# MOLECULAR DIAGNOSIS OF HEREDITARY NONPOLYPOSIS COLORECTAL CANCER (HNPCC)

Anu-Maria Loukola

Department of Medical Genetics
Haartman Institute
University of Helsinki
Finland

*Academic Dissertation*

*To be publicly discussed with the permission of the Medical Faculty of the University of Helsinki, in the large lecture hall of the Haartman Institute, Haartmaninkatu 3, Helsinki, on the 29th of September at 12 noon.*

Helsinki 2000

**Supervised by:**

Lauri A. Aaltonen, MD, PhD
Docent
Department of Medical Genetics
Haartman Institute
University of Helsinki
Finland


**Reviewed by:**

Charis Eng, MD, PhD
Director
Clinical Cancer Genetics Program, Comprehensive Cancer Center
and Division of Human Genetics, Department of Medicine
The Ohio State University
Columbus, Ohio
USA
and
Honorary Fellow
Cancer Research Campaign Human Cancer Genetics Research Group
University of Cambridge
United Kingdom


Marja-Liisa Savontaus, PhD
Professor
Department of Medical Genetics
Institute of Biomedicine
University of Turku
Finland


**Official opponent:**

Torben F. Ørntoft, MD, DMSci
Professor, Chief Physician
Department of Clinical Biochemistry
Aarhus University Hospital
Skejby
Denmark

*Imagine a single cell in your body, turning against you, becoming malignant,*
*and eventually causing a life-threatening disease.*

*Cancer is both fascinating and horrifying. For decades, scientists have battled to unravel the*
*underlying mechanisms. Although significant progress has been made,*
*the heart of the mystery remains unsolved.*

- AL -

# TABLE OF CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| A | adenine |
| AAPC | attenuated adenomatous polyposis coli |
| ACF | aberrant crypt foci |
| *APC* | *Adenomatous Polyposis Coli* |
| ASO | allele-specific oligonucleotide hybridization |
| *ATM* | *Ataxia Telangiectasia Mutated* |
| ATP | adenosine-triphosphate |
| BAX | BCL2-associated X protein |
| *BCL2* | B-cell lymphoma gene |
| *BLM* | Bloom syndrome gene |
| bp | base pair |
| *BRCA1* | *Breast and ovarian cancer gene –1* |
| *BRCA2* | *Breast and ovarian cancer gene –2* |
| C | cytosine |
| cDNA | complementary deoxyribonucleic acid |
| *CDK4* | *Cyclin-Dependent Kinase –4* |
| CGH | comparative genomic hybridization |
| *CHK1* | *Checkpoint kinase-1* |
| CIMP | CpG island methylator phenotype |
| CRC | colorectal cancer |
| dATP | deoxyadenine triphosphate |
| *DCC* | *Deleted in Colorectal Cancer* |
| dCTP | deoxycytosine triphosphate |
| dGTP | deoxyguanosine triphosphate |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| *DPC4* | *Deleted in Pancreatic Cancer –4* |
| dTTP | deoxythymidine triphosphate |
| *EXT* | *Exostosin* |
| FAP | Familial Adenomatous Polyposis |
| FISH | fluorescent *in situ* hybridization |
| G | guanine |
| GI | gastrointestinal |
| HNPCC | Hereditary Nonpolyposis Colorectal Cancer |
| IDL | insertion/deletion loop |
| ICG-HNPCC | The International Collaborative Group on HNPCC |
| *IGFIIR* | *Insulin-like Growth Factor Receptor type II* |
| JP | Juvenile Polyposis |
| *K-RAS* | proto-oncogene with a GTPase function |
| *LKB1* | a serine/threonine kinase defective in PJS |
| LOH | loss of heterozygosity |
| LOI | loss of imprinting |
| *MBD4* | *Methyl-CpG-Binding Domain –4* |
| *MED1* | *Methyl-CpG-Binding Endonuclease –1* |
| *MEN1* | *Multiple Endocrine Neoplasia Type 1 gene* |
| *MET* | gene for hereditary papillary renal cancer |
| *MLH1* | *human MutL Homolog –1* |

| | |
|---|---|
| *MLH3* | *human MutL Homolog –3* |
| *MMAC1* | *Mutated in Multiple Advanced Cancers* |
| M-MLV | Moloney murine leukemia virus |
| MMR | mismatch repair |
| *MSH2* | *human MutS Homolog –2* |
| *MSH3* | *human MutS Homolog –3* |
| *MSH6* | *human MutS Homolog –6* |
| MSI | microsatellite instability |
| MSI+ | microsatellite instable, MSI-positive |
| MSI-H | high microsatellite instability |
| MSI-L | low microsatellite instability |
| MSS | microsatellite stable |
| *MutH* | *mouse Mutator-H* |
| *MutL* | *mouse Mutator-L* |
| *MutS* | *mouse Mutator-S* |
| *NF* | *Neurofibromin* |
| *OBR* | leptin receptor gene |
| p | short arm of the chromosome |
| *p16* | *Cyclin-dependent kinase inhibitor-2A (CDKN2A)* |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| PJS | Peutz-Jeghers syndrome |
| *PMS1* | *human Postmeiotic Segregation increased -1* |
| *PMS2* | *human Postmeiotic Segregation increased –2* |
| *Pms2* | *mouse Postmeiotic segregation increased –2* |
| *PTEN* | *Phosphatase and Tensin homolog* |
| *PTCH* | human homolog of the Drosophila *patched* gene |
| q | long arm of the chromosome |
| *RB* | Retinoblastoma gene |
| *RECQL* | DNA helicase gene |
| RER | replication error |
| *RET* | *Rearranged during Transfection* |
| RIZ | Retinoblastoma Interacting Zinc finger protein |
| RNA | ribonucleic acid |
| RT-PCR | reverse transcription polymerase chain reaction |
| *SRC* | human homolog of the Rous sarcoma virus *v-src* gene |
| SSCP | single strand conformation polymorphism |
| *SMAD* | human homolog of Drosophila *Mad* gene |
| *STK11* | *Serine/Threonine Kinase -11* |
| T | thymidine |
| *TCF-4* | *T-cell transcription factor-4* |
| *TGF-b* | *Transforming Growth Factor-b* |
| *TGF-bRI* | *Transforming Growth Factor-b Receptor type I* |
| *TGF-bRII* | *Transforming Growth Factor-b Receptor type II* |
| *TP53* | *Tumor Protein 53* |
| *TSC* | gene for a protein called hamartin |
| UV | ultraviolet |
| *VHL* | von Hippel-Lindau syndrome gene |
| *WT1* | Wilms tumor gene |

# LIST OF ORIGINAL PUBLICATIONS

I      Salovaara R, **Loukola A**, Kristo P, Kääriäinen H, Ahtola H, Eskelinen M, Härkönen N, Julkunen R, Kangas E, Ojala S, Tulikoura J, Valkamo E, Järvinen H, Mecklin J-P, Aaltonen LA, and de la Chapelle A. A population-based molecular detection of hereditary nonpolyposis colorectal cancer. *Journal of Clinical Oncology* 18: 2193-2200, 2000

II      **Loukola A**, Salovaara R, Kristo P, Moisio A-L, Kääriäinen H, Ahtola H, Eskelinen M, Härkönen N, Julkunen R, Kangas E, Ojala S, Tulikoura J, Valkamo E, Järvinen H, Mecklin J-P, de la Chapelle A, and Aaltonen LA. Microsatellite instability in adenomas as a marker for hereditary nonpolyposis colorectal cancer. *American Journal of Pathology* 155: 1849-1853, 1999

III      **Loukola A**, de la Chapelle A, and Aaltonen LA. Strategies for screening for hereditary non-polyposis colorectal cancer. *Journal of Medical Genetics* 36: 819-822, 1999 (Correction at *Journal of Medical Genetics* 37: 479-480, 2000)

IV      Riccio A, Aaltonen LA, Godwin AK, **Loukola A**, Percesepe A, Salovaara R, Masciullo V, Genuardi M, Paravatou-Petsotas M, Bassi DE, Ruggeri BA, Klein-Szanto AJP, Testa JR, Neri G, and Bellacosa A. The DNA repair gene *MBD4* (*MED1)* is mutated in human carcinomas with microsatellite instability. *Nature Genetics* 23: 266-268, 1999

V      **Loukola A**, Vilkki S, Singh J, Launonen V, and Aaltonen LA. Germline and somatic mutation analysis of *MLH3* in MSI-positive colorectal cancer. *American Journal of Pathology* 157: 347-352, 2000

In the following text, these articles will be referred to by their Roman numerals. In addition, some unpublished data is presented.

# 1. INTRODUCTION

Cancer is one of the leading causes of death in the Western countries. Up to every third individual will develop cancer at some point in life. Typically cancer strikes elderly people. However, younger people also develop tumors, especially when having inherited a predisposing gene mutation. An estimated 5-15% of all cancers are hereditary (Houlston et al., 1992; Lynch & de la Chapelle, 1999). Several hereditary cancer syndromes have been identified, most of which are extremely rare.

One might question the relevance of studying rare cancer syndromes. The first obvious argument is that when dealing with an inherited disease, it is not just about that one affected patient, rather it concerns the whole family as well as relatives. By identifying the disease-causing mutation in the family, all at-risk relatives can be scrutinized and can either be taken into surveillance programs or be relieved of an excessive fear of cancer. The second argument is that in most cases the same genes underlying the inherited syndromes are also involved in the sporadic cases of the same cancer type. Thus, study of hereditary syndromes will enlighten the development and progression of the more common sporadic cancers.

Cancer is a devastating disease, but it is no longer a death sentence. Using advanced diagnostic methods and regular screenings, tumors can often be found at an early, pre-malignant stage, and surgery combined with radiotherapy and cytotoxic drugs cures a great deal of patients. Importantly, the earlier the diagnosis is made, the better the prognosis. In the future, more advanced therapies, including gene therapy, will hopefully further increase the cure rate.

The aim of this thesis was to improve the diagnosis of hereditary nonpolyposis colorectal cancer (HNPCC) by utilizing molecular methods. In HNPCC, accurate diagnosis is essential since patients have a high risk for synchronous and metachronous tumors, and since cancer deaths can be prevented by early removal of benign tumors and carcinomas (Järvinen et al., 2000).

# 2. REVIEW OF THE LITERATURE

## 2.1. Cancer

Progressing from normal to malignant cells involves multiple steps, during which several distinguishable properties are acquired. Most importantly, cancer cells lose the ability to control growth, and can proliferate almost inevitably. They are able to promote angiogenesis (Folkman, 1975; Taunton, 1997), and thus guarantee an unlimited supply of nutrients and oxygen. The telomerase enzyme is reactivated and maintains stable telomeres during repeated cycles of cell proliferation (de Lange, 1994). Cancer cells no longer respond to apoptotic signals (Rudin & Thompson, 1998). In the final stages of progression, cancer cells are able to detach from their original site, travel through blood or lymphatic veins to new destinations, and form new colonies, a property called metastasis.

