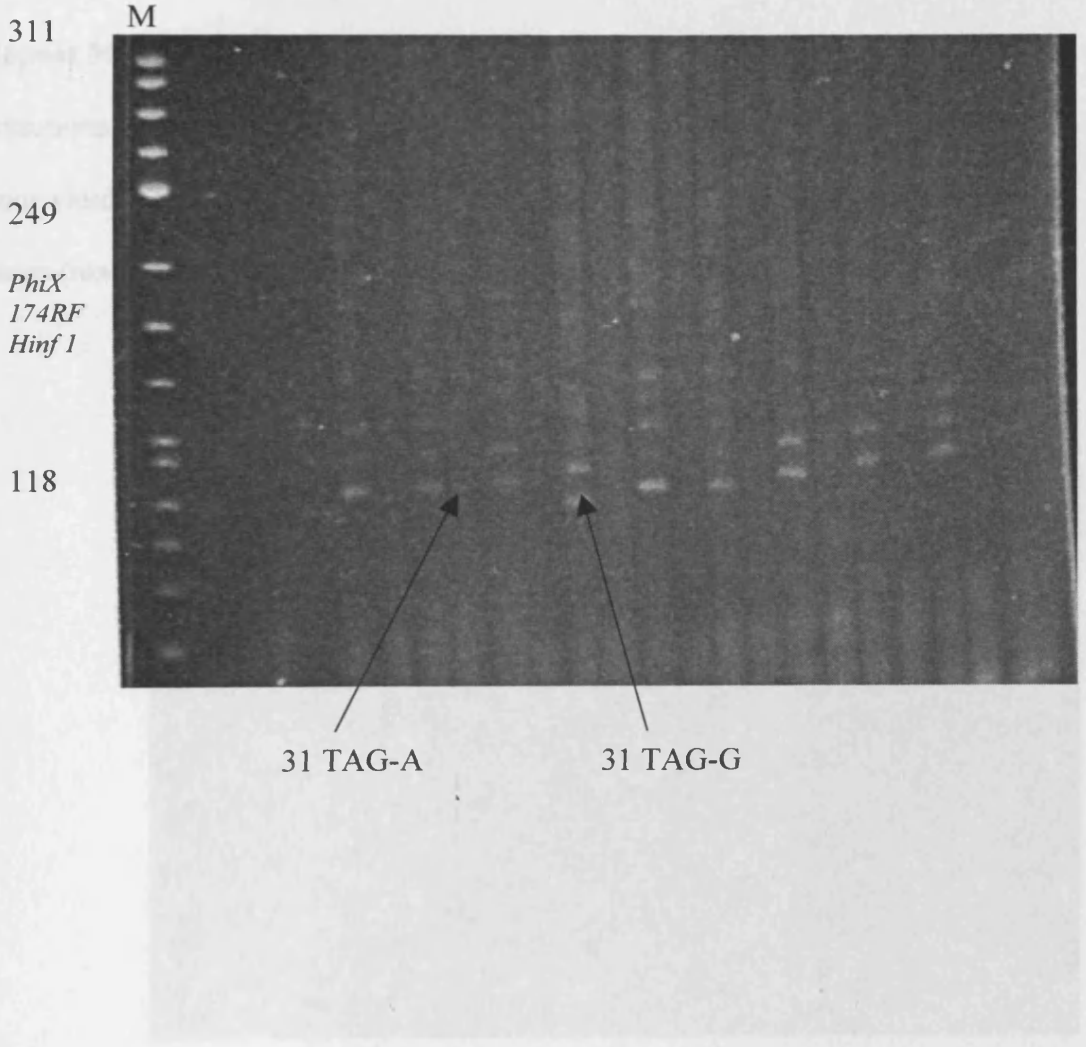


iii. The annealing temperature was decreased to 60°C from 64°C and the extension time decreased from sixty to thirty seconds. Once again there was no product from 31 TAG-G and the shorter bands were predominant (Figure 3.4). This still may have been due to internal priming.

Figure 3.4

Repeat MVR-PCR I for 5 tumour (T) / normal (N) tissue pairs

There was repeated failure of 31 TAG-G reactions and 31 TAG-A reactions once again favoured the generation of shorter product.

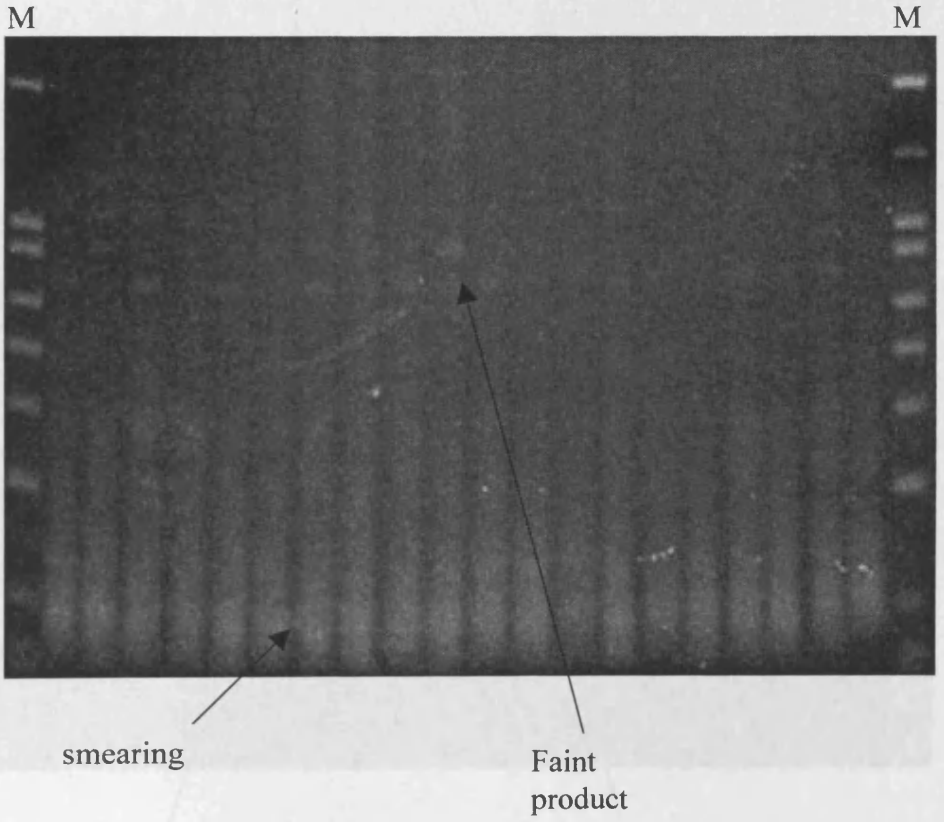


iv. The repeat unit specific primer concentrations were halved again. Bands appeared for 31 TAG-G but the overall yield was once again poor (Figure 3.5).

Figure 3.5

Repeat MVR-PCR I for 5 tumour (T) / normal (N) tissue pairs

Reactions with 31 TAG-G appeared to be successful in this instance but again with poor yield. The smearing at the bottom of very short products represents individual bases (marker not shown).

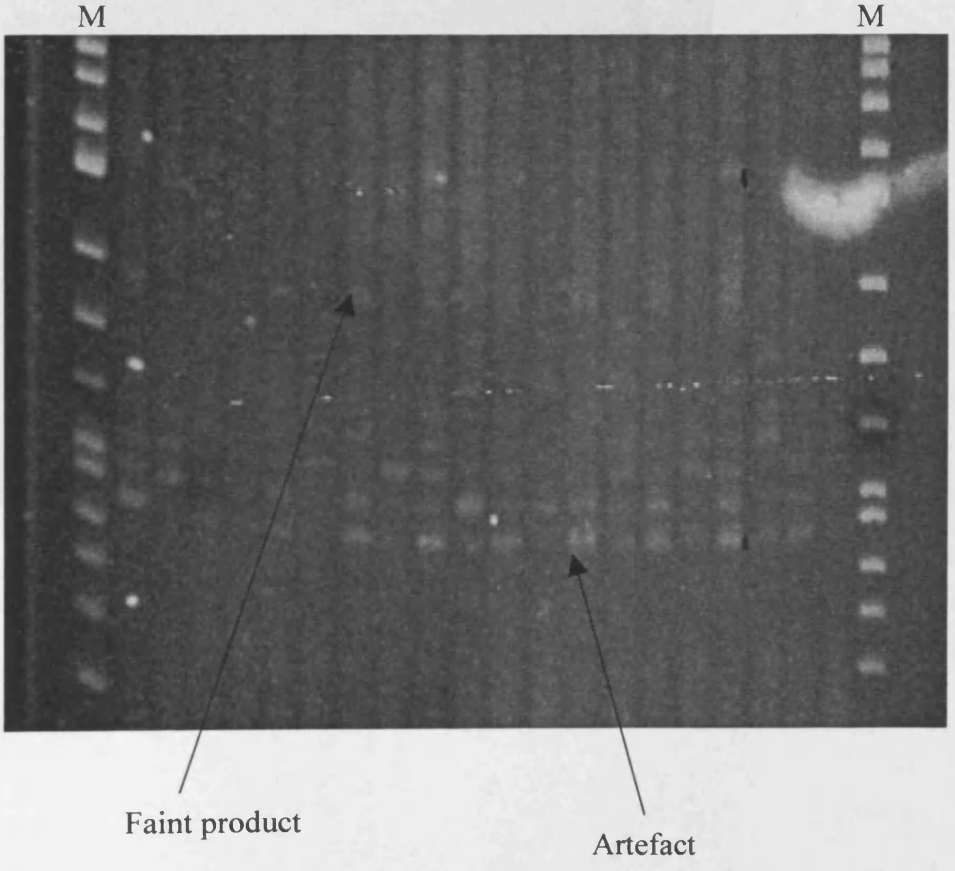


v. The original primer concentrations in the initial MVR-PCR I titration were used with the lower annealing temperature and elongation time (step iii). Once again yield, although improved, was poor (figure 3.6).

Figure 3.6

Repeat MVR-PCR I for 5 tumour (T) / normal (N) tissue pairs

Yield remained poor, though improved over previous reactions. Some faint bands representing longer PCR product (up to 300bp) appeared (arrowed). Artefacual product is also present as a result of amplification of primers (arrowed).

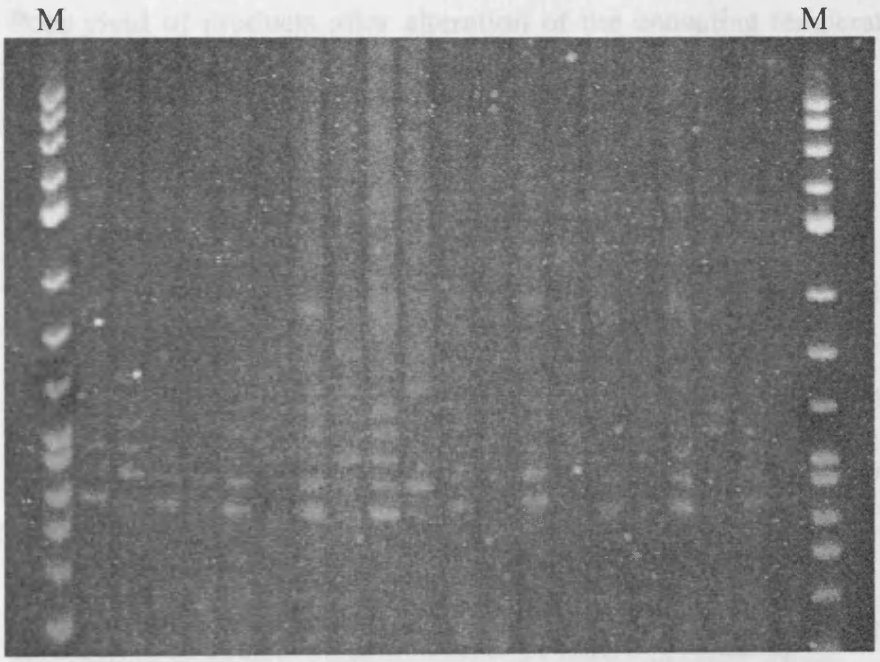


vi. Prior to adding to the PCR mixture the primers were heated in the Thermal cycler (Techne) to 94°C for 3 minutes, then plunged into ice. This was to ensure dis-aggregation of primers prior to PCR. The result, again, was poor (Figure 3.7).

Figure 3.7

Repeat MVR-PCR I for 5 tumour (T) / normal (N) tissue pairs

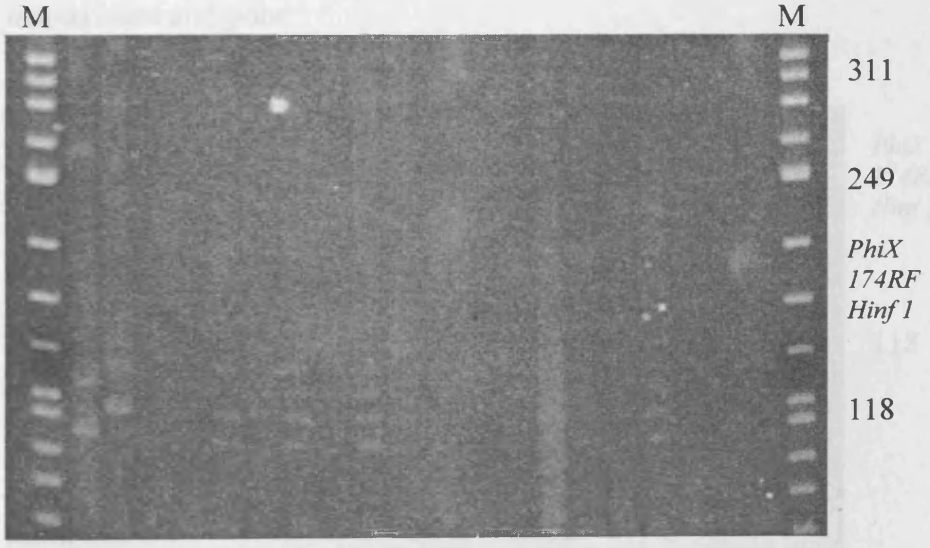
A poor result again with low yield and short products.



vii. Further alteration of thermal cycling by incrementing the annealing temperature on successive reactions by 2°C until annealing is no longer seen to occur to determine the optimum maximum temperature. Once again a poor yield was seen (Figure 3.8).