On the tissue level, cancers can be categorized on the basis of their origin. For example, sarcomas are derived from soft tissue and bone, whereas leukemias originate from blood cells. Carcinoma, derived from epithelial tissue, is the most common type of cancer. It typically starts within a benign, well-differentiated tumor (Muto, 1975), which has a structure resembling that of normal tissue. The next stage is carcinoma *in situ*, i. e., local tumor. After reaching the invasive stage, cancer is able to penetrate the basal membrane, and infiltrate the underlying tissue. Finally, the ability to metastasize is acquired. The clinical stages of carcinoma are illustrated in Figure 1.
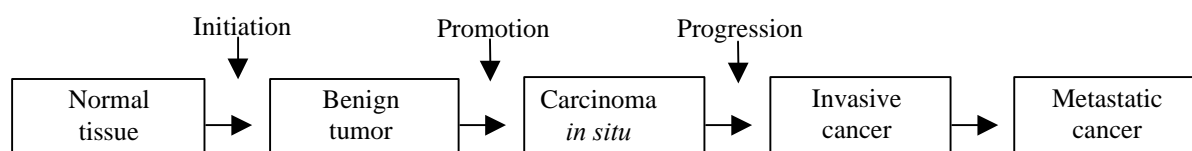


*Figure 1. Clinical stages of carcinomas. The underlying molecular events can be divided into initiation, promotion, and progression.*

Several well-known environmental factors increase cancer risk. Proven risk factors include smoking and asbestos for lung cancer, certain diet components for colorectal cancer, and UV-radiation for skin cancer. Viral infections have been associated with cervical cancer (Human

papilloma virus) (Marx, 1986) and hepatocarcinoma (Hepatitis B –virus) (Zuckerman, 1979; Hadziyannis, 1981). In addition, *Helicobacter pylori* infection increases the risk of gastric cancer (Markwick, 1990; Graham, 2000). In many cancers, however, the etiology remains obscure. The most common cancer types in males and females are presented in Table 1.

| Male | | | Female | | |
|---|---|---|---|---|---|
| Prostate | 2681 | (26%) | Breast | 3333 | (30%) |
| Lung | 1662 | (16%) | Colorectal | 1110 | (10%) |
| Colorectal | 950 | (9%) | Endometrial | 684 | (6%) |
| Bladder | 638 | (6%) | Ovarian | 586 | (5%) |
| Gastric | 527 | (5%) | Lung | 466 | (4%) |
| Total | 10358 cases | | Total | 11153 cases | |

*Table 1. Most common cancer types (Finnish Cancer Registry, 1997).*

## 2.2. Cancer Genes

Cancer is a disease caused by gene defects. The mutations can be hereditary (5-15% of cases) or acquired (85-95% of cases). In hereditary cases, the gene mutations are present in the germline, and thus, in every single cell of the body. The defect is passed from parents to children through gametes, resulting in accumulation of cancer in the family. In the acquired - or sporadic case, the gene mutation is induced, for example, by exposure to carcinogens. Since the defect happens in a single peripheral cell, it is passed only to this cell's descendants. Sporadic cancer thus happens by chance, and no familial clustering of cancer can be observed. Three different gene categories are known to be involved in cancer, both in the hereditary forms as well as in the sporadic cases. In addition to clear-cut mutations in clearly predisposing genes, a variety of polymorphisms and low penetrance-genes may contribute to cancer predisposition.

### 2.2.1. Proto-oncogenes and hereditary cancer

Proto-oncogenes are normal cellular components involved in controlling cell proliferation and differentiation. They can be classified according to their function into growth factors, growth factor receptors, signal transducers, cell cycle regulators, and transcription factors (Park, 1998). Activation of these genes results in uncontrolled cell proliferation. Mechanisms of proto-oncogene activation include point mutations, gene amplifications, and chromosomal translocations (Park, 1998). Oncogenes are dominant genes. Thus, mutation in either of the alleles is sufficient to cause intensive proliferation. So far, approximately 100 proto-oncogenes have been identified, but only a few of these have been associated with hereditary cancer (*RET* in thyroid cancer, *CDK4* in melanoma, and *MET* in papillary renal cell carcinoma) (Mulligan et al., 1993; Zuo et al., 1996; Schmidt et al., 1997).

### 2.2.2. Tumor suppressor genes and hereditary cancer

Tumor suppressor genes are negative regulators of cell growth (Marshall, 1991; Weinberg, 1991) and are thus often called "gatekeepers". They can be classified as cell cycle regulators, transcriptional regulators, or signaling proteins. According to the well-established two-hit hypothesis (Knudson, 1971), inactivation of both alleles of a tumor suppressor gene are needed for cancer formation. Several tumor suppressor genes are involved in hereditary cancer syndromes (see Table 2). In hereditary forms, one allele is already inactivated by the inherited mutation, and the second hit is typically due to large deletions visualized by loss of heterozygosity (LOH).

### 2.2.3. DNA repair genes and hereditary cancer

DNA repair genes are responsible for the recognition and repair of damaged DNA, and are thus often called "caretakers". Mistakes happening during DNA replication and due to exogenous factors need to be repaired in order to keep the genome intact. Inactivation of a caretaker gene results in a greatly increased mutation rate and is equivalent to a constant exposure to mutagens (Kinzler & Vogelstein, 1998), the result of which is a mutator phenotype (Perucho, 1996). DNA repair genes may, in a sense, be considered tumor suppressor genes since they also drive tumor formation through loss of function. These genes are recessive, meaning that both alleles need to be inactivated in order for a cell to acquire a mutator phenotype. In hereditary forms, one allele is already inactivated by the inherited

mutation, and the second hit can be due to, e.g., somatic mutation, loss of heterozygosity, or hypermethylation. Germline mismatch repair (MMR) gene mutations are associated with hereditary nonpolyposis colorectal cancer (HNPCC) (Kinzler & Vogelstein, 1996). Other examples of DNA repair gene involvement in hereditary cancer syndromes include *ATM*-gene in ataxia telangiectasia (Savitsky et al., 1995) and *BLM*-gene in Bloom syndrome (Ellis et al., 1995).

| Gene | Syndrome | Reference |
|---|---|---|
| *RB* | Familial Retinoblastoma | Bookstein et al., 1988 |
| *TP53* | Li-Fraumeni Syndrome | Malkin et al., 1990 |
| *WT1* | Familial Wilms Tumor | Call et al., 1990; Gessler et al., 1990 |
| *APC* | Familial Adenomatous Polyposis | Groden et al., 1991 |
| *NF1* | Neurofibromatosis Type 1 | Wallace et al., 1990 |
| *NF2* | Neurofibromatosis Type 2 | Rouleau et al., 1993 |
| *VHL* | von Hippel-Lindau Disease | Latif et al., 1993 |
| *BRCA1* | Familial Breast Cancer | Miki et al., 1994 |
| *BRCA2* | Familial Breast Cancer | Wooster et al., 1995 |
| *p16* | Familial Melanoma | Liu et al., 1995b |
| *EXT1* | Multiple Exostoses | Ahn et al., 1995 |
| *EXT2* | Multiple Exostoses | Stickens et al., 1996 |
| *PTCH* | Nevoid Basal Cell Carcinoma Syndrome | Hahn et al., 1996a |
| *PTEN / MMAC1* | Cowden Syndrome | Liaw et al., 1997 |
| *MEN1* | Multiple Endocrine Neoplasia Type 1 | Lemmens et al., 1997 |
| *TSC1* | Tuberous Sclerosis | van Slegtenhorst et al., 1997 |
| *TSC2* | Tuberous Sclerosis | * |
| *LKB1 / STK11* | Peutz-Jeghers Syndrome | Hemminki et al., 1998 |
| *SMAD4 / DPC4* | Juvenile Polyposis | Howe et al., 1998 |
| *E-CADHERIN* | Familial Gastric Cancer | Guilford et al., 1998 |
| *TGF-**b**RII* | Hereditary Nonpolyposis Colorectal Cancer | Lu et al., 1998 |

*Table 2. Tumor suppressor genes associated with inherited cancer syndromes.*

*\* European Chromosome 16 Tuberous Sclerosis Consortium, 1993*

# 2.3. Colorectal Cancer

Colorectal cancer (CRC) is the second most common malignancy as a cause of death in the Western countries (Dunlop, 1992). At least 50% of Western the population will develop a colorectal tumor by the age of 70, and in approximately 1 in 10 of these, progression will

5

proceed to malignancy (Kinzler & Vogelstein, 1996). Several environmental factors, such as diets containing high fat, excess of calories, low fiber, alcohol, or inadequate intake of vitamins and minerals, are implicated as risk factors for the development of colorectal cancer (Giovannucci & Willett, 1994; Nixon, 1995; Ferguson & Harris, 1996; Wargovich & Levin, 1996; Le Marchand et al., 1997). Furthermore, low physical activity has been associated with an increased risk for CRC (Sternfeld, 1992). A family history of CRC represents an independent risk factor and may account for up to 15% of all cases (Cannon-Albright et al., 1988; Houlston et al., 1992). The remaining 85% of colorectal cancer cases are considered "sporadic", i.e., without a clear inheritance pattern. However, the inheritability of cancer may sometimes remain unrecognized due to low-penetrance predisposing genes.

### 2.3.1. The adenoma-carcinoma sequence

Most colorectal cancers arise within an adenoma, a pre-neoplastic lesion (Muto, 1975; Jass, 1989). The progression from a benign adenoma to a malignant carcinoma passes through a series of well-defined histological stages, and may take 10 to 15 years (Muto, 1975). This progress is referred to as the adenoma-carcinoma sequence (Vogelstein et al., 1988). Colorectal adenomas are fairly common in the general population, but only 5-10% progress to a malignant tumor (Winawer et al., 1990).

Kinzler and Vogelstein (1996) demonstrated that colorectal tumor initiation and progression requires at least seven different somatic changes before a cell can develop into a carcinoma. Cytogenetic analysis of colorectal carcinomas has revealed that tumor cells undergo loss or gain of parts of chromosomes. The most frequently observed losses involve chromosomal regions 5q, 17p, and 18q (Vogelstein et al., 1988). These chromosomal segments are known to encompass important tumor suppressor genes including *APC* on 5q, *TP53* on 17p, and *DPC4* and *DCC* on 18q. Molecular characterization at different histopathological stages of the adenoma-carcinoma sequence has indicated that specific changes are associated with specific biological stages of tumor development. This suggests that not only the accumulation of these genetic lesions, but also their order of occurrence is important. These observations have led to the development of a genetic model for colorectal tumorigenesis (Fearon & Vogelstein, 1990; Kinzler & Vogelstein, 1996) (Figure 2).
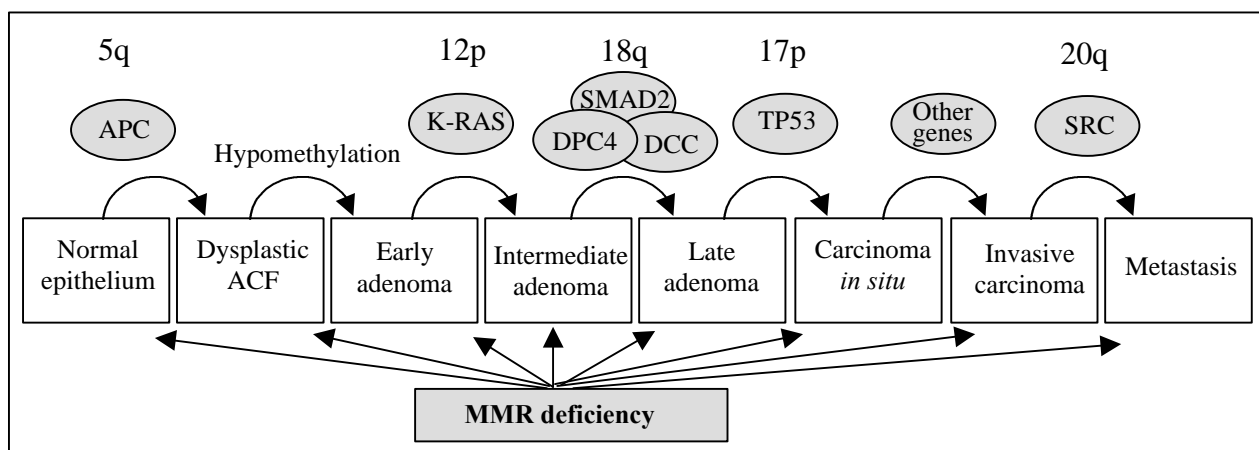
*Figure 2. "The adenoma-carcinoma sequence" (modified from Fearon & Vogelstein, 1990; Vogelstein & Kinzler, 1993; Kinzler & Vogelstein, 1996).*

In the majority of cases, the neoplastic process is initiated by mutations in the tumor suppressor gene *Adenomatous Polyposis Coli* (*APC*) (Powell et al., 1992). The inactivation of *APC* is already observed in the smallest precursor lesions of adenomas, the dysplastic aberrant crypt loci (ACF) (Jen et al., 1994; Smith et al., 1994). Therefore, *APC* is called the "gatekeeper" of colonic epithelial cell proliferation, as its inactivation is a rate-limiting event in the initiation of the adenoma-carcinoma sequence.

Mutations in the *K-RAS* proto-oncogene on chromosome 12p are thought to be involved in the progression of early adenomas (Vogelstein et al., 1988; Shibata et al., 1993). Although mutations in *K-RAS* are also frequently detected in early stages of progression, *K-RAS* does not seem to be involved in the initiation of adenomas. Colonic cells with *K-RAS* mutations will form foci of hyper-proliferating cells with normal intracellular and intercellular organization (Pretlow et al., 1993).

Loss of chromosome 18q occurs in about 50% of late adenomas and in 70% of colorectal carcinomas (Vogelstein et al., 1988; Fearon et al., 1990). Several putative tumor suppressor genes are thought to reside in chromosomal region 18q21, the most attractive candidate being *DPC4* (*Deleted in Pancreatic Cancer-4*) (Hahn et al., 1996b; Thiagalingam et al., 1996). *DCC* and *SMAD2* also reside in this region (Eppert et al., 1996, Thiagalingam et al., 1996). The *SMAD* genes play a role in transforming growth factor-β (TGF-β) signal transduction pathways (Massague et al., 1998). TGF-β is one of the most potent inhibitors of cell growth. Many malignancies of epithelial origin, such as those of the breast and colon, are resistant to

TGF-β, indicating an important role of this pathway in epithelial tumorigenesis. Tumors can become resistant to TGF-β either by loss of function of one of the two receptors, TGF-βRI and TGF-βRII, which interact with the TGF-β ligand (Lu et al., 1995; Markowitz et al., 1995), or by mutations in other genes, e.g., members of the *SMAD* family, involved in the downstream signaling cascade.

Loss of heterozygosity (LOH) at chromosome 17p and missense mutations in the *TP53* tumor suppressor gene on the remaining chromosome 17 are found in more than 80% of colorectal cancers and represent a late event in the adenoma-carcinoma sequence (Vogelstein et al., 1988; Baker et al., 1989, 1990). TP53 acts as a checkpoint control protein that determines cellular fate upon DNA damages (Kuerbitz et al., 1992). TP53 can delay the progression of the cell cycle from $G_1$ to S phase, thus allowing for repair of DNA damage (Kastan et al., 1991). Alternatively, TP53 can trigger apoptosis in response to DNA damage, most probably when the lesions are too extensive and DNA repair fails (Lane, 1993). Loss of TP53 tumor suppressor activity results in cells tolerating DNA damage, which eventually may lead to cancer formation due to inability to eliminate damaged cells by apoptosis.

Activating mutations in *SRC* have been detected in 12% of advanced colon cancers (Irby et al., 1999). Activation of this proto-oncogene is predicted to promote metastasis. Most likely a number of additional, as yet uncharacterized genes are involved in the adenoma-carcinoma sequence.

Beside mutations in proto-oncogenes and tumor suppressor genes, inactivation of genes involved in DNA repair are also associated with colorectal cancer (Leach et al., 1993; Fishel et al., 1993; Bronner et al., 1994; Papadopoulos et al., 1994; Nicolaides et al., 1994; Miyaki et al., 1997). Although mutations in these repair genes are not directly responsible for tumor initiation or progression, cells which are deficient in DNA mismatch repair accumulate genetic alterations at very high rates in the proto-oncogenes and tumor suppressor genes known to have significant roles in colorectal cancer (Ionov et al., 1993; Strickler et al., 1994; Uchida et al., 1994). The phenomenon of increased mutation rate due to genomic instability is called MSI (microsatellite instability). Approximately 10-15% of colorectal cancers are MSI-positive (Aaltonen et al., 1993, 1994; Ionov et al., 1993; Pedroni et al., 1999), in most sporadic cases due to *MLH1* promoter hypermethylation (Kane et al., 1997; Cunningham et al., 1998; Herman et al., 1998; Wheeler et al., 1999). Besides colorectal cancer, genomic

instability has been observed in several different types of sporadic tumors including endometrial carcinoma (Risinger et al., 1993; Burks et al., 1994; Duggan et al., 1994), pancreatic and gastric cancers (Han et al., 1993; Yamamoto et al., 1997), breast (Patel et al., 1994; Yee et al., 1994), ovarian (Orth et al., 1994), bladder (Gonzalez-Zulueta et al., 1993), prostate (Suzuki et al., 1995), and cervical cancers (Larson et al., 1996), and soft tissue sarcoma (Wooster et al., 1994).

## 2.3.2. Alternative pathways

The adenoma-carcinoma sequence proposed by Kinzler and Vogelstein (1996) is an excellent model for the sequence of events. However, it is not the only feasible one, and several modifications are bound to exist. Recent evidence has suggested that the order of events is not as straightforward as formerly believed. Dysplasia-carcinoma sequence is perhaps a better term, as it allows for the possibility that cells might acquire all genetic mutations necessary for malignant behavior without ever forming a visible adenomatous polyp (Lynch et al., 1995). Current studies have demonstrated MSI-positive adenomas and carcinomas showing similar degrees of genetic diversity, determined by microsatellite sequence mutation analysis (Shibata et al., 1996). Thus, an equally long period of progression has been proposed (Tsao et al., 2000). Adenomas may arise after long periods of occult progression, rather than simply being the starting points for carcinomas (Tsao et al., 2000).

One possible modification to the sequence of events is the so-called CpG island methylator phenotype (CIMP) (Toyota et al., 1999). CpG islands are short sequences rich in the CpG dinucleotide and can be found in the 5'-region of about half of all human genes (Larsen et al., 1992). Methylation of cytosine within 5'-CpGs is associated with loss of expression. Several tumor suppressor genes, such as *p16*, *RB*, *MLH1*, and *BRCA1* that have been shown to be mutated in the germline of patients with hereditary cancer syndrome have also been shown to be aberrantly methylated in some proportion of sporadic cancers (Jones, 1996; Kane et al., 1997; Baylin et al., 1998; Myöhänen et al., 1998; Esteller et al., 2000). CpG-island methylation has been proposed to serve as an alternative mechanism of gene inactivation in cancer (Jones & Laird, 1999).

The causes for aberrant methylation are still unclear. Aging could play a role in this process since the majority of CpG islands methylated in colon cancer are also methylated in a subset

of normal colonic cells during the process of aging (Issa et al., 1994). CIMP can lead to cancer development through the simultaneous inactivation of multiple tumor suppressor genes, such as *p16*, and induction of mismatch repair deficiency through inactivation of *MLH1*.

Methylation has important implications in tumorigenesis other than the methylator phenotype. CpG sites within genes are possible hot spots for inactivating mutations in tumor suppressor genes (Rideout et al., 1990). Approximately 25% of the point mutations in *p53* occur at CpG sites (Greenblatt et al., 1994). Spontaneous deamination of 5-methylcytosine results in formation of a T:G mismatch (Lindahl, 1993). Either side of the mismatch can be repaired, giving rise to a correct or incorrect sequence, respectively.

In addition to the CIMP phenotype, another possible factor in the sequence of events is loss of imprinting (LOI). Genomic imprinting is an epigenetic modification in the gamete or zygote that causes expression of a specific parental allele of a gene in somatic cells of the offspring (Cui et al., 1998). The mechanisms for the loss of this imprinting remain obscure, but may involve alteration in the methylation pattern of DNA (Miyaki, 1998). LOI can involve activation of the normally silent allele of a growth-promoting gene, or silencing of the normally expressed allele of a growth-inhibitory gene (Feinberg, 1998). LOI has been found both in cancer and normal tissues of a subset of cancer patients with MSI-positive tumors (Cui et al., 1998). Thus, LOI might have potential as a diagnostic marker, since LOI in normal mucosa and peripheral blood cells may be useful in identifying patients who will develop tumors with MSI (Cui et al., 1998; Miyaki, 1998).

MSI-positive colorectal cancers appear to follow a different path from MSI-negative ones. While MSI-positive cancers show infrequent LOH, as well as infrequent *APC*, *K-RAS* and *TP53* mutations, MSI-negative cancers frequently show the above-mentioned mutations and loss of 5q, 17p, and 18q (Konishi et al., 1996; Jass et al., 1999). An inverse relationship seems to exist between chromosomal and DNA instability. However, the two forms may co-exist in a tumor with mild instability (MSI-L) (Jass, 1999). An intriguing property of MSI-positive cancers is DNA hypermethylation (Jass, 1999). The mechanisms linking hypermethylation and microsatellite instability are obscure and need to be elucidated to enhance the understanding of the etiology and pathogenesis of colorectal cancer (Toyota et al., 1999).

## 2.3.3. Different hereditary syndromes

Inherited colorectal cancer syndromes are typically divided into the polyposis and nonpolyposis syndromes. The former are further divided into adenomatous and hamartomatous polyposis syndromes. In the following paragraphs, the most prominent syndromes will be briefly introduced.

### 2.3.3.1. Familial adenomatous polyposis

The most common polyposis syndrome is familial adenomatous polyposis (FAP), affecting approximately 1 in 10 000 individuals. FAP accounts for 0.5-1% of the total CRC burden (Mulvihill, 1983; Järvinen, 1992), and the gene underlying this hereditary syndrome is *APC* (Groden et al., 1991; Nishisho et al., 1991). The most prominent feature of FAP is the appearance of hundreds, or even thousands, of polyps in the colon. Unless these patients undergo prophylactic colectomy, they will develop colorectal cancer by the fourth decade of life (Bussey, 1975).

### 2.3.3.2. Turcot syndrome

Turcot syndrome is a genetically heterogenous disease characterized by the occurrence of tumors of the central nervous system as well as colorectal adenomas and carcinomas. A portion of Turcot patients have germline mutations in the *APC* gene and are characterized by a risk of CRC and medulloblastoma-type brain tumors (Hamilton et al., 1995). Another portion have germline mutations in mismatch repair genes and for those, the brain tumor is of the glioneural line, typically glioblastoma (Hamilton et al., 1995). In light of recent findings, Turcot syndrome might be a recessively inherited disease caused by homozygous or compound heterozygous low-penetrance mutations in *APC* and mismatch repair genes such as *PMS2* (De Rosa et al., 2000).