Figure 3.8 Repeat MVR-PCR I for 5 tumour (T) / normal (N) tissue pairs

Poor yield of products after alteration of the annealing temperature. Little, if any product can be seen.

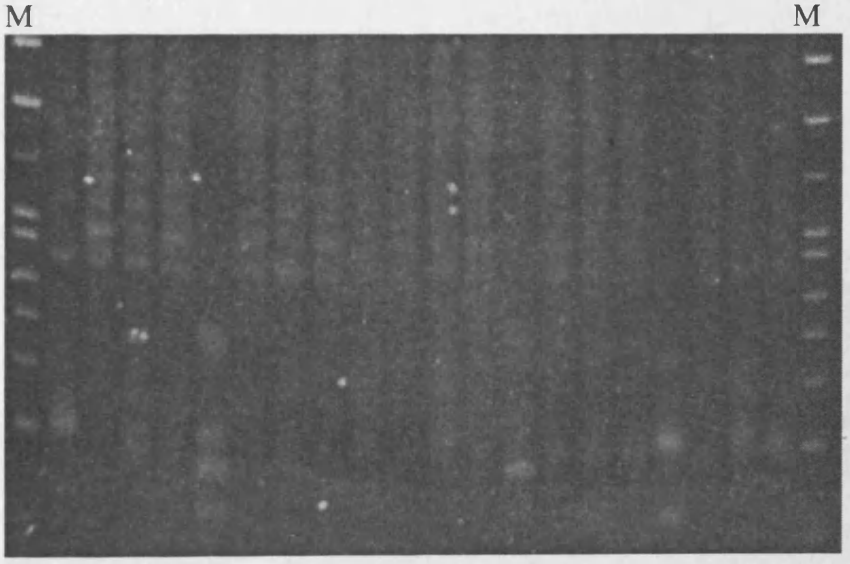


viii. The thermal cycling conditions described originally for MVR-PCR were used (Jeffreys *et al.*, 1991b). The TAG amplimer was omitted to generate a range of shorter products and the same concentrations of flanking primer (31A) and repeat unit specific primer (31 TAG-A or G) were used. There was zero yield of products (Figure 3.9).

Having altered all the variables affecting the process of MVR-PCR I without success, the primers were re-designed for MVR-PCR II.

Figure 3.9 Repeat MVR-PCR I for 5 tumour (T) / normal (N) tissue pairs

Results of further modulation of conditions for MVR-PCR I. Again the yield was inconsistent and poor.



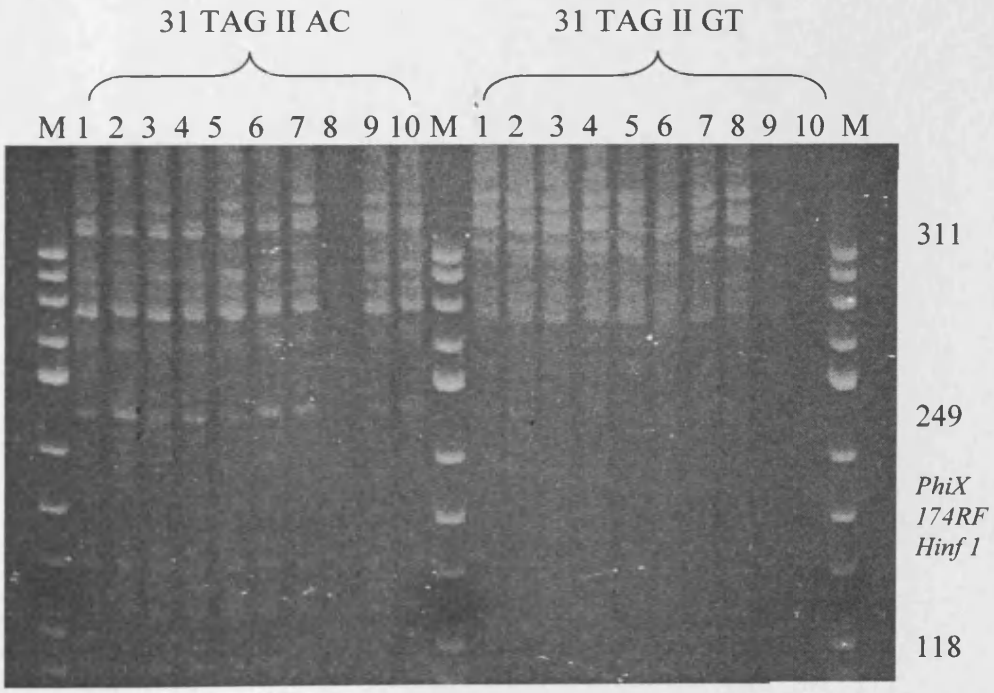
3.2 Development of MVR-PCR II

3.2.1 Titration of MVR-PCR II

The products from MVR-PCR II were of higher intensity and greater range of sizes than that from MVR-PCR I (Figure 3.10). Conditions were most favourable with 2% DMSO (0.5µl per reaction) and 1.5mM $MgCl_2$ and this combination was used in all subsequent reactions.

Figure 3.10 Titration of MVR PCR II (seeTable 2.5, Methods)

Ten reactions for each repeat unit primer (TAG II-AC and -GT), each containing different concentrations of 2% DMSO and $MgCl_2$ (tubes 1-10). There is equal yield for both and product sizes of between 150 and 700+bp for TAG II-AC and 350 and 700+bp for TAG II-GT. Conditions favour 2% DMSO and lower concentrations of $MgCl_2$.



3.2.2 MVR-PCR II for tumour / normal pairs

MVR-PCR II was used to compare DNA extracted from tumour and normal tissues from freshly frozen sporadic colorectal neoplasms with known replication error (RER) status.

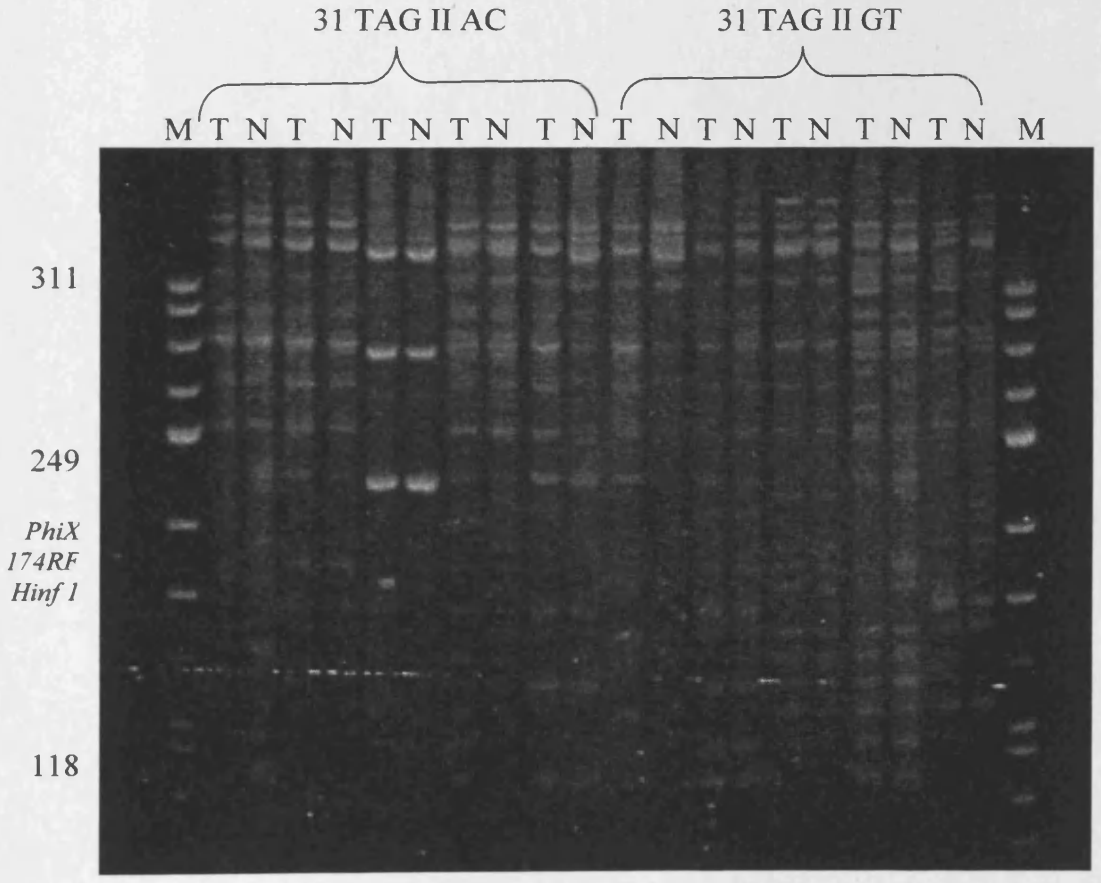
i. *Initial results*

In the first reaction DNA extracted from cases assessed to be RER negative were used to determine the initial result of MVR-PCR II (Figure 3.11). No differences were observed between normal and tumour DNA.

Figure 3.11

MVR-PCR II for MIN negative tumour / normal pairs

This photograph of a gel shows the first results of MVR-PCR II for five tumour (T) / normal (N) pairs. DNA extracted from cases 13, 14, 15, 16, and 23 were used (represented on the photograph in that order). All of these were MIN negative. Bands of between 120 and 500bp are consistently seen for both reactions. No differences were present between bands for tumour and normal DNA in either reaction. Bands of product did appear that were longer than 500bp but these were inconsistent and difficult to interpret.



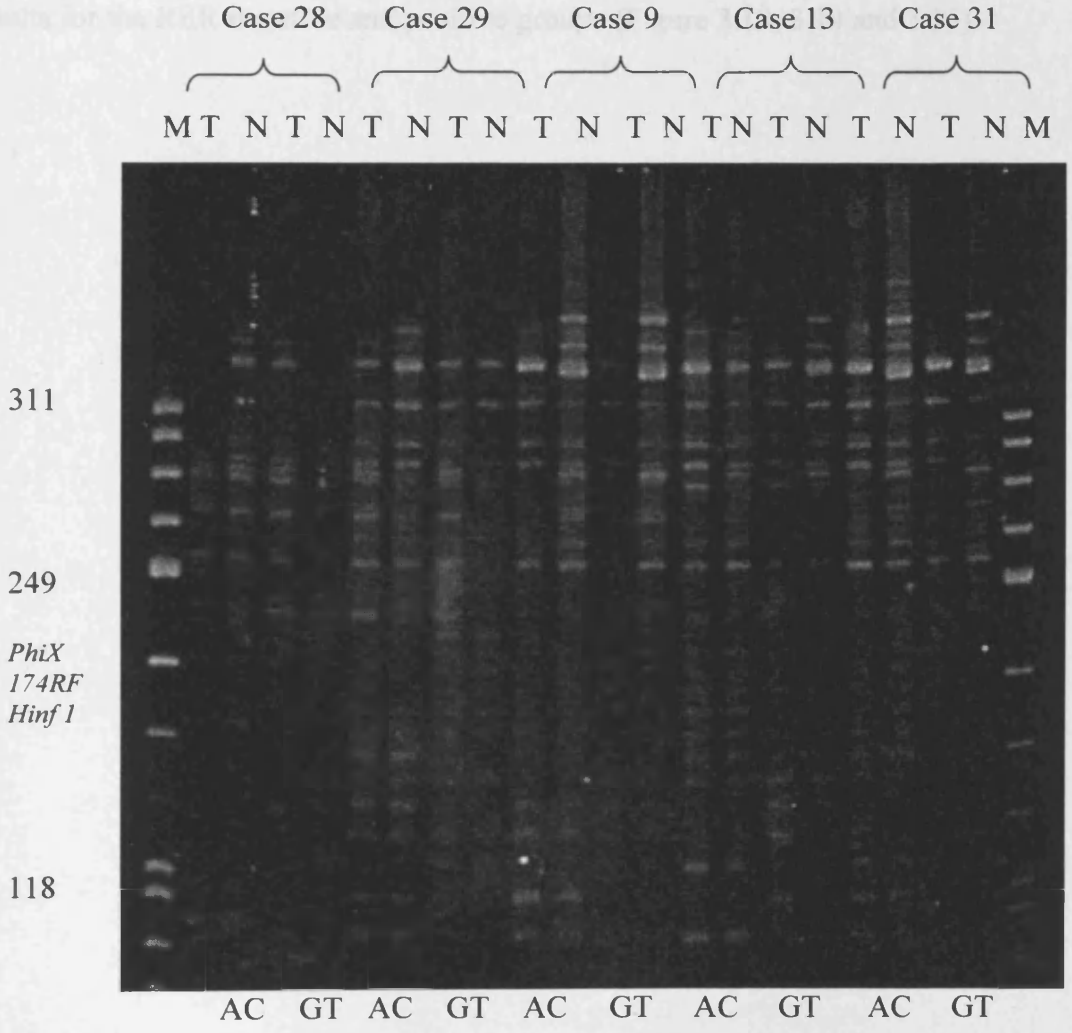
ii. *Reproducibility*

This result was repeated on several occasions for different patients to determine reproducibility (Figure 3.12). On each occasion identical bands occurred for tumour and normal DNA for RER negative cases for both reactions, hence cases that were known to be negative to microsatellite analysis for replication error could be used as controls in the same reaction as those known to be RER positive for comparative purposes.

Figure 3.12

MVR-PCR II for tumour / normal pairs - reproducibility

These gels represent MVR-PCR II performed on three separate occasions for the same paired tumour / normal DNA samples. This photograph is of a gel from MVR-PCR with the same cases and conditions as for Figure 3.11. It shows the same band pattern on each occasion for both reactions (31 TAG-II-AC and –GT).



iii. *Minisatellite instability*

Differences between the patterns of bands were observed when normal and tumour PCR products from microsatellite-determined RER positive tumours are compared (Figure 3.3.4). These differences exist in both reactions (31 TAG-II-AC and GT) and did not exist for RER negative cases. Results for RER negative and positive paired samples were re-assessed by blind observers (JNP and DME). Reactions were repeated on several different occasions to determine the reproducibility of results for the RER negative and positive groups (Figure 3.13, 3.14 and 3.15).

Figure 3.13 MVR-PCR II for tumour / normal pairs

DNA from fresh snap-frozen tumour (T) / normal (N) tissues of cases 9 and 13 (MSI stable), and cases 28, 29 and 31 of the HNPCC group (MSI high by microsatellite analysis). For Cases 9 and 13 no differences were observed between the tumour and normal bands for either reaction (31 TAG-II-AC or –GT). Poor band intensity for longer product of normal tissue with the GT marker is seen for Case 13 making interpretation difficult in this area. For cases 28, 29 and 31, both smearing and band loss is seen in the tumour DNA compared to the normal DNA. The marker (lanes 1 and 22) is PhiX174 Hinf I.

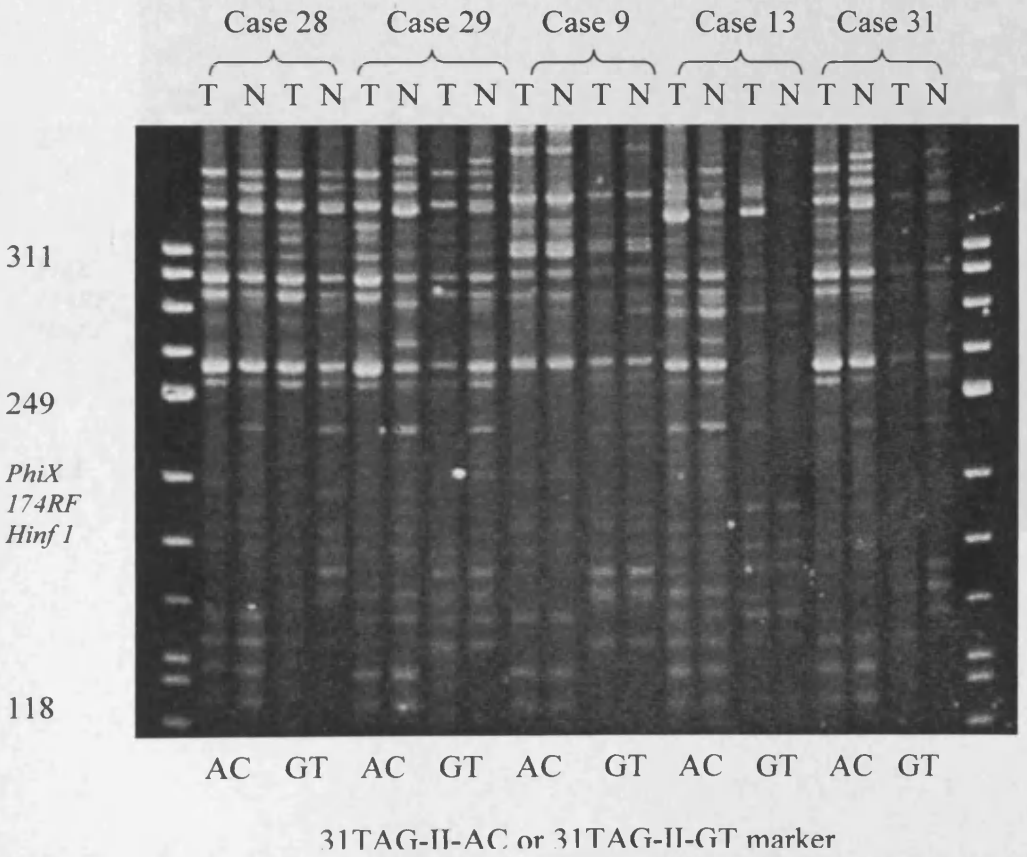


Figure 3.14

MVR-PCR II – detection of instability – reproducibility

MVR-PCR II for MSI negative tumours demonstrated no alterations compared to normal DNA on several occasions. Cases 5-9 are shown here. There was no product for the 31 TAG II GT for normal DNA due to failure of the reaction.

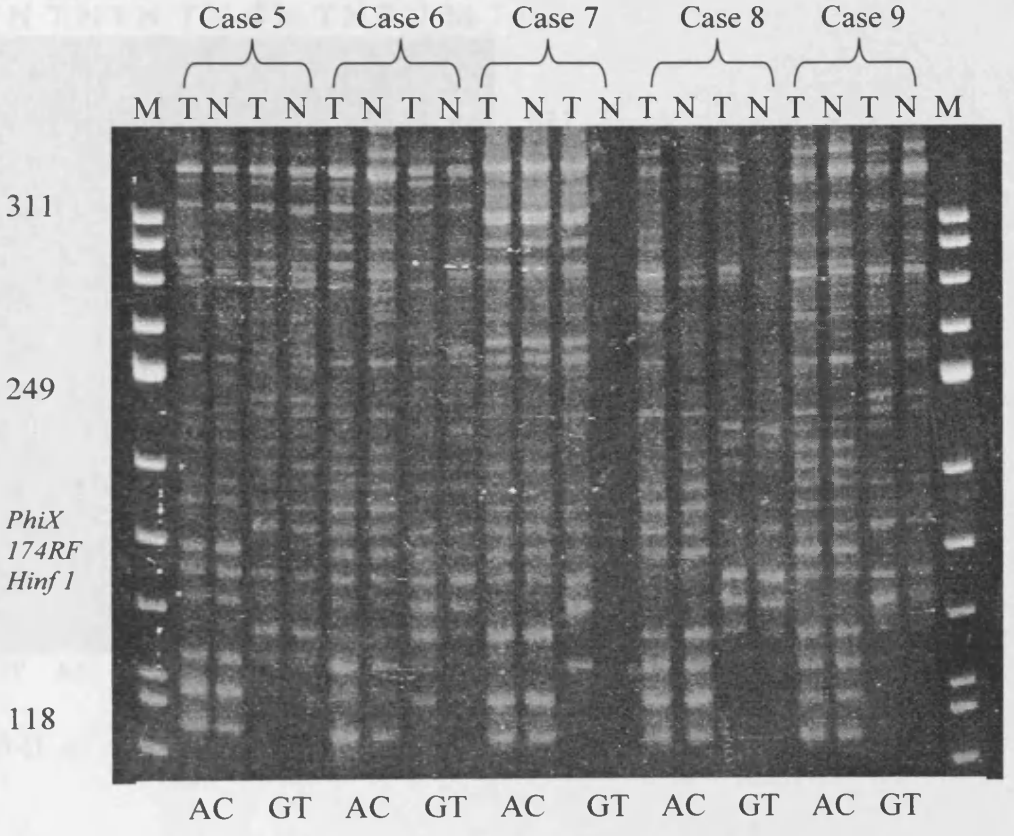
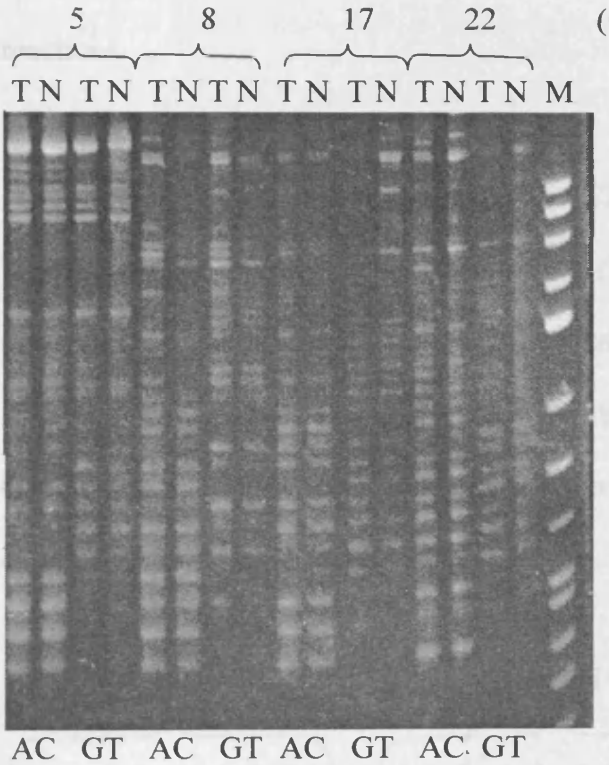


Figure 3.15 MVR PCR II for tumour/normal paired samples negative to analysis for MSI

Four cases negative for microsatellite analysis (numbers below, table 3.1). No difference between the tumour (T) and normal (N) bands were seen with MVR PCR II on repeated testing. M = size marker.



iv. *Non-radioactive MVR-PCR*

All the results obtained in these experiments were through the use of a non-radioactive technique. This avoids the use of radioactively labelled probes in the PCR mixture and autoradiography to visualise the products on a gel.

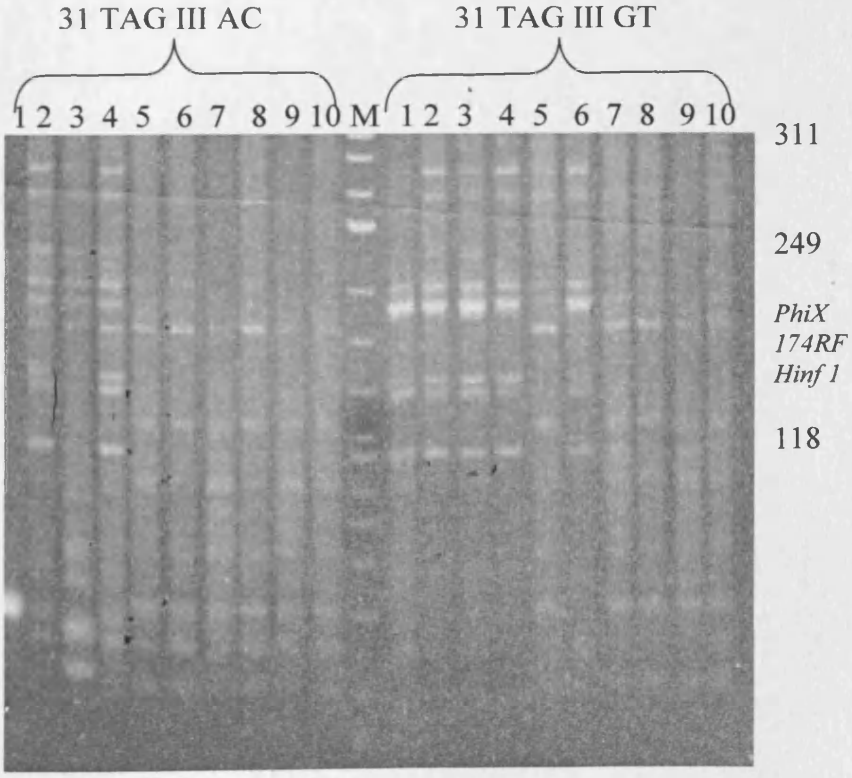
3.3 Development of MVR-PCR III

3.3.1 Titration of MVR-PCR III

The products from MVR-PCR III were of good spread in base pair size and intensity was sufficient (Figure 3.16). Conditions were most favourable with 2% DMSO (0.5µl per reaction) and 2.0mM $MgCl_2$ and these were used in subsequent reactions.

Figure 3.16 **Titration of MVR-PCR III** (See Methods table 2.6)

Ten reactions for each repeat unit primer (TAG III-AC and -GT) with varying concentrations of 2% DMSO and $MgCl_2$ (numbered 1-10). There is equal yield and size of products from 100 to 500bp for both TAG III-AC and TAG III-GT. Conditions favoured 2% DMSO and lower concentrations of $MgCl_2$ and these conditions were used in subsequent reactions.