### 2.3.3.3. Peutz-Jeghers syndrome

Peutz-Jeghers syndrome (PJS) is a rare cancer-predisposing syndrome with hamartoma-type polyps in the gastrointestinal (GI) tract. Another characteristic feature of PJS is mucocutaneous pigmentation of the lips and oral area (Peutz, 1921; Jeghers, 1949). The gene underlying PJS is *LKB1* (*STK11*), a serine/threonine kinase with a tumor suppressor function (Hemminki et al., 1998). PJS patients have an elevated risk for both gastrointestinal and extra-gastrointestinal tumors. The most commonly associated malignancies are cancers of the colon, stomach, small intestine, pancreas, breast, ovary, and testis (Giardiello et al., 1987;

Spigelman et al., 1989). The prevalence of PJS is estimated to be in the order of one twentieth that of FAP (Spigelman & Phillips, 1994).

### 2.3.3.4. Juvenile polyposis

Juvenile polyposis (JP) is characterized by the appearance of multiple hamartomatous polyps in the GI tract and an increased risk for gastrointestinal cancer. In addition, a variety of malformations have been associated with JP, including congenital heart disease, cleft lip/palate, and mental retardation (Shepherd & Bussey, 1990; Järvinen, 1993). JP is a rare disorder affecting approximately 1 in 100 000 people (Burt et al., 1993). The underlying gene for JP is *SMAD4* (*DPC4*) (Howe et al., 1998), a prominent member of the TGF-β signaling pathway (Zhang et al., 1997).

### 2.3.3.5. Cowden syndrome

Another hamartomatous polyposis is the Cowden syndrome, characterized by multiple lesions of the skin, mucous membranes, breast, thyroid, and gastrointestinal tract (Lloyd & Dennis, 1963). Cowden syndrome patients do not seem to have an elevated risk for colorectal cancer, but instead have a high risk for breast, thyroid, and endometrial cancers (Starink et al., 1986; Eng, 1997; Eng, in press). The gene predisposing to Cowden syndrome is *PTEN* (Liaw et al., 1997).

### 2.3.3.6. Nonpolyposis syndromes

Nonpolyposis syndromes can be distinguished from polyposis syndromes simply by the absence of numerous benign tumors in the GI tract. Nevertheless, some attenuated forms of polyposis syndromes, such as attenuated adenomatous polyposis coli (AAPC), may complicate this distinction. The following paragraphs will focus on the clinical and genetic features of hereditary nonpolyposis colorectal cancer (HNPCC).

# 2.4. Clinical features of HNPCC

Hereditary nonpolyposis colorectal cancer (HNPCC), originally called the "Cancer Family Syndrome", and also known as the Lynch syndrome, is an autosomal dominantly inherited cancer syndrome. It was originally described by Warthin in 1913, in a report of a family with an aggregation of cancers of the colon, endometrium, and stomach (Warthin, 1913). In the

past few years, the genetic basis of this disease has been partly unraveled, providing valuable insights into the process of colorectal tumorigenesis.

HNPCC accounts for 1-5% of all colorectal cancers (Mecklin, 1987; Kee & Collins, 1991; Ponz de Leon, 1994; Aaltonen et al., 1998). The population frequency of HNPCC is at least 1:1000, which makes it one of the most common hereditary syndromes. HNPCC is characterized by familial aggregation of colorectal cancer that is often diagnosed at an early age (mean age 42 years), has a predominance of proximal location, and an excess of synchronous and metachronous tumors. Microsatellite instability (MSI) is found in more than 85% of the cases (Aaltonen et al., 1994; Liu et al., 1996; Pedroni et al., 1999).

In addition to colorectal cancer, some extracolonic tumors are often associated with HNPCC, and particularly cancers of the endometrium, stomach, small bowel, urinary tract, biliary tract, renal pelvis, ovary, and brain are frequently observed in HNPCC families (Watson & Lynch, 1993; Vasen et al., 1999). The absence or presence of extracolonic tumors has been the basis for subdividing HNPCC into Lynch syndrome I (CRC only) and Lynch syndrome II (CRC and extracolonic tumors) (Lynch et al., 1988). This division is somewhat artificial, even though there is some evidence of a genotype/phenotype correlation (Vasen et al., 1996; Jäger et al., 1997; Lin et al., 1998). The cumulative risks for the most common cancers in HNPCC patients are indicated in Table 3.

| Cancer type | Cumulative risk | Order of magnitude |
|---|---|---|
| Colorectal | 78% | 150x (at the age of 40 years |
| Endometrial | 43% | 10x |
| Gastric | 19% | 4x |
| Biliary tract | 18% | 5x |
| Urinary tract | 10% | 22x |
| Ovarian | 9% | 3.5x |
| Small bowel | small | 25x |

*Table 3. Cumulative risk (by 80 years of age) for the most common HNPCC-associated cancers (Watson & Lynch, 1993; Aarnio et al., 1995, Voskuil et al., 1997).*

HNPCC tumors do not harbor any pathognomonic clinical features that could be used for diagnosis. In general, HNPCC patients exhibit an increased proportion of mucinous tumors, poorly differentiated tumors, and tumors with marked host-lymphocyte infiltration and

lymphoid aggregation at the tumor margin (Kim et al., 1994). In addition, HNPCC tumors are often diploid or nearly diploid (Kouri et al., 1990).

The penetrance of HNPCC is very high, but it does not quite reach 100% (Vasen et al., 1996). The 5-year survival rate of patients with HNPCC is significantly better as compared with that of sporadic CRC patients (65% vs. 44%, respectively) (Sankila et al., 1996). The reason for the better survival rate is somewhat obscure. One theory is that immunological defense mechanisms may for some reason be more active in HNPCC, as indicated by the presence of a lymphoid reaction around the tumor (Kim et al., 1994). Another theory suggests that the high mutation rate in MSI-positive tumors is incompatible with tumor survival (Shibata, 1994). The better survival could also simply be due to the diploidy observed in HNPCC tumors, since diploid tumors generally have better prognosis (Kokal et al., 1986).

HNPCC patients form adenomas only at a slightly higher rate than the general population (Järvinen et al., 2000). Adenomas in HNPCC tend to be large and show a villous architecture and high-grade dysplasia (Jass & Stewart, 1992). It is possible that the mutator phenotype characteristic of HNPCC tumors drives the promotion of adenoma to carcinoma. Consistent with this hypothesis, adenomas in HNPCC patients are slightly more likely to progress to carcinoma than are adenomas in the general population (Jass et al., 1994; Lynch et al., 1995). It is estimated that in HNPCC patients a colorectal adenoma may evolve into carcinoma in only 2-3 years, whereas in non-HNPCC patients it may take as long as 8-10 years (Jass & Stewart, 1992). MSI can be seen at an early stage in HNPCC tumors, but is usually observed at later stages in sporadic colorectal tumorigenesis (Konishi et al., 1996). Indeed, adenomas from patients with HNPCC frequently show MSI as opposed to 0-3% of apparently sporadic colorectal adenomas (Young et al., 1993; Aaltonen et al., 1994).

Colonoscopic screening in individuals with HNPCC gene mutations provides an efficient method of cancer prevention (Järvinen et al., 1995). In a recently completed 15-year screening program focused on HNPCC mutation-positive individuals, both early detection of cancer and removal of premalignant adenomas resulted in a 65% reduction in cancer mortality (Järvinen et al., 2000). Similar but less dramatic benefits of colonoscopic cancer surveillance in the general population have been observed before (Winawer et al., 1993). Given the high (approximately 80%) risk of colorectal cancer in individuals who have germline HNPCC mutations (Watson & Lynch, 1993; Aarnio et al., 1995; Lynch & Smyrk,

1998) and the common occurrence of metachronous tumors in HNPCC patients, it is highly desirable to determine which patients with colorectal adenomas and carcinomas have HNPCC.

# 2.5. Genetic features of HNPCC

## 2.5.1. Predisposing genes

A unique approach was utilized during the cloning of HNPCC genes. First, a predisposing locus was mapped to chromosome 2p in several families with classical HNPCC features (Peltomäki et al., 1993). Tumors in patients with linkage to 2p showed widespread alterations in short repeated DNA sequences, suggesting that numerous replication errors had taken place during tumorigenesis (Aaltonen et al., 1993). A candidate gene was identified in chromosomal region 2p16 by virtue of its homology to bacterial *MutS* mismatch repair genes, previously shown to produce microsatellite instability when defective (Levinson & Gutman, 1987; Strand et al., 1993). Finally, germline mutations of the gene, named *MSH2*, were discovered in HNPCC patients (Leach et al., 1993, Fishel et al., 1993). Similarly, another HNPCC locus was first assigned to chromosome 3p by linkage studies (Lindblom et al., 1993), and the predisposing gene, *MLH1*, with homology to the bacterial DNA mismatch repair gene *MutL*, was identified (Bronner et al., 1994; Papadopoulos et al., 1994).

Three more mismatch repair genes have been cloned, and to date germline mutations in at least five different genes, i.e., *MSH2*, *MLH1*, *PMS1*, *PMS2*, and *MSH6* are known to cause HNPCC (Leach et al., 1993; Fishel et al., 1993; Bronner et al., 1994; Papadopoulos et al., 1994; Nicolaides et al., 1994; Miyaki et al., 1997). Approximately 90% of mutations so far reported in HNPCC affect *MLH1* and *MSH2* (Peltomäki & Vasen, 1997; http//www.nfdht.nl/), perhaps because most studies have focused on these genes. Table 4 presents the number of different mutations found in HNPCC worldwide and in Finland.

The inheritance of HNPCC is dominant despite the recessive nature of the underlying MMR-genes. No clear explanation exists, but this inconsistency can be reasoned. Since one allele is already defective due to the inherited mutation, all that is needed is for the other allele to mutate as well. This is very likely to happen sooner or later in cells that divide frequently, as

in the epithelium of the gastrointestinal tract. As soon as the other allele is silenced, the cell can no longer repair replication errors and begins to accumulate mutations. Eventually mutations strike tumor suppressor genes or oncogenes, and cancer starts to develop.

| Gene | Mutations worldwide | Mutations in Finland |
|------|---------------------|----------------------|
| *MLH1* | 151 | 8 |
| *MSH2* | 119 | 3 |
| *MSH6* | 25 | - |
| *PMS1* | 1 | - |
| *PMS2* | 2 | - |
| *TGF-**b**RII* | 1 | - |

*Table 4. Number of mutations found in HNPCC genes worldwide and in Finland (according to the 31-AUG-1999 update of the HNPCC mutation database at: http//www.nfdht.nl/).*

Mutations in different genes can cause slightly different phenotypes. There is one report of a family with a germline *MSH6* mutation and a non-typical HNPCC with an older age of onset, excess of gynecologic cancers, and only a weak family history of gastrointestinal tumors (Akiyama et al., 1997b). A clear example of genetic heterogeneity of this disease is the discovery of a germline *TGF-**b**RII* mutation in an HNPCC family with MSI-negative tumors and a late onset (Lu et al., 1998). Quite possibly more HNPCC genes remain to be discovered.

A strong founder effect has been detected in HNPCC in Finland. Finns were founded some 2000 years ago (de la Chapelle & Wright, 1998) by a very limited number of individuals. Most likely due to the small founder population, two *MLH1* founder mutations have become enriched in the population, accounting for more than 50% of all mutations detected in Finland (Nyström-Lahti et al., 1996; Aaltonen et al., 1998). Such a strong founder effect has implications in genetic testing, and makes screening for HNPCC feasible.

### 2.5.2. MSI phenomenon

DNA mismatch repair (MMR) genes encode enzymes that survey the newly replicated DNA for errors and repair all mismatched bases. Defects in MMR-genes result in replication errors and genetic instability (de la Chapelle & Peltomäki, 1995), which can easily be observed in

repetitive sequences such as microsatellites (Ionov et al., 1993), hence referred to as microsatellite instability (MSI).