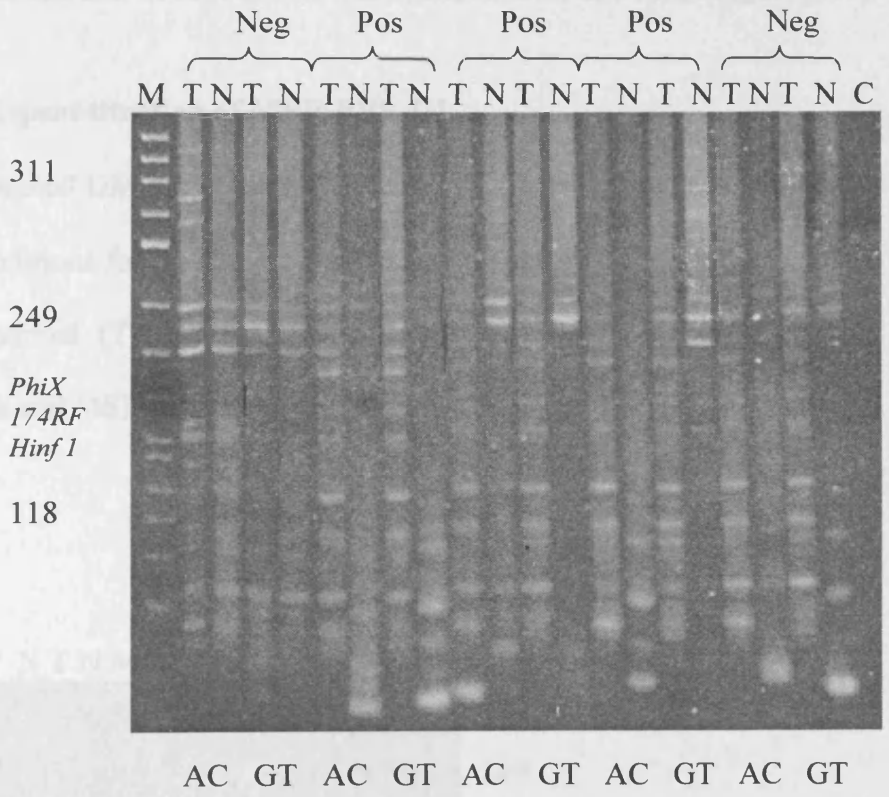
3.3.2 MVR-PCR III for tumour / normal pairs

MVR-PCR II was used to compare DNA extracted from tumour and normal tissues from freshly frozen sporadic colorectal neoplasms with known MSI status.

Whilst interpretable bands appeared in titration of MVR-PCR III, they were inconsistent in the assessment of DNA from tumour / normal pairs. Cases known to be MSI negative by microsatellite analysis demonstrated similarity between the products of tumour and normal DNA as would be expected, but there was excessive homogeneity in the band positions between the DNAs of different individuals suggesting a spurious result (Figure 3.17).

Figure 3.17 MVR PCR III for tumour/normal pairs

DNA from fresh snap-frozen tumour (T) / normal (N) tissues. The marker (M, lane 1) is PhiX174 Hinf I. C = control: no DNA. Neg = DNA from cases that tested negative for MSI. Pos = DNA from cases that tested positive for MSI (MSI-high).

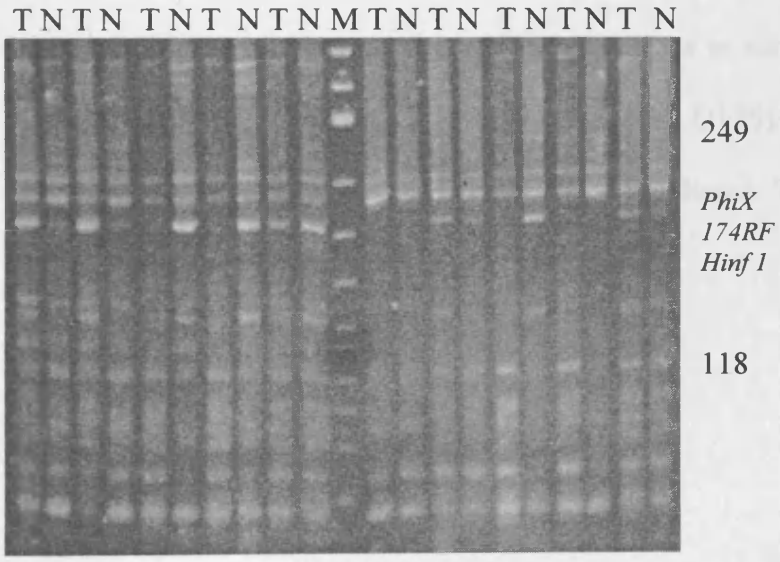


3.3.3 Repeat titration of MVR-PCR III

Conditions favoured reactions containing 2.0 or 2.5 µl $MgCl_2$ and no DMSO but the yield of products was generally poor. Attempts to use the optimal conditions failed on repeated occasions and MVR PCR III was abandoned on this basis (Figure 3.18).

Figure 3.18 Repeat titration of MVR PVR III

No reaction contained DMSO; either 2.0 or 2.5 µl $MgCl_2$ per reaction is used as per the optimum conditions for the previous titration (Figure 3.16). DNA extracted from paired tumour/normal (T/N) cases. No differences could be observed between known MSI-high and MSI negative cases. M= Marker.



3.4 Analysis Of DNA From Snap Frozen Tissues

3.4.1 Sporadic Colorectal cancer cases (Table 3.1)

3.4.2 Microsatellite Analysis

Titration of conditions was carried out for each microsatellite marker with varied concentrations of the reagents MgCl and DMSO (Figure 3.19; Methods section 2.3; table 2.1).

There were 25 tumour-normal pairs from cases of sporadic carcinoma of the colon or rectum. These were analysed with 10 microsatellite markers. Two (8 %) revealed differences in the electrophoretic mobility between PCR-amplified microsatellite fragments of tumour and normal tissue for at least two microsatellite markers analysed. In case number ten there were alterations in six out of ten microsatellite markers: D5S404, D17S787, D11S904, D15S120, D13S175, and D10S197.In case number 25 markers D8S255 and D10S197 were altered. These alterations imply a MSI positive phenotype.

Figure 3.19 Microsatellite Titration for markers D2S123 and D7S519 using control DNA.

Lanes 1-10 represent different concentrations of DMSO and $MgCl_2$ to determine the optimum (Table 2.1, methods). The middle lane contains the DNA size marker (M, PhiX 174RF, Promega). For D2S123 conditions favoured 2.5 mM $MgCl_2$ and 1% DMSO (Lane 6; Table 2.1). For D7S519, Optimum conditions were unaffected by DMSO and were best for 2.0 mM $MgCl_2$ (Lane 3).

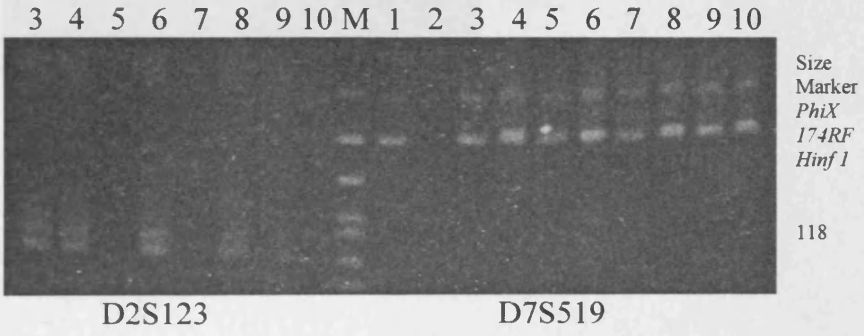


Figure 3.20 Microsatellite analysis at D5S404 for four tumour/normal (T/N) pairs.

C = control lane with no DNA. M = size marker (ψX174 HinfI (Promega, UK)). No differences are observed between tumour and normal DNAs (MSI negative).

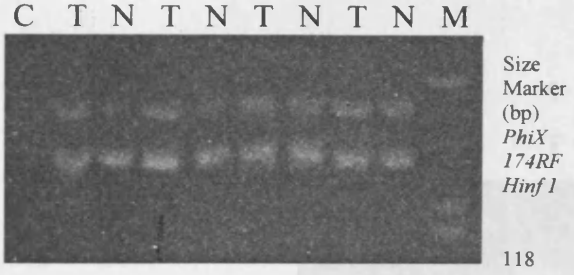


Figure 3.21 Microsatellite analysis at D11S904 (11) and D17S787 (17) for six tumour/normal (T/N) pairs.

M = size marker (ψX174 HinfI (Promega, UK)). No differences are observed between tumour and normal DNAs (MSI negative).

Faint product and no product at the arrowed sites were thought to be technical failure rather that representative of MSI or LOH.

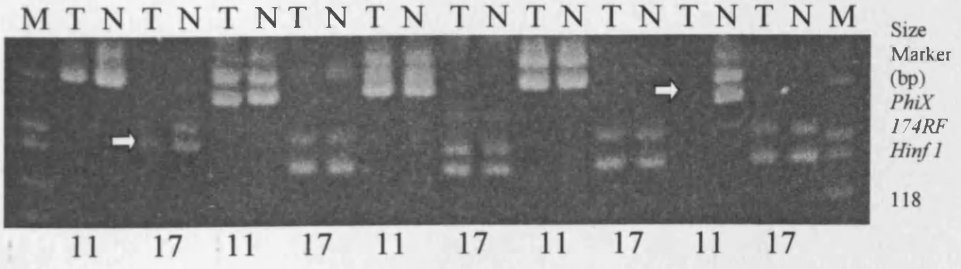
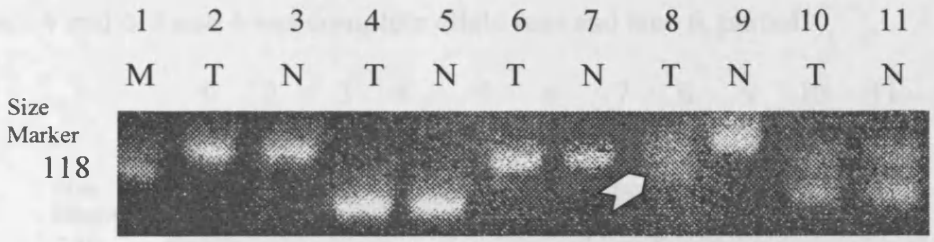


Figure 3.22 Microsatellite analysis of 5 tumour/normal (T/N) pairs at D8S255.

Lane 1 contains a size marker (M, φx174 Hinf (Promega)). Lane 8 contains a tumour with the band smeared over a range of sizes (arrow). This change implies microsatellite instability at this locus.



3.4.3 Loss of heterozygosity

Complete or partial allelic deletions could be analysed in those tumours not showing microsatellite instability, as LOH interpretation is difficult in this context. Nine tumours exhibited LOH in at least one marker and LOH was most frequently observed in chromosomes 5 and 8 (three tumours), and in chromosomes 11 and 17 (two tumours) Figure 3.24).

Figure 3.23 Microsatellite analysis at D11S904 for 5 tumour / normal (T/N) pairs.

Lane 1 contains a size marker (M, ψX174 Hinf I (Promega, UK)). No replication error was found here, however there is evidence of loss of heterozygosity shown by the disappearance of an allele in the lane represented by tumour tissue when compared to normal tissue. This represents loss of heterozygosity (LOH) and is evident in lanes 4 and 6. Lane 4 has complete allele loss and lane 6, partial.

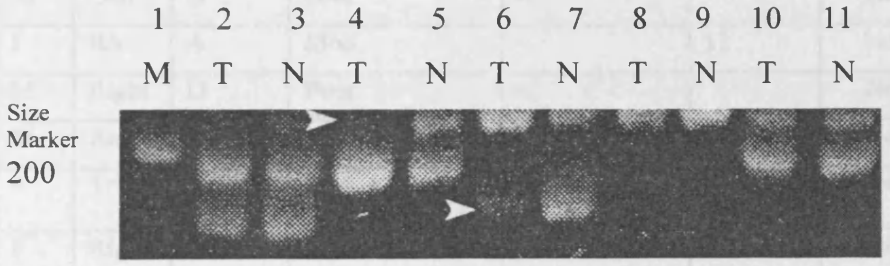


Table 3.1 Results for sporadic colorectal cancers.

Site (of tumour): Sig. = sigmoid, Rec. = rectum, R.S. = recto-sigmoid, Tr. = transverse. Grade: Poor = poorly differentiated, Mod. = moderately differentiated, well = well differentiated, Dyspl. T.A.. = dysplastic tubulovillous adenoma, Dyspl. Ad. = dysplastic adenoma. MSI / LOH LOCI: 2 = D2S123, 5 = D5S404, 7 = D7S519, 8 = D8S255, 10 = D10S197, 11 = D11S904, 13 = D13S175, 15 = D15S120, 17 = D17S787, 18 = D18S58.

Patient Number	Age	Sex	Site	Dukes' Stage	Tumour differentiation	MSI (altered loci)	LOH (loci)	MVR-PCR
1	79	M	Sig	C	Poor	-		Neg
2	83	M	Rec	C	Mod	-	11	Neg
3	80	F	Rec	B	Mod	-		Neg
4	77	F	Rec	B	Well	-		Neg
5	76	F	Rec	B	Mod	-		Neg
6	75	M	Left	B	Poor	-		Neg
7	67	F	RS	A	Mod	-	11	Neg
8	79	M	Right	D	Poor	-		Neg
9	72	M	Rec	B	Mod	-	5	Neg
10	84	F	Tr	C	Mod	2,5,8,10,11, 13,15,17,18		Pos
11	69	F	Right	B	Mod	-		Neg
12	75	M	Left	C	Poor	-	5,8,17	Neg
13	80	F	Right	B	Well	-		Neg
14	67	M	Sig	B	Poor	-		Neg
15	74	M	Rec	C	Mod	-		Neg
16	68	F	Rec	C	Mod	-		Neg
17	50	F	Rec	Polyp	Dyspl TA	-		Neg
18	75	M	RS	D	Mod	-		Neg
19	69	M	Tr	B	Well	-	5	Neg
20	77	M	Sig	C	Mod	-	8,17	Neg
21	57	M	Sig	Polyp	Dyspl Ad	-		Neg
22	58	F	Rec	D	Poor	-		Neg
23	65	M	Rec	C	Mod	-	8	Neg
24	67	M	Rec	C	Poor	8,15		Neg
25	78	F	Tr	B	Mod	-		Neg
26	63	F	Right	C	Mod	7,15,17,18	2,13	Pos
27	77	M	Sig	D	Poor	10,13,15,18	2,8	Pos

Tumour and normal mucosa was collected from 33 cases of colorectal neoplasia. Of these, 27 were sporadic and 6 from suspected HNPCC patients (most either MMR gene mutation or Amsterdam criteria positive family history).

3.4.4 The Sporadic CRC Group

In this group the average age of patients at operation was 71.8 years (range 50 to 84). There were 15 males (55.5%) and 12 females. Eleven of the neoplasms were in the rectum (41%), with 7 (26%) in the sigmoid or recto-sigmoid, 2 (7%) in the left, 3 (11%) in the transverse and 4 (15%) in the right colon. There was one Dukes' A carcinoma. Both adenomas had features of severe dysplasia. Of the remainder, 10 were Dukes' B (37%), 10 Dukes' C (37%), and 4 Dukes' D (15%) (Table 3.1).

Analysis of 10 microsatellite markers in the 27 sporadic tumours revealed 3 (11%) to demonstrate MSI+ in equal to or greater than 40% of loci (MSI-high) (Table 3.1). Loss of heterozygosity (LOH) was also observed with some markers (Table 3.1). An additional one tumour (3%) demonstrated MSI in less than 40% of markers (MSI-low).

After separation of the products of MVR-PCR on a gel by electrophoresis and visualisation, complimentary ladders are revealed from each of the two repeat unit specific primers, for normal and tumour DNA. Therefore four lanes for each patient are seen on the gel (Figure 3.13). MVR-PCR for the 3 MSI-high sporadic tumours demonstrated consistent alterations compared with normal DNA on several occasions. These alterations included smearing and band loss of the products from tumour DNA. LOH at the D7S21 locus in the tumours can be ruled out as in some positions in the gel both AC or GT bands co-exist. This, the co-migration of AC and GT bands is only possible if 2 distinct products of the same size are produced from

2 loci. With the 23 MSI negative tumours and the one MSI-low tumour the above marked changes were not seen between the products from tumour and normal DNA (Figure 3.3.4) although an occasional aberrant band was observed in tumour DNA.

3.4.5 The HNPCC Group

In the 6 cases with suspected HNPCC, 3 were shown by sequencing to have mutations in the MMR gene hMLH1 and 1 in hMSH2 (Table 3.2). In the remaining 2 a mutation could not be identified in spite of intensive analysis, including hPMS2. One had a kindred satisfying the Amsterdam criteria (Liu, Parsons, Papadopoulous, *et al.*, 1996) and the remaining one had familial and phenotypic features highly suggestive of HNPCC but a family history failing to satisfy the Amsterdam criteria.

Five of the 6 suspected HNPCC cases yielded positive results from analysis by MVR-PCR II (Figure 3.13 and Table 3.2) with band loss and smearing of product from tumour DNA. Only 2 of the 6 demonstrated MSI in 4 out of 10 loci (MSI-high) and an additional one in 3 out of 10 (MSI-low). Of the three that were MSI stable, 2 had mutations in hMLH1 (Table 3.2). The one patient in this group who was stable on MVR-PCR was MSI stable and no mutation could be detected.

In summary, therefore, 8 out of the 33 patients had mismatch repair defects as demonstrated by either MSI or MMR mutation. All 8 of these patients demonstrated minisatellite instability using MVR-PCR.

Table 3.2 Results of MVR-PCR and microsatellite analysis on patients with suspected HNPCC. All except patient 33 demonstrated features suggesting instability at minisatellite loci. Two of the three cases with MMR gene mutations were MSI stable (neg). Of the two Amsterdam positive (MMR negative) cases, one was MSI high. For MSI loci see Table 2.

Patient number	Age	Sex	Site	Dukes Stage	Amsterdam Criteria	MSI (loci aletered)	MMR Gene mutations	MVR -PCR
28	30	M	R colon	B Later liver metastases	Neg	2,7, 11,15	None detected	Pos
29	68	F	R colon	C	Pos	7,13,15	HMLH1 exon 19 stop mutation G > A @ base 2135	Pos
30	48	F	R colon	A	Pos	Neg	HMLH1 exon 1 2 bp insertion +AA @ base 105	Pos
31	62	M	R colon	B	Pos	2,10, 11,13, 15,18	HMSH2 exon 15 7bp deletion CTAATTTCC C to CCC @ codon 836	Pos
32	35	M	R colon	B	Neg	Neg	HMLH1 exon 16 3bp deletion GAAGAAGA AG to GAAGAAG @ codon 616/617/618	Pos
33	61	F	Sig. colon	N/K	Pos	Neg	None detected	Neg

3.5 *Analysis of DNA extracted from wax blocks*

3.5.1 Introduction

There were 41 sets of wax block-embedded specimens from 41 consecutive cases undergoing surgery for colorectal adenocarcinoma whose family history was as yet undetermined. The average age at operation was 71.7 years old (range 38 to 86). The sites of tumours were right colon, 8 (20%), left colon, 1 (2%), sigmoid colon, 5 (12%) and rectum or recto-sigmoid, 27 (66%). The pathological staging was: Dukes' stage A, 9 (22%), Dukes' stage B, 16 (39%), Dukes' stage C, 9 (22%) and Dukes' stage'D', 7 (17%). The wax blocks of 24 out of 25 Dukes stage A and B cases could be segregated macroscopically in to blocks containing either tumour or normal tissue by a consultant pathologist (C.duB.).

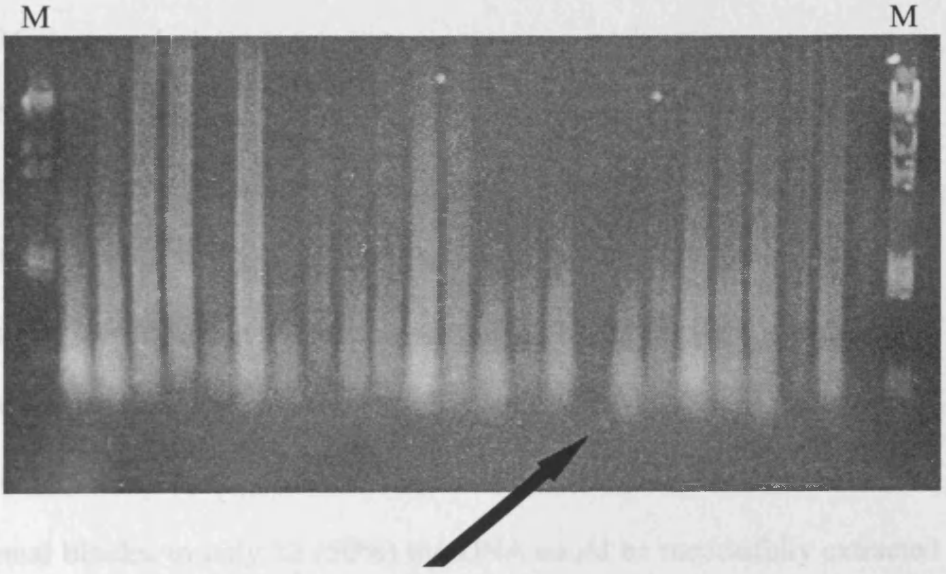
3.5.2 Quantitative analysis

Extracted samples of DNA were separated on an agarose gel by eletcrophoresis and visualised by immersion in an ethidium bromide solution and ultraviolet excitation (Figure 3.24). This showed that extraction had failed in some cases. Yield was variable in the remainder. Product size was spread over a wide range. DNA extraction from both tumour and normal tissue appeared to be successful in terms of sufficient yield of products of appropriate sizes for analysis in 13 of 24 cases (54%). Of the remainder, a further 6 (25%) contained some tumour and normal DNA evident but not sufficient for further analysis and 5 (21%) either tumour or normal DNA for each case.

Figure 3.24

Quantitative analysis of DNA extraction by separation on agarose gel with electrophoresis

A photograph of an agarose gel under ultraviolet light. Each lane contains DNA fragments of all sizes separated by electrophoresis. The empty lanes are those in which extraction has failed (labelled). The marker is Lamda Hind (M).



3.5.3 Qualitative analysis

i. *GAPDH gene analysis*

Control PCR for the GAPDH gene gave poor results with very few visible bands of low intensity (Figure 3.25).

ii. *CYP2D6 gene analysis*

Of the 26 DNA samples from 13 tumour / normal tissue pairs that showed adequate quantities of DNA by electrophoresis on agarose, 10 (77%) had successful PCR reactions at locus CYP2D6 in that bands could be seen (Figure 3.26). Of those with poor quantitative results from extraction, 2 out of 6 results were positive. The remainder were not analysed as both tumour and normal DNA is required for comparison in microsatellite or minisatellite instability analysis. Therefore out of 24 tumour / normal blocks, in only 12 (50%) the DNA could be successfully extracted from both tumour and normal tissue in terms of its quantity and quality.

Figure 3.25

Qualitative analysis of DNA extraction by amplification of the GAPDH gene.

A photograph of a gel showing the results from tumour / normal pairs. Faint bands only are present for some of the lanes (arrowed). The marker (M) is ψX174 HinfI (Promega, UK).

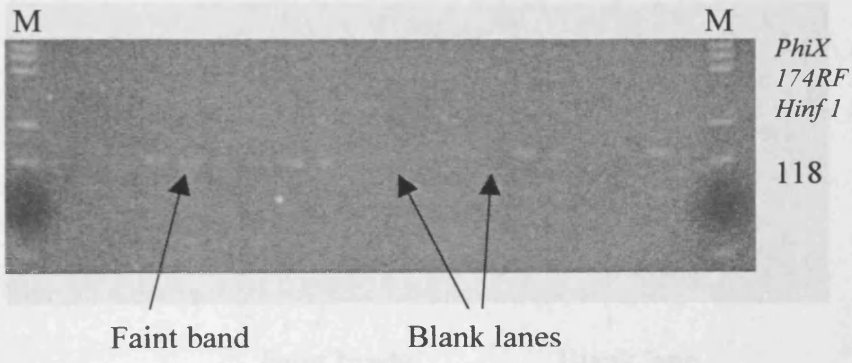
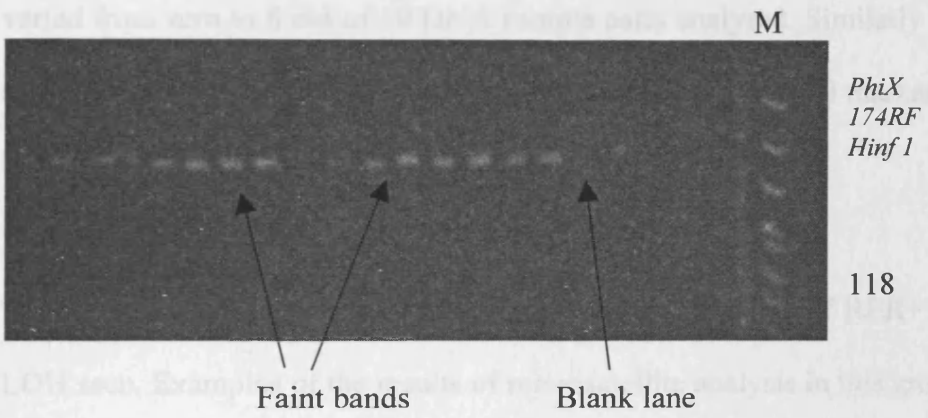


Figure 3.26

Qualitative analysis of DNA extraction by amplification of the CYP2D6 gene.

A photograph of a gel showing the same tumour / normal pairs as figure 3.25. More definite bands are present in lanes with faint bands are present in other positions. The marker (M) is ψX174 HinfI (Promega, UK).



3.5.4 Microsatellite analysis

DNA successfully extracted form 10 tumour / normal tissue pairs was subjected to microsatellite analysis by PCR using the 10 markers used in other experiments in this project. The results in terms of the number of visible bands on a gel was variable (Table 3.3). PCR at loci D7S519, D15S120 and D18S58 yielded no products after PAGE. The number of interpretable results per microsatellite marker varied from zero to 8 out of 10 DNA sample pairs analysed. Similarly microsatellite analysis was possible in even the best case for only 5 out of 10 markers (case no. 1, table 3.3).

In no case were any alterations in tumour DNA suggestive of RER+ phenotype or LOH seen. Examples of the results of microsatellite analysis in this group are shown in figure 3.27

Table 3.3 Quantitative results of microsatellite analysis of DNA from 10 tumour / normal tissue pairs at 7 microsatellite loci.

This table documents the yield of interpretable results Those cases where analysis resulted in visible bands for both tumour and normal DNA are represented by a '+'. In no case was LOH or evidence of RER+ phenotype seen. '+/-' is an equivocal result, '-' is a negative result. No results were obtained at D7S517, D13S175 or D18S58 which were excluded from this table.