Microsatellite DNA sequences are short tandem-repeats that are distributed throughout the human genome, covering at least 1% of the sequence (Strachan & Read, 1999). Typically, the tandem sequences consist of DNA repeats of six base pairs or fewer, and the total length of the stretch is less than 100 base pairs. In humans, the most common repeat sequences are $(A)_n/(T)_n$ and $(CA)_n/(GT)_n$, which are characteristically located within non-coding DNA sequences.

Microsatellite instability (MSI), also known as replication error (RER), is defined as a change of length due to either insertion or deletion of repeated units in a microsatellite within a tumor as compared with normal tissue. Mono-, di-, tri-, and tetranucleotide markers can be used for studying MSI. In 1997, "The International Workshop on Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition" proposed a panel of five microsatellite markers to be used in MSI analysis (Boland et al., 1998). For the purpose of providing some uniformity, two mononucleotide repeats (BAT25 and BAT26) and three dinucleotide repeats (D2S123, D5S346, and D17S250) were recommended. Using this reference panel, tumors having instability in two or more markers are defined as MSI-H (high instability), tumors having instability in one marker are defined as MSI-L (low instability), and tumors where none of the markers exhibit MSI are defined as MSS (microsatellite stable).

The mononucleotide marker BAT26 has some advantages over other markers in MSI analysis. It is extremely sensitive (Hoang et al., 1997; Aaltonen et al., 1998; Samowitz et al., 1999; Sutter et al., 1999) and shows negligible size variation between both alleles of one individual and among individuals (Hoang et al., 1997). Several studies support the use of BAT26 on tumor DNA alone, without matching normal DNA (Hoang et al., 1997; Aaltonen et al., 1998; Zhou et al., 1998). However, a germline polymorphism in the BAT26 locus has been detected in 7.7-12.6% of African Americans (Pyatt et al., 1999; Samowitz et al., 1999) and in 0.08% of Caucasians (Samowitz et al., 1999). Thus, the presence of allelic variations, although rare, emphasizes the need for the matching normal DNA to avoid misclassification.

17

Sixty to seventy percent of colorectal tumors fall into the group lacking MSI, and the remaining tumors are nearly evenly split between the MSI-H and MSI-L groups (Boland et al., 1998). Arguments in favor of combining the MSI-L and MSS groups include the fact that the baseline mutation rate for microsatellites in apparently stable colorectal cancers is not precisely known. The distinction between these two groups is highly dependent on both the type and the number of microsatellites analyzed. Given the use of enough markers, it may be that all colorectal cancers will exhibit some level of MSI (Boland et al., 1998).

Typically MSI-positive tumors acquire somatic deletions and insertions in simple repeated sequences. Several genes involved in tumorigenesis contain mononucleotide repeats in their coding regions. Somatic deletions have been seen in the *Insulin-like Growth Factor Receptor type II* (*IGFIIR*) (Souza et al., 1996) and in the *BCL2*-associated protein *BAX* involved in the regulation of apoptosis (Rampino et al., 1997). One specific mutation is almost invariably found in mismatch repair-deficient colorectal tumors. About 90% of the DNA mismatch repair-deficient colorectal carcinomas display mutations in the poly-A tract present in the coding sequence of the *Transforming Growth Factor-b Receptor type II gene* (*TGF-b RII*) (Lu et al., 1995; Markowitz et al., 1995; Parsons et al., 1995; Akiyama et al., 1997a). Moreover, the mismatch repair genes *MSH3* and *MSH6* frequently show somatic deletions (Malkhosyan et al., 1996), Accumulation of somatic mutations in these mismatch repair genes is believed to further increase genetic instability. The target for mutations in *MSH3* is an $(A)_8$ repeat, whereas *MSH6* has a $(C)_8$ repeat. Both genes have confirmed roles in human mismatch repair, and germline *MSH6* mutations are associated with a minor subset of HNPCC cases (Akiyama et al., 1997b; Miyaki et al., 1997; Verma et al., 1999). Although these genes have no prominent roles in hereditary susceptibility to MSI-positive tumors, they appear to be targets for secondary hits.

# 2.6. DNA mismatch repair machinery

In general, there are three major types of errors that alter DNA. First, environmental factors can damage single nucleotides. Exposure to UV light can lead to cross-linking of pyrimidine residues. The resulting thymidine dimers block normal replication of DNA. Spontaneous deamination of cytosine residues into uracil residues is another example of injury. These errors are corrected by a system called nucleotide excision repair (Chung & Rustgi, 1995; de

Boer & Hoeijmakers, 2000). The second type of error is due to genetic recombination producing regions of heteroduplex DNA that may contain mismatched nucleotides. Third, misincorporation of nucleotides during DNA replication can yield mismatched base pairs and nucleotide insertions and deletions. Although the DNA polymerase is very precise, it is not 100% accurate (Chung & Rustgi, 1995). A unique situation involves repetitive sequences of DNA that are more prone to replication errors. The system responsible for correcting these defects is the DNA mismatch repair system.

The human mismatch repair machinery is complex. Mismatches and insertion/deletion loops (IDLs) are recognized by MSH2/MSH6 heterodimers (Kolodner, 1996; Jiricny, 2000). In the absence of MSH6, the recognition of IDLs can be mediated by MSH2/MSH3 heterodimers, indicating redundant functions of MSH6 and MSH3 (Marsischky et al., 1996; Umar et al., 1998; Edelmann et al., 2000; Jiricny, 2000). The actual mismatch repair is mediated by MLH1/PMS2 heterodimers (Prolla et al., 1994; Li et al., 1995). Figure 3 illustrates a schematic representation of the human mismatch repair machinery.

The mismatch repair complex probably includes a number of additional components. Similar to the repair in bacteria, exonuclease, helicase, polymerase, and ligase activities are likely needed to complete the repair (Kolodner, 1996). *PMS1* has been identified on the basis of sequence homology with bacterial *MutL* genes. A germline mutation in *PMS1* has been detected in a HNPCC kindred (Nicolaides et al., 1994), but little is known about its biochemical function in DNA mismatch repair (Kolodner, 1995). A novel DNA mismatch repair gene *MBD4*, also known as *MED1*, has recently been cloned (Bellacosa et al., 1999), but its function in DNA mismatch repair is obscure. Similarly, the possible role of another novel DNA mismatch repair gene, *MLH3* (Lipkin et al., 2000), is unclear.

A critical aspect of DNA mismatch repair is strand-specificity, i.e., incision of the newly synthesized DNA strand carrying the mutation. In bacteria, the newly synthesized strand is recognized by MutH proteins on the basis of its hemimethylated status (Kolodner, 1995; Modrich, 1997). The mechanisms for strand-specificity in eukaryotes are still unknown. The novel MMR-protein MBD4 forms a complex with MLH1, binds to methyl-CpG-containing DNA and fails to bind unmethylated DNA, and has single- and double-strand endonuclease activity (Bellacosa et al., 1999). Thus, MBD4 is a possible mediator of methylation-based strand-specificity in eukaryotic mismatch repair.
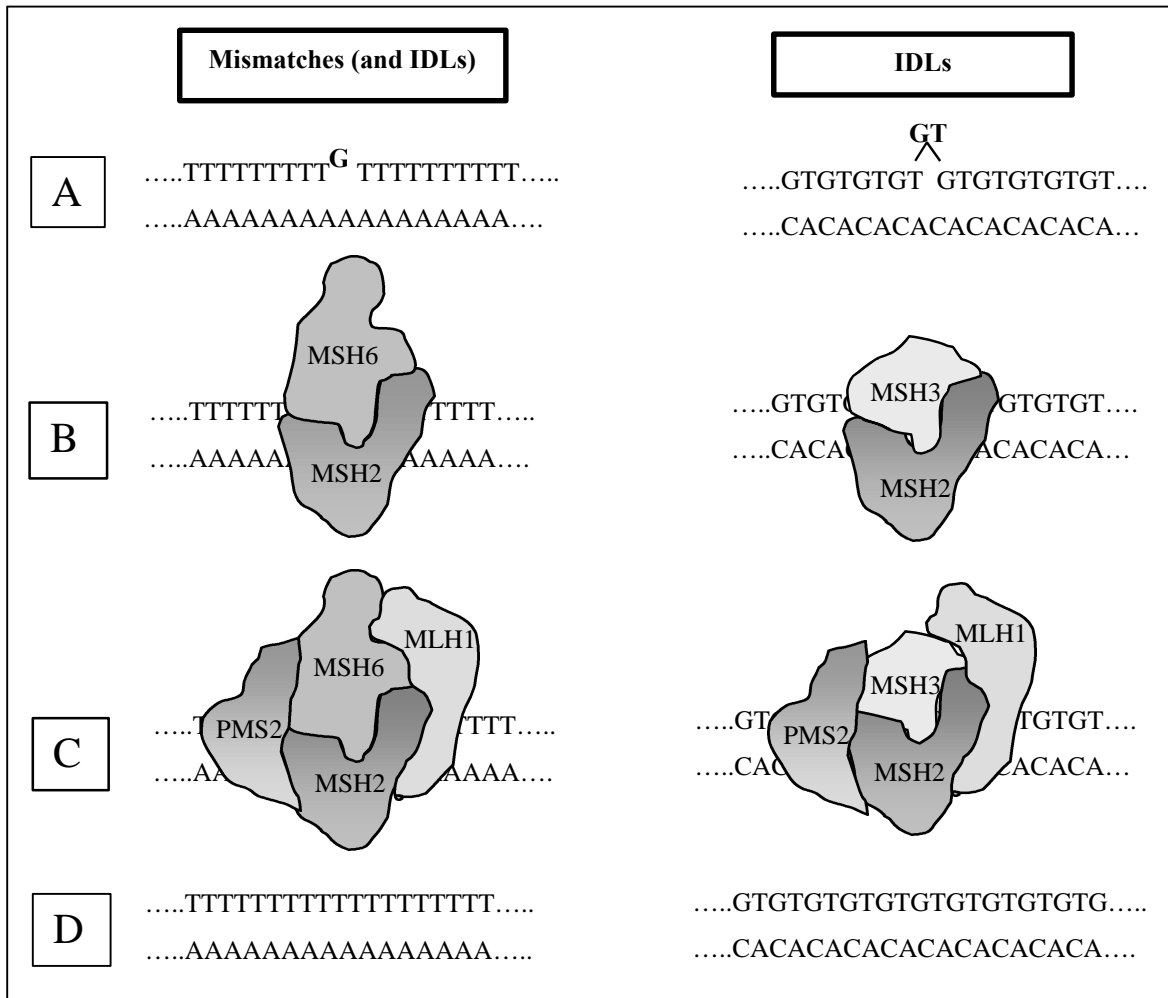
*Figure 3. The human mismatch repair machinery. (A) Sequence with a mismatch; on the left a mismatched base pair, and on the right an IDL. (B) Mismatch recognition. A* MSH2/MSH6 *heterodimer can recognize both mismatches and IDLs, while the* MSH2/MSH3 *heterodimer preferentially recognizes IDLs. (C) Mismatch repair is mediated by the* MLH1/PMS2 *heterodimer. (D) Repaired sequence where the wild-type sequence has been retained.*

An association between *MLH3* and DNA mismatch repair can be proposed based on the following evidence: *MLH3* is homologous to bacterial *MutL* genes, is predicted to have an MLH1 interaction domain, and is believed to be involved in mismatch repair in yeast (Lipkin et al., 2000). Furthermore, overexpressed dominant negative *MLH3* induces MSI in mammalian cell culture (Lipkin et al., 2000). The predicted MLH1 interaction domain indicates that MLH3 might replace PMS2 in the mismatch repair complex. Very few germline mutations have been found in *PMS2*, and *Pms2*-/- mice do not display colon cancer susceptibility (Prolla et al., 1998). However, *MLH3* might have a unique role in mismatch repair, since it does not show great similarity to *PMS2*.