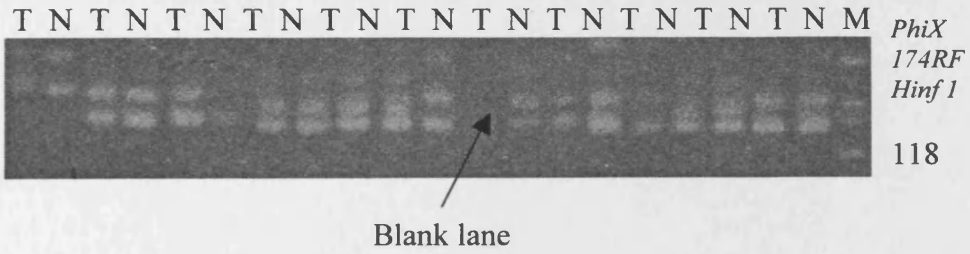
Case number	D2S123	D5S404	D11S904	D17S787	D10S197	D8S255	D13S175
1	-	+	+	+	+	+/-	+
2	+	+	+	+	-	-	+
3	-	-	-	+/-	-	-	-
4	-	+	+	+	-	+/-	+
5	-	+	+	+	+/-	+/-	-
6	-	-	-	-	-	-	+/-
7	-	-	+	+	+/-	-	+/-
8	-	-	-	+	-	-	-
9	-	+	-	+	+	-	-
10	-	-	+	+	+	+/-	-

Figure 3.27

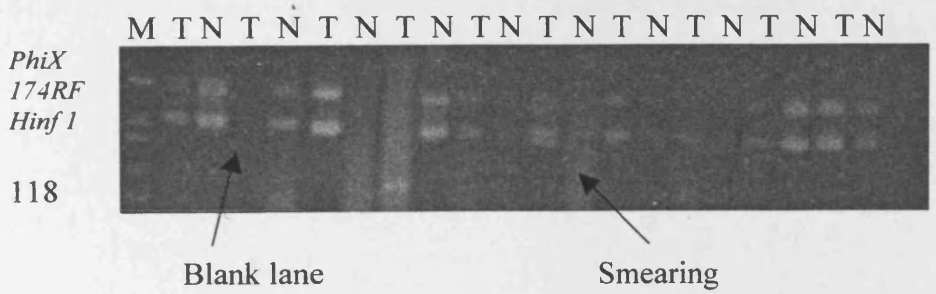
Microsatellite analysis of DNA extracted from wax blocks

Three photographs of gels showing the results of microsatellite analysis at (a) D17S787 of 11 tumour/normal pairs (T/N) (b) D10S197 of 10 tumour/normal pairs (T/N) and (c) D5S404 of 4 tumour/normal pairs (T/N). The band intensity in all cases is poor and there are several blank lanes. The marker (M) is ψX174 HinfI (Promega, UK).

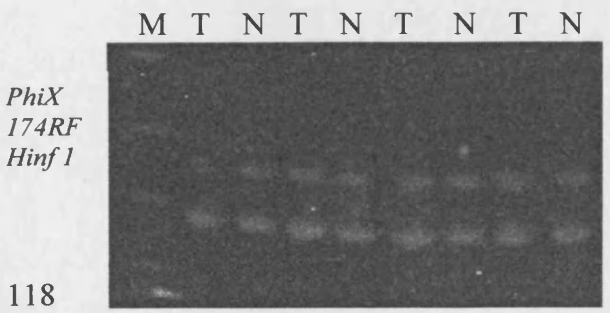
(a) D17S787



(b) D10S197



(c) D5S404



3.5.5 MVR-PCR

MVR-PCR II was carried out for the DNA extracted from the 10 tumour / normal tissue pairs with the most consistent results from extraction and microsatellite analysis. No visible bands were apparent after PAGE (photograph not shown).

3.6 Results of mutation analysis in suspected HNPCC

108 were analysed during the study period. These were all close relatives of affected cases who were offered colonoscopic screening. DNA extracted from peripheral blood was used in 106 cases. In the remaining 2, affected relatives were deceased; so colonic normal mucosa was used in one case and in the other, only tumour tissue was available. Of the 108, 17 (16 per cent) had detectable mutations in one of 17 variants of hMLH1 or hMSH2, of which 11 (10 per cent) were protein truncating. Among those with Amsterdam positive family histories (n=25), 10 mutations were detected. The yield for mutation detection in Amsterdam positive families was higher (Methods 2.5.2) with 32 per cent having clinically significant mutations (Table 3.4)

Table 3.4 Mutation analysis in relatives referred to the Wessex Regional Genetics service who were offered colonoscopy (Section 2.5.2)

Category	All cases where close relatives offered screening coloncoscopy	Group 1 Amst +	Group 2 Amst +	Group 3 FH +	Group 4 Isolated early onset	Group 5 Multiple neoplasia
Total studied	108	25	13	30	26	14
Clinically useful mutations detected (%)	17 (16%)	10 (40%)	3 (23%)	3 (10%)	1 (4%)	0
Mutations detected (%)	11 (10%)	8 (32%)	2 (15%)	0	1 (4%)	0

Chapter Four

Discussion and Conclusions

4.1 Introduction

Colorectal cancer develops as a result of a series of imbalances in cellular growth and alterations in differentiation. These critical characteristics are controlled by numerous positive and negative regulators of cell growth and include the proteins encoded by tumour suppressor genes. Mutational de-activation of tumour suppressor genes results in malignant transformation. By contrast, oncogenes which are normally inactive, when mutationally activated during carcinogenesis also lead to malignant transformation when they are inappropriately expressed. Alterations in oncogenes and tumour suppressor genes form the basis of the adenoma to carcinoma sequence.

Subsets of colorectal cancers, including those due to HNPCC manifest genome-wide DNA instability. This is conventionally detected by PCR amplification of regions of DNA that contain short repeated (mono- di- or tri- nucleotide) sequences of base pairs known as microsatellites. In those cancers that are affected by microsatellite instability, amplification reveals characteristic differences between tumour DNA and DNA extracted from normal tissues. Microsatellite instability (MSI) is a molecular phenotype often caused by defects in mismatch repair genes. Mutations in MMR genes are inherited in an autosomal dominant fashion in the germlines of families with HNPCC. In sporadic CRC that have MSI, it is thought to arise through spontaneous germline mutations or somatic mutations. Whilst DNA repair mechanisms that are controlled by MMR genes have been shown to function in vitro in sequences where the repeated length is up to 4 base pairs long, DNA instability has been discovered in longer repeats in colorectal cancers, including

minisatellites (Hoff-Olsen, Meling, and Olaisen, 1995). Therefore MMR genes or similar processes may influence the excision and repair of damage during replication in longer DNA repeats. Therefore it is a logical step to extend the study of genomic instability from microsatellites, to longer repeats or minisatellites.

4.2 Microsatellite instability (MSI)

MSI is a feature of HNPCC associated tumours and some sporadic colorectal carcinomas indicating a defect in DNA mismatch repair in that tumour. It consists of alterations in length of repeated sequences due to strand slippages during replication that are not repaired. They may be detected by PCR amplification of a microsatellite locus.

Conventionally the DNA instability observed in colorectal cancer is detected by the PCR amplification of microsatellites which are di- and tri-nucleotide repeats found scattered throughout the human genome, characteristically, in non-coding DNA sequences (Lindblom, Tannergård, Werelius, & Nordenskjöld, 1993). Their high degree of polymorphism has proved invaluable as a tool for a wide variety of genetic applications (Wooster, Cleton-Jansen, Collins, *et al.*, 1994). Currently over 2000 microsatellite loci have been found in the human genome and this number continues to increase (Weissenbach, Gyapay, Dib, *et al.*, 1992). Since microsatellite alleles are short in sequence length (up to 100bps), individual loci may not necessarily manifest DNA instability even though the genome as a whole may be affected. Therefore several microsatellite loci are amplified and analysed per case and if a proportion manifest differences between tumour and normal DNA,

then microsatellite instability (MSI) is inferred. The choice and number of microsatellite loci selected for analysis is likely to affect the sensitivity of the assay. Until recently (Boland, Thibodeau, Hamilton, *et al.*, 1998), there has been no consensus as to how many microsatellite loci must be altered to diagnose the MSI phenotype. The likelihood of alteration in microsatellites varies from 55 to 91 per cent in different loci in familial colon cancer (Lothe, Peltomäki, Meling, *et al.*, 1993), and between 12 and 28 per cent in sporadic colorectal tumours (Aaltonen, Peltomäki, Leach, *et al.*, 1993; Thibodeau, Bren, and Schaid, 1993). MMR gene mutations can be found in approximately 70% of patients with familial colorectal cancer (Liu, Parsons, Papadopoulos, *et al.*, 1996). In sporadic colorectal cancer that are MSI-high, constitutional MMR gene mutations are not commonly found but there normally mutational inactivation of MMR genes. This results in loss of hMLH1 expression and is due to epigenetic transcriptional silencing by methylation of CpG in the hMLH1 promoter (Deng, Chen, Hong, Chae, and Kim, 1999; Herman, Umar, Polyak, *et al.*, 1998). This may explain the somatic acquisition of defects in MMR genes observed in some sporadic CRC.

The finding of MSI in the tumours of suspected HNPCC cases is not sufficient for diagnosis but it may be useful as an initial screening tool, as the widespread use of direct sequence analysis is time and cost-consuming (Bocker, Diermann, Friedl, *et al.*, 1997).

Before the NCI consensus there was no general agreement as to the number and type of microsatellites required to classify tumours with possible MSI (Bocker, Diermann, Friedl, *et al.*, 1997). A number of publications classified tumours as MSI

when one of two loci were altered (Bocker, Diermann, Friedl, *et al.*, 1997). This would now be defined as MSI-low (Boland, Thibodeau, Hamilton, 1998). Most leading authors in this field used at least four microsatellite markers (Table 1.3, Introduction) to detect MSI and diagnosed a positive result (MSI-high) when at least two of the markers show a difference between tumour and normal DNA. More rigorous studies have defined microsatellite instability if at least 20% of no fewer than seven markers were altered. In this study where 10 markers were used, MSI-high was defined when at least three markers were altered, as in most other studies. This work was carried out before the recent consensus that suggested a reference panel of 5 microsatellite markers (Boland, Thibodeau, Hamilton, 1998). The utility of the consensus approach has yet to be fully determined, but 5 of the 10 markers in this study were either part of the reference panel (D2S123), or part of the 19 additional loci (D18S58, D10S197, D13S175, D7S519). The additional loci were recommended where clarification between MSI-negative and MSI-low is required, for example, where strong evidence (Amsterdam positive family history, positive MMR gene mutation) is found to suggest the presence of MSI, but none is found using the reference panel.

The microsatellite markers used in this study were already in use in our laboratory and in the Wessex Regional Genetics laboratory. Since the NCI recommendations, the reference panel has been used, with the additional markers where appropriate.

A variety of techniques have been applied to the detection of microsatellite instability (Bocker, Diermann, Friedl, *et al.*, 1997). PCR products are visualised on a gel by silver or ethidium bromide staining or as a radioactive PCR product using

^{32}P-labelled primers. Automated methods are also described using a DNA sequencer. Inevitably a variety of techniques lead to differences in the interpretation of band pattern, which cannot be explained entirely on the basis of technique.

The PCR assay used in this study consisted of PCR followed by non-denaturing PAGE and staining with ethidium bromide. This differs from many other groups who use denaturing PAGE and radio-labelling for band visualisation. Our technique proved relatively simple to perform and removes the need for radioactivity and autoradiography.

MSI-high colorectal tumours have distinct pathological characteristics. These include a predilection to the right colon, poor differentiation, extracellular mucin production and Crohn's like lymphoid reaction. These characteristics are shared between sporadic CRC and those associated with HNPCC. Therefore the MSI in sporadic CRC may, as in HNPCC, be also the result of either *denovo* germline or somatic mutations in mismatch repair genes. Survival among patients with sporadic CRC whose tumours show two or more microsatellite alterations compared to their germline is better than survival of MSI negative colon cancer patients (Thibodeau, Bren & Schaid, 1993, Lothe *et al.*, 1993). Both of these studies however were among patients with Dukes' stage B and C disease and did not compare survival for stage matched MSI-high and MSI-negative colorectal cancer.

MSI status may also be of importance in chemotherapeutic drug resistance. MSI positive cancers may also show increased drug resistance to alkylating agents (Barnch, Aquilina, Bignami & Karran, 1993; Kat, Thilly, Fang, Longley, Li &

Modrich, 1993). Further studies are needed to determine the differential responses of colorectal cancers to standard therapies such as 5-Fluorouracil-based chemotherapy.

Most patients with sporadic CRC do not have germline mutations in any of the recognised mismatch repair genes (Liu, Nicolaides, Markowitz, *et al.*, 1995a) but detection of instability may represent a useful marker for a subset of patient more likely to harbour a germline mutation in a mismatch repair gene. In this study 15% of sporadic CRC manifested instability but only 10% of these had a hMLH1 or hMSH2 mutation (Liu, Nicolaides, Markowitz, *et al.*, 1995a). Defects in other mismatch repair systems may therefore be involved in the remainder.

In this study, three of the 27 sporadic CRC cases were found to be MSI-high. One tumour was positive in 9 out of 10 markers and the other in two in four loci. Two of these tumours were located proximal to the splenic flexure – a feature typical of sporadic tumours with MSI. In view of the patients age (84 and 78) they are unlikely to represent undetected HNPCC kindreds. The other was in the sigmoid colon of a 77 year old. Amongst the six putative and proven HNPCC cases, three were found to be MSI-high. We may have detected MSI in the negative cases if the consensus panel markers had been used. In particular we (unpublished) and others (Shitoh, Konishi, Masubuchi, Senba, Tsukamoto and Kanazawa, 1998) have found the BAT26 locus, a mononucleotide repeat, informative in confirming the presence of MSI. Nevertheless the conclusions of this study would have been unaffected by using these probes as we found that microsatellite instability was only associated with MSI-high tumours.

4.3 Mismatch repair and DNA instability

Mutations in genes responsible for DNA mismatch repair are a feature of HNPCC and some sporadic colorectal cancers. DNA mismatches occur during mitotic replication either by incorrect base pairings or by slippage of DNA polymerase on the template strand. Slippage is most likely to occur during replication of long repeating sequences and results in more or fewer copies of the repeat. (Strand, Prolla, Liskay, and Petes, 1993). The mechanisms for DNA mismatch repair have been extensively characterized in prokaryotes (*E. coli*) (Cleaver, 1994). The mutS protein recognizes and binds to mismatched DNA sequences. The mutL and mutH then function with the bound mutS protein. MutH finds a single strand nick in the strand containing the incorrect nucleotide, which is followed by helicase II unwinding the DNA. Thereafter a bi-directional nuclease removes the bases between the nick and the mismatched pairs, and the DNA polymerase fills in the gap with the correct sequence. Homologous repair systems have also been identified in eukaryotes (Prolla, Christie and Liskay, 1994). These repair processes have also been found in human cell extracts (Thomas, Roberts, and Kunkel, 1991) where they have the ability to repair mismatch loops up to 16 bases (Umar, Boyer, and Kunkel, 1994). Loops of this size may develop if the mismatched sequence is longer than the 2 to 3 base pair sequences observed in bacteria. Therefore, it is known that accurate strand specific DNA mismatch repair mechanism exist in humans that have been shown in vitro to repair loops of up to 16 bases. While the effects of defective mismatch repair have not previously reported in minisatellite loci, mechanisms exist for the repair of DNA sequences longer than that observed in microsatellites. Furthermore, alterations in minisatellites alleles have been observed in tumours,

particularly colorectal carcinomas (Hoff-Olsen, Meling, and Olaisen, 1995). To date only a limited numbers of genetic alterations have been examined in the context of MMR deficiency, specifically those associated with bi and tri-nucleotide repeats. Although the mechanisms may not be fully understood it appears that MMR deficiency may influence sequence heterogeneity in longer repeat sequences, such as those detected by MVR-PCR. In this study using MS31A, the repeats were still relatively short (20bps). Further work is required to determine whether the same phenomenon may be observed in other minisatellites, including those with a longer repeat sequence. This will give insight into whether the range of genetic alterations found in MMR deficiency need to be revised.

4.4 MVR-PCR

4.4.1 Introduction

Minisatellites, like microsatellites are also regions of DNA repeats which feature hypervariability in allele length also making them suitable for linkage analysis and DNA fingerprinting (Jeffreys, Wilson and Thein, 1985b). By contrast to microsatellites the DNA repeats in minisatellite loci are 9-45bps in length but in both their allelic variability is a feature of the number of tandem repeats (Wong, Wilson, Patel, Povey, and Jeffreys, 1987). Minisatellites have a total array size of 0.5-30kb and are also widespread in the human genome (Jeffreys, Wilson and Thein, 1985a). Multiple or single minisatellite loci may be amplified by PCR (Jeffreys, Wilson, Neumann, and Keyte, 1988). The usefulness of the information generated is limited by error prone estimates of allele length and equivocal allele identification (Jeffreys, MacLeod, Tamaki, Neil, and Monckton, 1991b). An alternative method of typing minisatellite loci is to assay the sequence variation of tandem repeat units. Minisatellite alleles vary not only in the repeat copy number but also in the interspersion pattern of variant repeat units along alleles (Jeffreys, Neumann, & Wilson, 1990). In certain rare loci two classes of repeat unit differ only by a single base substitution that either creates or destroys a *Hae*III restriction endonuclease site (Neil and Jeffreys, 1993). MVR-PCR is a further means of analysis of DNA polymorphism in minisatellite alleles by the study of repeat unit sequence variation (Jeffreys, MacLeod, Tamaki, Neil, and Monckton, 1991b).

Repeat unit mapping (MVR-PCR) assays the interspersion pattern of variant repeat units along minisatellite alleles (Jeffreys, Neumann & Wilson, 1990). It uses two separate reactions which each generate a stable set of products of variable length which can be used to create, after separation by electrophoresis, an extraordinarily variable but unambiguous pattern used in a variety of applications (Jeffreys, MacLeod, Tamaki, Neil and Monckton, 1991b). For maximum informativeness in a MVR locus, the two classes of repeat unit need to be of similar frequency along the allele, and they need to be substantially intermingled (Jeffreys, Neumann & Wilson, 1990). There also needs to be sufficiently high allele variability in the interspersion pattern such that the locus is useful in generating individual profiles for forensic analysis. The MS31A locus conforms to these requirements (Neil & Jeffreys, 1993).

4.4.2 MVR-PCR I

The repeat unit specific primer sequences for MVR locus MS31A (Neil & Jeffreys, 1993) were used initially to evaluate the technique (Appendix 7). Titration was performed by alteration of reagent concentrations and PCR conditions to improve the result in terms of product yield. Although some generation of shorter products was observed, the results were generally inconsistent and poor, favouring the shorter bands and indicating internal priming. The first annealing cycle of MVR-PCR consumes all the repeat unit specific primer, which is at low concentration. If any remains some short generation product may be formed. Despite lowering the concentration further, and altering other parameters internal priming still occurred and no products longer than 300bps were generated. Primers for MS31A were therefore redesigned for MVR-PCR II (Appendix 8).

4.4.3 MVR-PCR II

MVR-PCR is the choice for DNA fingerprinting and can also be used for the detection of DNA instability as present in many tumours (Coleman, Gough, Bunyan & Primrose, 1997) However the current technique is lengthy and labour intensive requiring blotting and autoradiography (Jeffreys, MacLeod, Tamaki, Neil & Monckton, 1991; Neil, & Jeffreys, 1993). Ostensibly this is because of the design of the assay only resulting in very small amounts of the product. At first it appears that the paucity of the product is an inevitable consequence of the need to amplify all products equally, independently of length. However by judicious redesign of the TAG portion of the repeat specific primers and hence the TAG primer itself, it has been possible to subdivide the PCR amplification into two distinct but contiguous parts: the first is performed at a low annealing temperature (52 °C), which is below the melting temperature (Tm) of all primers. Although this allows amplification of all available targets, possible selection of the smallest products is stopped by changing the annealing temperature during the exponential phase of amplification to 62 °C which stops priming of the repeat specific primers but allows the TAG and 31A primers to prime and amplification to occur. The design of all primers was specifically carried out to ensure this was the case; in particular the TAG portion of the repeat specific primers (and therefore the TAG II primer itself) was designed so as to ensure no binding could occur between it and the next repeat which would affect the Tm. The new primers are pivotal and allow this technique to be robust and reproducible.

Consistent good results were obtained having redesigned the repeat unit specific primers and driver primer for minisatellite locus MS31A. Amplification of this allele revealed, after ethidium bromide staining of the polyacrylamide gel, at least

20 of the first repeat units in the form of a ladder representing the interspersion pattern of those units along MS31A. For both AC and GT, interpretative comparisons could be made between individual's constitutional DNA and between tumour and normal DNA.

4.4.4 Analysis of sporadic CRC by MVR-PCR II

MVR-PCR II was used in this study to compare DNA extracted from normal and tumour tissue in sporadic colorectal cancer. Cases negative and positive to testing by microsatellite analysis were analysed. No differences between tumour and normal DNA were observed for the MSI negative tumours or the single MSI-low tumour for MVR-PCR II. Consistent alterations of tumour DNA compared to normal DNA were observed for the 3 MSI-high cases. These alterations included smearing and band losses suggesting loss or alterations of sub-units, which we interpret as being instability at this minisatellite locus. These alterations were consistently found on repetition of the technique therefore making artefact unlikely. LOH at the D7S21 locus can be ruled out as in some positions in the gel both AC and GT bands co-exist. This, the co-migration of AC and GT bands is only possible if 2 distinct products of the same size are produced from 2 loci.

4.4.5 Analysis of HNPCC tumours by MVR-PCR II

Of the group of 6 cases with either suspected HNPCC on grounds of family history or with a confirmed MMR gene mutation, three were MSI-high. 2 of these had MMR gene mutations and all three had features suggesting instability on MVR-PCR. The case with MSI and instability on MVR-PCR but no detectable mutation in

hMLH1, hMSH2, hPMS 1 or 2 had no family history of note, but was 30 at the time of presentation with a Dukes' B stage carcinoma of the right colon and, later, liver metastases. This suggests strongly that a defect in mismatch repair exists in this case due to an undetected MMR gene mutation that was either acquired somatically or as a *denovo* constitutional mutation. In the MSI negative group, there were three cases. Two of these had germline MMR gene mutations, but both consistently had features suggestive of instability at the minisatellite locus. Therefore either the DNA instability present in these particular tumours did not affect shorter tandem repeats (STRs) or that the STRs studied here happened not to be affected by the instability affecting other microsatellite loci. This finding together with the variable rate of microsatellite instability detected in both sporadic CRC and those associated with HNPCC imply that MVR-PCR may be a more sensitive technique for the detection of DNA instability in cancer. The third case in this group was was suspected of having HNPCC with an Amsterdam positive history of cancer but no mutation found in either hMLH1 , hMSH2 or hPMS2. There was no evidence for DNA instability for either microsatellite analysis or MVR-PCR. This family may have a genetic predisposition to CRC that is not the result of a MMR gene mutation with consequent genomic instability.

These findings may provide additional insight into the nature of DNA instability in tumours with MMR deficiency.

4.5 Minisatellites and DNA instability

Although germline and spontaneous mutation in minisatellites is common, minisatellite instability as a feature of colorectal cancers is not widely recognised. Minisatellite instability has been previously described in colorectal cancer cell populations (Armour Patel, Thein, Fey and Jeffreys, 1989a; Hoff-Olsen, Meling & Olaisen, 1995). Armour *et al.* (1989a) found that minisatellite mutations occur in gastrointestinal tumours (gastric and colorectal). There were of the form of alterations in allele length at several minisatellite loci. 15 of 51 (29%) of primary gastrointestinal tumours studied displayed mutations at least one minisatellite locus. Three quarters were from colorectal tumours and all but one of the remainder, stomach. The minisatellite loci studied included MS31 (D7S21) and MS32 (D1S8) (Armour *et al.*, 1989). No record was made of the family history of these cases and presumably they were all sporadic which would correspond approximately with the proportion of abnormal alleles.

In the other study of minisatellite instability in 224 cases of CRC, the locus MS1 (D1S7) allele length was probed and 12% were found to be mutated, again in terms of allele length (Hoff-Olsen, Meling & Olaisen, 1995). The repeat units at this particular locus are 9 bp. The majority of mutations took the form of loss of repeat units and this correlated strongly with parallel analysis of tetranucleotide repeats in the same study.

Both of these studies assessed allele length alterations in minisatellites and they establish the phenomenon of instability affecting minisatellites in colorectal cancer. The latter study associates it with instability in short tandem repeats. Importantly,

both studies assessed the length of the minisatellite allele rather than the interspersion pattern of repeat units, but they nevertheless reinforce our theory that instability may not be a feature that affects only microsatellites.

4.6 MVR-PCR II Conclusions

In this study we have shown that MSI-high sporadic colorectal tumours and tumours from patients with germline hMLH1 mutations or other convincing features of HNPCC invariably manifest minisatellite instability by the application of MVR-PCR. This is easily visualised as smearing and band loss comparing normal and tumour DNA. No MSI negative or MSI-low sporadic tumour exhibited these features. Further, in 2 patients with demonstrated hMLH1 mutations and minisatellite instability, MSI was not found even with the use of 10 microsatellite markers. The only patient suspected of having HNPCC, on the basis of satisfying the Amsterdam criteria, who did not manifest instability on MVR-PCR had a left sided cancer, was MSI stable and had no detectable mutation in hMLH1, hMSH2, hPMS 1 or 2. Given the absolute correlation observed between MMR deficiency and MVR detectable abnormalities in the remaining 32 tumours, the absence of MVR-PCR abnormalities provides strong evidence that this patient familial predisposition that does not relate to MMR deficiency.

These observations raise the possibility that MMR deficiency may be easily and reliably detected by MVR-PCR, a technique that utilises a pair of PCR reactions per DNA sample, or 4 per tumour/normal pair. The findings of this study also may give additional insight into the nature of DNA instability in tumours with MMR deficiency.

This study supports the further evaluation of MVR-PCR, which is a reproducible technique involving 4 PCR reactions, as compared to the 10 required (2 per tumour/normal pair) if the consensus panel is used (Boland, Thibodeau, Hamilton,

et al., 1998). However this study was performed using frozen tissue. We are currently performing work to determine whether the technique may be equally applied to formalin fixed tissue, essential if it is to have a widespread application.

4.7 MVR-PCR III

The use of MVR-PCR III to amplify the repeat units of the MS32 locus was of limited success. The results appeared to confirm no differences between normal and tumour DNA in cases found to be MSI negative by analysis with microsatellite markers and with MVR-PCR II but findings in many cases were difficult to interpret and were therefore omitted from overall analysis of MSI status.

4.8 Clinical implications of MVR-PCR

Many symptomatic and asymptomatic individuals are referred to clinicians because of a positive family history of colorectal neoplasia. Single gene mutation analysis in this context is inefficient and ineffective as a tool in the primary assessment of all at-risk individuals because of the low yield of positive results, the costs and the time involved per test. Detection of DNA instability is a means of identifying individuals likely to have an underlying MMR gene mutation thereby improving the yield from MMR gene mutation analysis and limiting the numbers of unnecessary tests.

DNA instability also has implications for prognosis and response to cytotoxic chemotherapy. Various studies have shown that the presence of MSI has a positive influence on prognosis and response to agents such as 5-fluorouracil, camptothecin and etoposide (the latter two are topoisomerase inhibitors) (Esaleh, Powell, McCaul, *et al.,* 2001, Rosty, Chazal, Etienne, *et al.,* 2001, Jacob, Aguado, Fallik, *et al.,* 2001).

It is possible, therefore, that the detection of MSI may have a clinical role in determining prognosis for patients presenting with colorectal cancer and predicting possible response to chemotherapy.

Given that this study demonstrates features suggesting instability in colorectal tumours, and that instability in minisatellites has been demonstrated in such neoplasms, MVR-PCR could therefore offer an additional means to MSI detection to identify cases for possible MMR gene analysis, for prognostic stratification and for potential sensitivity to chemotherapy.