# 2.7. Diagnosis of HNPCC

## 2.7.1. Utilizing family history

In the absence of clinical hallmarks, the diagnosis of HNPCC has mainly been based on family history of cancer. In an attempt to standardize the diagnosis, the so-called Amsterdam criteria were established by the International Collaborative Group on HNPCC (ICG-HNPCC) (Vasen et al., 1991). These criteria include the presence of histologically verified colorectal cancer in at least three relatives (one of whom must be a first-degree relative of the other two), the presence of at least two affected successive generations, and an age at onset of colorectal cancer of less than 50 years in at least one of the patients. In addition, Familial adenomatous polyposis (FAP) must be excluded. Considerable criticism has been aimed at these criteria excluding some classic HNPCC families because they fail to take into account the extracolonic cancers that are part of the syndrome. An estimated 20% of HNPCC families with germline MMR gene mutations do not meet the Amsterdam criteria (Rodriguez-Bigas et al., 1997). To improve the selection criteria, the ICG-HNPCC introduced new selection criteria (Vasen et al., 1999), known as Amsterdam criteria II. These criteria are otherwise identical to the previous ones, except that all HNPCC-associated cancers (CRC, cancer of the endometrium, small bowel, ureter, and renal pelvis) are included. Since the Amsterdam criteria are quite strict especially for small families, the utility of these criteria in diagnosis is questionable.

As an attempt to improve diagnosis based on family criteria, a mathematical algorithm was introduced by Wijnen and colleagues (1998b). This algorithm is based on simple variables (mean age of CRC diagnosis in the family, fulfillment of Amsterdam criteria I, and presence of endometrial cancer in the family), and can be easily used to calculate the probability of finding a deleterious *MLH1* or *MSH2* mutation. A probability of 20% or higher has been considered sufficient to justify mutation analyses (Wijnen et al., 1998b). An alternative algorithm to be used in small families was also proposed, including the mean age of CRC in the family, the number of patients with CRC in the family, and the number of patients with endometrial cancer in the family. This algorithm was retrospectively established based on 184 Dutch HNPCC or HNPCC-like kindreds, and has not been prospectively evaluated.

Recently, selection criteria for molecular diagnosis of HNPCC were proposed (Aaltonen et al., 1998). These simple criteria include: (1) age of onset of CRC less than 50 years, or (2) synchronous or metachronous CRC or endometrial cancer, or (3) at least one first-degree relative with CRC or endometrial cancer. According to Aaltonen and colleagues (1998), criteria-positive patients are analyzed for microsatellite instability, and MSI-positive patients are included in *MLH1* and *MSH2* mutation analyses. These selection criteria have been retrospectively established utilizing a series of 509 unselected colorectal cancer patients including 10 patients with a germline *MLH1* or *MSH2* mutation, and have not so far been prospectively evaluated.

### 2.7.2. Utilizing MSI analysis followed by mutation analysis

Microsatellite instability (MSI) has potential use as a marker of patients and families who need a more detailed study of germline DNA to identify HNPCC individuals. Eighty-five to ninety percent of HNPCC patients show MSI (Aaltonen et al., 1994, Pedroni et al., 1999), whereas only 10-15% of sporadic colorectal tumors do so (Ionov et al., 1993; Aaltonen et al., 1994; Pedroni et al., 1999). Thus, MSI is a relatively sensitive, but unspecific marker for HNPCC.

MSI analysis is typically performed by amplifying microsatellite markers either with fluorescently labeled primers or with radioactive labeling of the PCR product. A necessity for a successful MSI analysis is a representative tumor sample with a high tumor cell percentage and thus a careful histological evaluation of the tumor specimen is needed. One possibility is to microdissect tumor tissue to enrich the percentage of tumor cells in the specimen. Another important factor is an optimal marker panel. The recommended panel of five microsatellite markers (Boland et al., 1998) has not been empirically evaluated and most groups use panels of their own. One of the recommended markers, a mononucleotide marker called BAT26, is perhaps considered to be the most sensitive of all known microsatellite markers (Hoang et al., 1997; Iacopetta & Hamelin, 1998; Bradshaw et al., 1999; Cravo et al., 1999; Samowitz et al., 1999) and is widely used in MSI analysis.

Another feasible way to identify MSI-positive tumors is analysis of MLH1 and MSH2 protein expression by immunohistochemistry. Specific antibodies have been raised against these proteins, and immunohistochemistry can be utilized in screening tumor sections for loss

of expression in a cost-effective and fast way (Marcus et al., 1999; Debniak et al., 2000). No need for DNA extraction, PCR amplifications or enrichment of tumor cells exists, since the expression patterns can be compared with the histology of the section. Immunohistochemical analysis of tumors may possibly substitute the PCR-based MSI analysis in a considerable percentage of the HNPCC cases (Thibodeau et al., 1998; Cawkwell et al., 1999; Jass, 1999).

The presence of a mutation in a mismatch repair gene confirms the diagnosis. Even though recent data implicates a possible involvement of *MSH6* in a considerable proportion of HNPCC cases (Miyaki et al., 1997; Akiyama et al., 1997b; Verma et al., 1999), over 90% of mutations identified to date have been detected in *MLH1* and *MSH2*, and mutation searches typically concentrate on these genes. However, genes responsible for HNPCC are not yet fully known, and some might be associated with more attenuated phenotypes (Boland et al., 1998).

# 3. AIMS OF THE STUDY

1. To evaluate the frequency of hereditary nonpolyposis colorectal cancer (HNPCC) and the common founder mutations in Finnish unselected colorectal cancer and adenoma patients

2. To test whether MSI analysis can be used in prescreening unselected colorectal cancer and adenoma patients for HNPCC

3. To evaluate the recently established (Aaltonen et al., 1998) selection criteria for molecular analysis of HNPCC, and to test the power of a mathematical model proposed by Wijnen and colleagues (1998b) in predicting the probability of finding a deleterious *MLH1* or *MSH2* mutation in unselected colorectal cancer specimens

4. To evaluate the possible role of two novel DNA mismatch repair genes, *MBD4* and *MLH3*, in HNPCC and MSI-positive colorectal cancer

# 4. MATERIALS AND METHODS

## 4.1. Colorectal carcinoma patients (I,III,IV,V)

Altogether 1044 unselected colorectal cancer patients were included in the study. Fresh-frozen tumor tissue was obtained from the Finnish central hospitals in Helsinki, Joensuu, Jyväskylä, Kajaani, Kotka, Kuopio, Lappeenranta, Mikkeli, and Savonlinna between May 1994 and June 1998. All tumor samples were histologically evaluated by a pathologist at the Department of Pathology, University of Helsinki. The study protocol was approved by the Ethics committee of the Department of Medical Genetics, University of Helsinki. Informed consent was obtained from the patients. Either peripheral blood or fresh-frozen tissue of the normal colonic epithelium was available in most cases. The specimens representing normal mucosa were always derived from a separate site, not from tumor margins. Family history of first-degree relatives (parents, siblings, and children) was obtained through official population registries. On average, eight first-degree relatives per family were identified. Data on patients and relatives were cross-linked with the Finnish Cancer Registry, as well as the Finnish HNPCC registry to reveal possible connections to previously identified HNPCC families.

## 4.2. Colorectal adenoma patients (II)

In all, 402 colorectal adenoma samples from 378 patients were obtained from nine central hospitals in Eastern Finland between June 1994 and June 1998. The study protocol was approved by the Ethics committee of the Department of Medical Genetics, University of Helsinki. Informed consent was obtained from the patients. The number of adenomas obtained from one individual varied from one to seven, but was typically one. Most (84%) of the samples were obtained during colonoscopy and some (11%) during surgery (information not available in 19 cases). All samples were evaluated by a pathologist at the respective hospital. The patient data were cross-linked with the Finnish HNPCC registry data, as well as the Finnish Cancer Registry data. The series was found to contain 11 patients from known HNPCC-families. Seven families segregate characterized *MLH1* mutations. Either peripheral blood or fresh-frozen tissue from the normal colonic epithelium was available in most cases.

## 4.3. Endometrial and pancreatic tumors, and cell lines (IV)

In all, 45 endometrial carcinoma samples (9 MSI-positive and 36 MSS), 46 pancreatic tumors (5 MSI-positive and 41 MSS), 6 MSI-positive cell lines, and 8 MSS cell lines were included in study IV in addition to 42 MSI-positive and 39 MSS colorectal carcinomas, previously described in detail in section 4.1. Corresponding normal DNA was available from endometrial and pancreatic cancer patients, as well as from colorectal carcinoma patients.

## 4.4. Cancer-free control individuals (I,II,V)

Blood samples from anonymous cancer-free individuals were obtained from the Finnish Red Cross, Helsinki. These control individuals were used in studies I, II, and V to verify the nature and population frequency of several sequence variants.

## 4.5. DNA and RNA extraction (I,II,III,IV,V)

DNA was extracted from fresh-frozen tissue and peripheral blood by a standard non-enzymatic procedure (Lahiri & Nürnberger, 1991). In study II, a rapid method to lyse white cells from 0.5 ml of whole blood (Higuchi, 1989) was used for anonymous blood donors. Total cellular RNA was extracted from fresh-frozen tissue by RNA extraction kit (Qiagen GmbH, Hilden, Germany).

## 4.6. Microsatellite instability (MSI) analysis (I,II)

In the series of 1044 unselected colorectal cancer patients, MSI analysis and subsequent *MLH1* and *MSH2* mutation analysis had already been performed for 509 samples (Aaltonen et al., 1998). In this study, MSI analysis was performed for 535 colorectal cancer samples and for 402 colorectal adenoma samples utilizing mononucleotide markers BAT26 $[(A)_{26}]$ and *TGF**b**RII* $[(A)_{10}]$. The procedure is explained in detail in studies I and II. Briefly, microsatellite markers were amplified from the tumor DNA utilizing fluorescently labeled

primers, and PCR products were loaded on 6% polyacrylamide gels and run on an ABI PRISM 377 DNA Sequencer (Perkin Elmer Applied Biosystems, Foster City, CA, USA). The data were collected automatically and analyzed by GeneScan 3.1 software (Perkin Elmer). The results were evaluated visually by two reviewers. All tumors showing aberrant patterns were re-analyzed with the matching normal DNA to confirm the somatic origin of the aberrant alleles. Patients whose tumors showed alleles that were not present in the corresponding normal DNA were acknowledged as MSI-positive (MSI+).