4.9 Non-radioactive MVR-PCR

All previous techniques described for MVR-PCR have used radioactively labelled probes and autoradiography to visualise the PCR products on a gel. This is a lengthy procedure that requires blotting and takes overnight incubation before a result can be obtained. By using non-radioactive primers of modified design and staining with ethidium bromide, we have been able to simplify the technique successfully to one that is safer, less expensive and takes approximately 3 hours to obtain a result.

4.10 Loss of heterozygosity

Loss of heterozygosity was also observed in a number of cases, at microsatellite loci. This took the form of complete loss of one allele of a marker but could be also observed by a partial loss perhaps indicating the presence of contaminating normal cells within the tumour sample or indicating that the tumour was heterogeneous. The question could be resolved by analysis of micro-dissected tumours.

Loss of heterozygosity is a common mechanism of tumour suppressor gene loss in a developing tumour. In this study we found losses on chromosomes 5, 8, 17, 11 and 19. Losses on chromosomes 5 and 17 may include the *APC* gene and p53 gene respectively, both of which are known to be intimately involved in the development of colorectal cancer.

4.11 HNPCC

A family history that conforms to the Amsterdam criteria is the best predictor of the presence of a mismatch repair gene mutation (Nyström-Lahti, Wu, Moisio, *et* al., 1996; Wijnen, Meera Khan, Vasen, *et al.*, 1997; Beck, Homfray, Frayling, Hodgson, Harocopos and Bodmer, 1997). The presence or absence of a clear-cut disease causing mutation is the only clinically useful indicator of the need for screening colonoscopy or prophylactic subtotal colectomy in families where a mutation is identified. Although analysis for MSI may be a useful indicator, as may immuno-histochemical analysis for the absence of hMLH1 or hMSH2 protein (Kim, Piao, Kim, *et al.*, 1998), neither can be used as the basis for decision making in affected families. Analysis for MSI may, however be useful in identifying cases for mutation screening regardless of family history.

For mutation analysis in this study there was a low pick-up rate for mutation analysis among even Amsterdam positive families. 25 of 108 cases referred were Amsterdam criteria positive. Even in this group, only 32 per cent had clinically significant mutations that were detectable in the two commonly affected loci examined. In the other groups examined for MMR gene mutations, the yield of clinically useful mutations was much poorer. All these cases were, on the basis of risk assessment, offered colonoscopic screening. The results of colonoscopy are not available, but if neoplasia is discovered (adenoma or carcinoma). This group, in the absence of a detectable MMR gene mutation could undergo analysis for DNA instability. In order to avoid unnecessary mutation analysis in risk groups that do not fulfil the Amsterdam criteria, testing for DNA instability may help to target those in whom the yield of testing is likely to be higher (Lamberti, Kruse, Ruelfs, *et al.*, 1999).

MVR-PCR may therefore provide an additional option in testing neoplasms within a possible HNPCC kindred or high-risk group. Further analysis of tumours in these groups is required using MVR-PCR to further validate the technique.

4.12 DNA extraction from wax blocks

The wax blocks containing tissue from colorectal cancers were from a consecutive series of 41 cases of CRC from The North Hampshire Hospital, Basingstoke (with permission from R.J.H). There were a disproportionately high number of rectal cancers (66%) probably reflecting the sub-speciality interest of the Colorectal Unit in that hospital. Quantitatively and qualitatively, the results of DNA extraction in this group were disappointing. This may be a reflection of the extraction technique but the methods were repeated on several times with additional measures added aimed at eliminating contamination without significant improvement in outcome. It is possible to account for these problems in terms of DNA degradation. At the time of analysis none of the blocks were more than 3 years old so significant degradation should not have taken place in storage. In some cases the specimens may, at the time of surgery, have been left too long prior to immersion in Formaldehyde solution such that degradation may have occured, or that immersion of a specimen whose lumen was sealed took place such that the tissues on the luminal aspect were fixed less rapidly and degradation could occur.

Of the 41 cases, 24 were Dukes' stage A or B and were selected for initial analysis. Of these interpretable results were obtained from microsatellite analysis for DNA extracted from tumour and normal tissues but no evidence of alterations suggesting microsatellite instability was found. This may have reflected the lack of staining and

microscopic analysis of each block such that tumour and normal tissues could be separated accurately. This however is unlikely for two reasons. First, only Dukes' stage A and B tumours were used initially such that DNA extracted from lymph nodes could be confidently defined as normal. Secondly, tumour blocks were carefully examined by a consultant pathologist (C.duB.) to allow sectioning of those containing only tumour. After the poor results from DNA extraction and microsatellite analysis in the first cohort, work on the Dukes' stage C and D cases was abandoned.

MVR-PCR II analysis of the initial cohort resulted in no interpretable results. This probably reflects the sensitivity of the technique and its requirement for good quality DNA in adequate amounts. Microsatellite analysis is usually possible where DNA fragments of 100-200 bp are present in extracted DNA, such that the DNA extracted from formalin-fixed, paraffin-embedded tissue can easily be used. The fact that the results from microsatellite analysis were poor in this cohort make the success of MVR-PCR here, less likely.

Other studies have shown that it is possible to extract workable DNA from formalin fixed, paraffin embedded tissue sections. More work is required on such DNA samples to determine the effectiveness of MVR-PCR in this context.

Conclusions

In this study we have shown that MSI-high sporadic colorectal tumours and tumours from patients with germline MMR gene mutations or other convincing features of HNPCC invariably manifest minisatellite instability by the application of MVR-PCR.

MVR-PCR at least in some instances appears to be more sensitive markers for the presence of instability.

MVR-PCR may be a useful adjunct to testing for MSI where suspicion of MSI in a tumour exists based on age, family history , histopathology etc.

We have accomplished the technique without radioactively labelled primers or autoradiography to visualise the products of PCR, which is simpler, cheaper and safer.

MVR-PCR alone in the analysis of paired tumour/normal DNA samples for MSI is at least half as cheap financially as using microsatellites using the NCI recommended panel. In this study it was a quarter of the price.

The extraction of DNA of sufficient length and quality for MVR-PCR from wax blocks was not possible in this study but further work is required and is underway.

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Appendix 1

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Appendix 2

1M $Mg\ Cl_2$

Dissolve 203.3g of $Mg\ Cl_3.6H_20$ (Biogene, UK) in 800ml of dH_20. Adjust volume to 1 litre with dH_20.

1M TRIS

121g Tris base (ICN, UK) was dissolved in 800ml of distilled water (dH_20). The Ph was adjusted to 7.6 by adding 60 ml of concentrated HCl at room temperature. The volume was then adjusted to 1 litre with dH_20.

1 x TE (Tris/EDTA)

10 mM Tris HCl (ICN, UK) and 1 Mm EDTA (Ethylene-di-amine-tetra-acetic acid di-sodium salt, BDH, UK) (pH 8.0).

Equilibration of Phenol

Before use, phenol must be equilibrated to pH > 7.8 because DNA will partition into the organic phase at acid pH.

i. Add equal volume of 0.5M Tris.HCl (pH 8.0). Mix well.
ii. Remove upper (aqueous) layer.
iii. Add equal volume of 0.1M Tris.HCl (pH 8.0). Mix well and remove upper layer as before.
iv. Repeat until pH of phenolic layer is pH 7.8.
v. Store under 100mM Tris.HCl (pH 8.0) in a light-tight bottle at 4^0C.

Tris Acetate EDTA (TAE) (Mixture equilibrated to pH 8)

48.4g	TRIS base (ICN, UK)
11.4 ml	Glacial Acetic acid (BDH, UK).
20ml(0.5M)	EDTA

Appendix 3

8% polyacrylamide gel (PAGE)

10ml of a 50:50 solution mixture of acrylamide and acrylamide: bis acrylamide (Anachem, UK)

10ml 5xTBE (54g TRIS, 27.5g Boric acid (BDH, UK), 20ml 0.5M EDTA, 1litre dH_2O)

30 ml dH_2O

200µl Ammonium persulphate (Sigma, UK)

50µl T-MED (N.N.N.N. Tetramethyl Ethylenediamine, Sigma, UK)

0.6% Agarose gel

0.45g agarose (Sigma, UK) and 75ml 1 x TAE

Mix well and microwave on medium for ~ 2 minutes

Allow to cool to lukewarm and pour into mould, allow to set.

Ethidium Bromide solution

2,7 Di-amino-10 ethyl- 9 phenylanthridinium bromide (95%, Sigma, UK), diluted to 100µg/ml.

di Nucleotide triphosphates (dNTPs)

Stock solution (Pharmacia Biotech, UK), at 100mM, diluted by a factor of 10 with dH_2O

Appendix 4

GAPDH gene sequences (Cruachem, UK)

Forward

5'-AGTACGCTGCAGGGCCTCACTCCTT-3'

Reverse

3'-AAGAGCCAGTCTCTGGCCCCAGCCA-5'

CYP2D6 primer sequences (Gough *et al.*, 1990, Oswel, UK)

2D6i4u

AAATCCTGCTCTTCCGAGGC

2D6C3D

CGCCTTCGCCAACCACTCCG

Appendix 5

Primer sequences for microsatellite analysis

D2S123 forward

ACATTGCTGGAAGTTCTGGC

D2S123 reverse

CCTTTCTGACTTGGATACCA

D7S519 forward

ACAGACCAGGACTCAACGCAG

D7S519 reverse

ACAGCCAAGCATTTCTGCTG

D8S255 forward

TTTTGGAATTTCTAGCCTCC

D8S255 reverse

TGAAACCCACAGATATTGGG

D18S58 forward

GCTCCCGGCTGGTTTT

D18S58 reverse

GCAGGAAATCGCAGGAACTT

Appendix 6

Bromophenol blue

15% phycol 40 000 (Pharmacia, UK) in 1x TAE with 1ng Bromophenol blue.

Hydrolink gel mix

2 x MDE gel solution (FMC Bioproducts, UK)	20 mls
5 x TBE (Sigma, UK)	4.8 mls
Distilled water	15.2 mls
10% APS (Ammonium persulphate, Sigma, UK)	240 µl
TEMED (Sigma, UK)	30 µl

Appendix 7

Primer sequences for MVR-PCR I (5' to 3')

31-TAG-A (repeat unit specific primer)

TCATGCGTCCATGGTCCGGAGTGTCTGTGGGAGGTGGA

TAG region

31-TAG-G (repeat unit specific primer)

TCATGCGTCCATGGTCCGGAGTGTCTGTGGGAGGTGGG

TAG region

31-A (flanking site primer)

CCCTTTGCACGCTGGACGGTGGCG

31-TAG (driver primer)

TCATGCGTCCATGGTCCGGA

Appendix 8

Primer sequences for MVR-PCR II (5' to 3')

TAG II

GACACTCACAAAGAACAACGGACA

31-TAG-II-AC

GACACTCACAAAGAACAACGGACATCTGTGGGAGGTGGAC

TAG region

31-TAG-II-GT

GACACTCACAAAGAACAACGGACATCTGTGGGAGGTGGGT

TAG region

Appendix 9

Primer sequences for MVR-PCR III (5' to 3')

32-TAG-III-C

CTGACAAAAGTTGACAAGTAGAACGTCTGAGTCACCCCTGGC

TAG region

32-TAG-III-T

CTGACAAAAGTTGACAAGTAGAACGTCTGAGTCACCCCTGGT

TAG region

TAG-III

CTGACAAAAGTTGACAAGTAGAACG

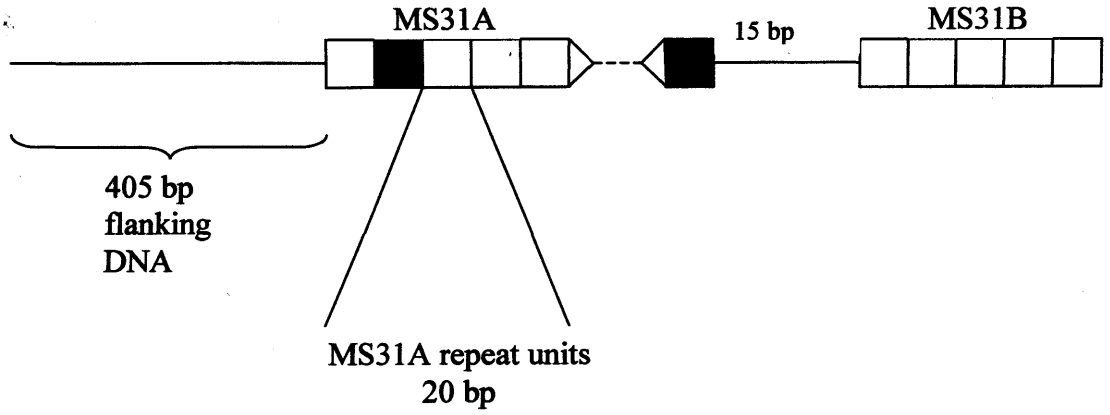
32-D

CGACTCGCAGATGGAGCAATGG

Appendix 10

Organisation of the D7S21 Locus

Variant repeat units at MS31A are indicated by filled and empty boxes; dashed lines indicate the presence of varying numbers of repeats.



Appendix 11

Abbreviations

APC	Adenomatous polyposis coli
AXIS	Adjuvant X-ray and Infusion Study
bp(s)	base pair(s)
CRC	Colorectal Cancer
DNA	Deoxyribonucleic acid
FAP	Familial adenomatous polyposis
GAPDH	Glyceraldehyde-Phosphate-Dehydrogenase
HNPCC	Hereditary nonpolyposis colorectal cancer
LOH	Loss of heterozygosity
$MgCl_2$	Magnesium Chloride
MIN	Microsatellite instability
MSI	Microsatellite instability
MVR	Minisatellite variant repeat unit
MVR-PCR	Minisatellite variant repeat unit by the polymerase chain reaction
nt	Nucleotide
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RER	Replication error