# 4.7. Mutation analysis

## 4.7.1. HNPCC founder mutation analysis (I,II)

All 535 CRC patients and 378 adenoma patients were analyzed for the two most common Finnish HNPCC founder mutations. MUTATION 1 is a 3.5-kb genomic deletion causing an in-frame 165-bp deletion comprising *MLH1* exon 16. It was analyzed by PCR, followed by agarose gel electrophoresis (Nyström-Lahti et al., 1995). Three primers, (1) GAGCCTCCAA TACAATGTTGAATAGAAG, (2) ACATATGTGACATCCTCTCCACTCG, and (3) GTCA ATCAGAAGGGGTAATCCAATCAG, were used in a single amplification reaction. Primers (1) and (2) amplify a fragment of 634 bp specific for the mutated allele (in the normal allele the distance between these primers is about 4 kb, and therefore no product is obtained by conventional PCR). Primers (2) and (3) amplify a 475-bp fragment specific for the normal allele. PCR was carried out in a 50-µl volume containing 100 ng of genomic DNA, 1 x PCR buffer (Finnzymes, Espoo, Finland), 200 µM of each dNTP (Finnzymes), 0.4 µM of each primer, and 1 unit of Dynazyme polymerase (Finnzymes). The $MgCl_2$ concentration was 1.5 mM. The following PCR cycles were used for amplification: 94° for 5 min, 35 cycles of 94° for 1 min, 60° for 1 min, and 72° for 1 min. The final extension was 72° for 10 min. The PCR products were run on a 1% agarose gel (NuSieve, FMC BioProducts, Rockland, Maine, USA).

MUTATION 2 is a splice acceptor mutation in *MLH1* (exon 6, G→A at 454 – 1) leading to the deletion of exon 6, and causing frame-shift and a premature stop codon. It was analyzed by allele-specific oligonucleotide hybridization (ASO) (Nyström-Lahti et al., 1995). The primer sequences used were: (F): CTTTTGCCAGGACATCTTG and (R): ACAAATCTCA

GAGACCCAC. PCR was carried out in a 25-µl volume containing 100 ng of genomic DNA, 1 x PCR buffer (Perkin Elmer), 200 µM of each dNTP (Finnzymes), 0.4 µM of each primer, and 1.5 units of AmpliTaq polymerase (Perkin Elmer). The $MgCl_2$ concentration was 1.5 mM. The following PCR cycles were used for amplification: 94° for 5 min, 35 cycles of 94° for 1 min, 58° for 1 min, and 72° for 1 min. The final extension was 72° for 10 min. PCR fragments were run on 2% agarose gel to verify the amplification, thus avoiding the need for hybridization with the wild-type probe. PCR products from three individuals were pooled together into the filter. A probe containing the mutant sequence (5´-CTTCTGTTCAAGTGG AGGAC-3´) was labeled with γ-P$^{32}$ATP utilizing T4-polynucleotide kinase (New England BioLabs, Schwalbach, Germany). Filters were hybridized at +65°C with the mutant probe. If a positive signal was obtained, the respective samples were rehybridized separately in a new filter.

In addition, a rapid screening method was developed for the third most common Finnish mutation, founder MUTATION 3 [*MLH1* exon 4 missense type of change 320T→G (Ile→Arg)], utilizing ASO. All 376 adenoma patients were analyzed for this founder mutation. This mutation has been studied functionally and shown to be pathogenic (Shimodaira et al., 1998). The primers used were: (F): CAGATAACCTTTCCCTTTGGTG and (R): TATGCACACTT TCCATCAGC, and the PCR-reactions were carried out in a 20-µl reaction volume containing 100 ng of genomic DNA, 1 x PCR buffer (Perkin Elmer), 500 µM of each dNTP (Finnzymes), 0.7 µM of each primer, and 1 unit of AmpliTaqGOLD polymerase (Perkin Elmer). The $MgCl_2$ concentration was 2.75 mM. The following PCR cycles were used for amplification: 95° for 10 min, 40 cycles of 95° for 1 min, 57° for 1 min, and 72° for 1 min. Final extension was 72° for 10 min. PCR products were run on 2% agarose (NuSieve) gel to verify the amplification, thus avoiding the need for hybridization with a wild-type probe. PCR products from three individuals were pooled together into the filter. Filters were hybridized at +62°C with a probe containing the mutant sequence (5'-CTT TGGCCAGCAGAAGCCAT-3'). If a positive signal was obtained, the respective samples were rehybridized separately in a new filter.

In study II, DNA derived from 2497 anonymous blood donors was analyzed by semi-automated screening methods for mutation 1 and 2. The aim was to roughly estimate the population frequency of these mutations. Details of this analysis are described in study II and by Syvänen and colleagues (1992).

### 4.7.2. Analysis of other MLH1 and MSH2 germline mutations (I, II)

If neither of the two founder mutations were found in MSI-positive CRC samples, or if none of the three mutations were found in MSI-positive adenoma samples, a thorough *MLH1* and *MSH2* mutation analysis was performed by direct genomic sequencing. Each exon as well as the promoter region was amplified separately utilizing PCR amplification kits containing primers, nucleotides, and buffer (Perkin Elmer, commercial kit, unpublished data), and AmpliTaqGOLD polymerase (Perkin Elmer). Direct sequencing was performed utilizing the ABI PRISM Dye Terminator or ABI PRISM dRhodamine cycle sequencing kits (Perkin Elmer). Sequencing products were electrophoresed on 6% Long Ranger gels (FMC BioProducts) and analyzed on an Applied Biosystems model 373A or 377 DNA sequencer (Perkin Elmer).

### 4.7.3. Restriction enzyme analyses and ASO in evaluating sequence variants (I)

Restriction enzyme digestion was used to screen cancer-free controls for sequence variants of *MLH1* and *MSH2* in study I. The *MLH1* exon 4, 378C→G (Tyr→Stop) nonsense change was analyzed in 90 cancer-free individuals by *Mnl*I (New England BioLabs) digestion. The primers used were: (F): GGTGACCCAGCAGTGAGTTT and (R): TTACTCTGAGACCTA GGCAAAAA. PCR reactions were carried out in a 20-μl reaction volume containing 50 ng of genomic DNA, 1 x PCR buffer (Perkin Elmer), 300 μM of each dNTP (Finnzymes), 0.6 μM of each primer, and 1 unit of AmpliTaqGOLD polymerase (Perkin Elmer). The $MgCl_2$ concentration was 2.1 mM. The following PCR cycles were used for amplification: 95° for 10 min, 40 cycles of 95° for 1 min, 58° for 1 min, and 72° for 1 min. Final extension was 72° for 10 min. PCR products were run on 2% agarose (NuSieve) gel to verify amplification. The digestions were performed in 1xNEBuffer (New England BioLabs) at +37°C overnight. After digestion, the PCR products were run on 3% agarose gel. *Mnl*I cuts the PCR fragment (196 bp), which contains the substitution, into two fragments of approximately equal size, whereas the wild-type PCR fragment lacks the restriction site and is not digested.

A rapid screening method based on ASO was established to evaluate the occurrence of the *MSH2* exon 12, 1808G→A (Asp→Asn) missense change in 90 cancer-free individuals. Details of this analysis are presented in study I.

A missense change 965G→A (Gly→Asp) in *MSH2* exon 6 has been reported both as a pathogenic mutation and as a polymorphism (http://www.nfdht.nl/database/mdbchoice.htm). The presence of this change was evaluated in 89 cancer-free individuals by *Hinf*I (New England BioLabs) digestion. Details of this analysis are presented in study I.

### 4.7.4. MBD4 mutation analysis (IV)

In study IV, somatic deletions and insertions in four [(A$_6$)–(A$_{10}$)] poly-A tracts residing in the coding region of *MBD4* were analyzed in 56 MSI tumors (42 colorectal carcinomas, nine endometrial carcinomas, and five pancreatic tumors) by SSCP. The same repeats were analyzed in 116 MSS tumors (39 colorectal, 36 endometrial, and 41 pancreatic tumors) as well as in six MSI-positive cell lines and eight MSS cell lines. All aberrant samples were cloned utilizing the pGEM$^®$-T Easy Vector System (Promega, Madison, WI, USA) and sequenced to study the mutations. The corresponding normal DNA was analyzed to confirm the somatic origin of the changes. An (A)$_8$ tract in *PMS2* and (A)$_9$ tracts in DNA helicase genes *BLM* and *RECQL* were analyzed in all 56 MSI-positive tumors as controls for the overall mutation rate.

Possible biallelic inactivation was studied in six colorectal carcinomas showing somatic deletions of *MBD4*. For LOH analysis, *MBD4* was first localized by FISH (fluorescent *in situ* hybridization), as previously described (Fan et al., 1990), to chromosomal region 3q21-22, and markers were identified in this region by screening the GeneMap database (http://www.ncbi.nlm.nih.gov/genemap98/). LOH analysis was performed by radioactive polyacrylamide gel electrophoresis (PAGE) utilizing 11 markers residing in this chromosomal region.

### 4.7.5. MSH6 mutation analysis (unpublished data)

In study IV, five MSI-positive CRC patients, in which germline *MLH1* and *MSH2* mutations had been excluded by direct sequencing, were found to have somatic *MBD4* mutations. To confirm the absence of germline HNPCC predisposition, mutations in *MSH6* were scrutinized by RT-PCR and direct sequencing. Fresh-frozen tissue was available in four cases. 20 μl of cDNA was created from 0.8 μg of both normal and tumor RNA using standard random priming methods with 200 units of M-MLV reverse transcriptase (Promega), 1 x reaction

buffer (Promega), 10 μM of random hexamer and 60 units of RNAse inhibitor (Promega). The reaction was carried out at 42°C for 45 min, and then 95°C for 10 min.

The gene was amplified in 13 overlapping fragments using primers designed by utilizing the Primer3 server (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). The primer sequences used were (F1): AGATGCGGTGCTTTTAGGAG, (R1): GTAAACCAGACAA GGCCACC; (F2): ACCAGGAGATTTGGTTTGGG, (R2): CGTAAGTTGTGCCTACCTC CA; (F3): GGCTTGAATTGGCAGTTTGT, (R3): AGGGCGTTTCCTTCCTAGAG; (F4): ATGGTGACTGGAAATGGCTC, (R4): CCCCACCTTGTAACAGATGAC; (F5): ATGAG GAAGTGGTGGCAGAT, (R5): TTACTGTAGTTCTCAGAGGGATCA; (F6): TGATAGA GTGGTGAGGAGGGA, (R6): GAGCCGGGTATCAGACCTTC; (F7): TCTAAAGAGTTC ATTGTCCTGTTCTC, (R7): AGCACCATTCGTTGATAGGC; (F8): ATATTCCCTTGGA TTCTGACAC, (R8): GCTGTCTGGGTGGTTCTGAC; (F9): AAGACCTCATGGTTGTGC CT, (R9): TTTCGAGCCTTTTCATGGTC; (F10): TCCTGAAGGTCGTTTTCCTG, (R10): CTTCAATGATACATCCCTCCG; (F11): CCAAGAAGGGCTGTAAACGA, (R11): CATA AGCGTAGACTTGCCCC; (F12): CTGTGAGGAAGAGGAGCAGG, (R12): CTAGGCGC ACAGCAACATT; and (F13): GTCGTACATTATTTTCAACTCACTACC, (R13): TTGTC AGAAGTCAACTCAAAGC.

The PCR reactions were carried out in a 50-μl volume containing 3 μl of cDNA, 1 x PCR buffer (Perkin Elmer), 250 μM of each dNTP (Finnzymes), 0.8 μM of each primer, and 2.5 units of AmpliTaqGOLD polymerase (Perkin Elmer). The $MgCl_2$ concentration was 1.5 mM (2.5 mM for fragments 5 and 10). The following PCR cycles were used for amplification: 95° for 10 min, 40 cycles of 95° for 45 s, 57° for 45 s (56° for 45 s for fragments 1 and 13), and 72° for 45 s. Final extension was 72° for 10 min.  PCR products were run on a 3% agarose gel (NuSieve) to verify the specificity of the reaction. PCR products were purified using the QIAquick PCR purification kit (Qiagen). Direct sequencing of the purified PCR products was performed as previously described. Mutation analysis was performed on tumor cDNA, and for all aberrant sequences, the corresponding normal cDNA was analyzed to unravel the origin of the variants.

### 4.7.6. *MLH3* mutation analysis (V)

#### 4.7.6.1. *MLH3* germline mutation analysis

In study V, 46 MSI-positive and six MSI-negative colorectal cancer patients from the series of 1044 unselected CRC patients were analyzed for germline *MLH3* mutations by SSCP analysis. The SSCP procedure and PCR conditions are described in detail in study V. In short, *MLH3* was amplified in 24 fragments including all coding regions and exon-intron boundaries. Primers were designed utilizing the Primer3 server (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). PCR fragments were denatured and run on 0.6 x MDE gel (FMC BioProducts) for 14-34 hours to separate the complementary strands. MDE gels were silver-stained according to standard procedure. All aberrant bands were sequenced. All sequence variants were screened in cancer free-individuals to determine their nature.

#### 4.7.6.2. *MLH3* somatic mutation analysis

Ninety-three MSI-positive colorectal cancer patients from the series of 1044 unselected CRC patients were analyzed for somatic mutations in the eight poly-A repeats [$(A_6)$–$(A_9)$] in the coding region of *MLH3* by radioactive PAGE analysis. Primers for short PCR fragments (100 bp or less) were designed utilizing the Primer3 server (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) to maximize the separation on polyacrylamide gel. Details of the PAGE-analysis as well as the PCR conditions are described in study V. In short, 10 µl of radioactive PCR product was mixed with 7 µl of loading buffer, and run on 6% polyacrylamide gels with a constant power of 80 watts. After electrophoresis, the gels were dried and exposed to Kodak Scientific Imaging films (Eastman Kodak Company, Rochester, NY, USA). All aberrant PAGE bands were sequenced, and in all aberrant cases the corresponding normal DNA was analyzed to confirm the somatic nature of the mutation.

To estimate the background poly-A mutation rate, the same 93 tumor samples were analyzed for somatic deletions and insertions in $(A)_8$ and $(A)_9$ repeats in the intron of *OBR*-gene by radioactive PAGE analysis. The following primers were used: $(A_8F)$: CAATTATTTTCCAC CAACAGACC, $(A_8R)$: AAAATCTTTCTGTTGCCACATTC, $(A_9F)$: TGTATGCAGATTG ATGCAGATTT, and $(A_9R)$: TTTTCAATACCACTGTAAACAGGAA. PCR reactions were carried out in a 10-µl volume containing 50 ng of genomic DNA, 1 x PCR buffer (Perkin Elmer), dCTP at 20 µM, 500 µM each of dATP, dGTP, and dTTP (Amersham Pharmacia

Biotech, Piscataway, NJ), 1 µM of each primer, and 1 unit of AmpliTaqGOLD polymerase (Perkin Elmer). The $MgCl_2$ concentration was 4 mM for the $(A)_8$ repeat and 11.5 mM for the $(A)_9$ repeat. The following PCR cycles were used for amplification: 95° for 10 min, 38 cycles of 95° for 45 s, 57° for 45 s $[(A)_8]$ or 61° for 45 s $[(A)_9]$, and 72° for 45 s. Final extension was 72° for 10 min.

## 4.8. Evaluation of selection criteria for the molecular analysis (I)

Selection criteria for the molecular diagnosis of HNPCC have recently been proposed (Aaltonen et al., 1998). These simple criteria include: (1) age of onset of CRC less than 50 years, or (2) synchronous or metachronous CRC or endometrial cancer, or (3) at least one first-degree relative with CRC or endometrial cancer. We prospectively evaluated these criteria in a series of 535 unselected colorectal cancer patients.

## 4.9. Mathematical analysis (III)

Wijnen and colleagues (1998b) proposed an algorithm to be used for predicting the probability of finding a deleterious germline *MLH1* or *MSH2* mutation in patients with HNPCC or an HNPCC-like condition. Details of this mathematical analysis are described in chapter 2.7.1 and in study III. We prospectively tested this algorithm in our series of 509 unselected CRC patients, including 10 mutation-positive patients, to find out whether it could be used for screening for HNPCC. An error was made in the formula used in the original publication of study III presenting data on 509 patients. The error was corrected with results on an additional 535 patients, yielding results on a total of 1044 CRC patients, including 28 mutation-positive patients. Pedigrees comprising first-degree relatives (on average, 8 family members identified) were available for all patients, and extended pedigrees (on average, 38 family members identified) were available on all *MLH1* or *MSH2* germline mutation-positive patients.

# 4.10. Gene expression studies (unpublished data)

*MLH1* expression was analyzed in four MSI-positive colorectal tumors, in which no germline *MLH1* or *MSH2* mutation had been detected, by RT-PCR. A colon cDNA library (Clontech, Palo Alto, CA, USA) was used as a control template. The following primers were used to amplify 236 base pairs of the 5'-end of *MLH1*: (F): AGATCCAAGACAATGGCACC and (R): AGGGGCTTTCAGTTTTCCAT, and as a control for amplification strength, 446 base pairs of the 3'-end of *MSH6*: (F): CTGTGAGGAAGAGGAGCAGG and (R): CTAGGCGC ACAGCAACATT. PCR reactions were carried out in a 50-µl volume containing 5 µl of cDNA, 1 x PCR buffer (Perkin Elmer), 200 µM of each dNTP (Finnzymes), 0.4 µM of each of the four primers, and 2.5 units of AmpliTaqGOLD polymerase (Perkin Elmer). The $MgCl_2$ concentration was 1.5 mM. The following PCR cycles were used for amplification: 95° for 10 min, 40 cycles of 95° for 1 min, 57° for 1 min, and 72° for 1 min. The final extension was 72° for 10 min. The PCR products were run on a 3% agarose gel, and the PCR bands representing both genes were compared with the colon library cDNA. In addition, *MLH1* and *MSH2* expression was scrutinized in tumor sections by immunohistochemistry.

# 5. RESULTS

## 5.1. MSI analysis (I,II)

Of the 535 unselected colorectal cancer patients, 66 (12%) had an MSI-positive tumor. All showed deletions in BAT26, and 58 of these 66 (88%) also showed *TGF-bRII* deletions. Of the 378 adenoma patients, 6 (1.6%) showed MSI. One patient had two independent MSI-positive adenomas. All seven MSI-positive adenomas displayed instability at the BAT26 locus, and three displayed *TGF-bRII* poly-A deletions. BAT26 and *TGF-bRII* results of an MSI-positive CRC patient are illustrated in Figure 4.
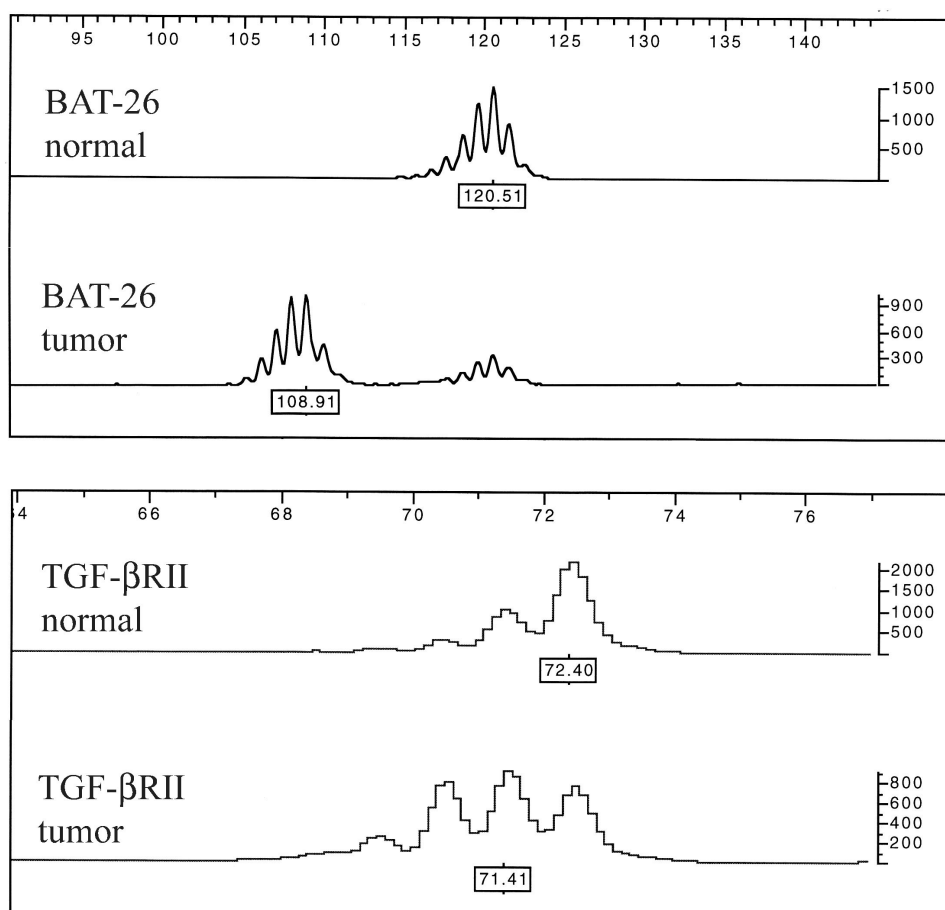


*Figure 4. MSI analysis of an MSI-positive patient utilizing BAT26 and TGF-**b**RII mononucleotide markers. The length of the PCR product is indicated in base pairs, and the intensity of the amplification is indicated on the right. MSI is detected as novel alleles in the tumor DNA that are absent in the normal DNA.*

## 5.2. *MLH1* and *MSH2* mutation analysis (I,II)

Among 535 colorectal cancer patients, 18 (3.4%) had an *MLH1* or *MSH2* mutation. Nine patients had founder mutation 1, and four had mutation 2. Three patients were found to have founder mutation 3 [*MLH1* exon 4, 320T→G (Ile→Arg)]. In addition, two novel mutations were found: *MLH1* exon 4, 378C→G (Tyr→Stop) and *MSH2* exon 12, 1808G→A (Asp→Asn). These novel variants were not present in the 90 cancer-free individuals. All mutations occurred in MSI-positive patients. None of the MSI-negative patients had mutation 1 or mutation 2. A previously described sequence variant of unclear nature [*MSH2* exon 6, 965G→A (Gly→Asp)] was confirmed as a polymorphism.

Among 378 adenoma patients, five (1.3%) had an *MLH1* mutation. Two patients had founder mutation 1, one patient had founder mutation 2, and one patient had mutation 3. In addition, a previously described (Nyström-Lahti et al., 1996) *MLH1* exon 17 nonsense mutation 1975C→T (Arg→Stop) was detected in one patient. No *MSH2* mutations were found. All mutations occurred in MSI-positive patients and none of the MSI-negative patients had mutation 1, mutation 2, or mutation 3.

None of the 2497 anonymous blood donors had mutation 1 or 2. The carrier frequency of these mutations in the cancer-free population appears to be low, perhaps less than 1/2500 or 0.0004 in the geographic area of highest HNPCC incidence in Finland.

## 5.3. Evaluation of selection criteria for molecular diagnosis (I)

Evaluation of the newly proposed selection criteria for molecular diagnosis (Aaltonen et al., 1998) in a series of 535 unselected colorectal cancer patients confirmed the practicality of these criteria. In all, 117 of 535 patients fulfilled at least one of the criteria, 34 of these criteria-positive patients were MSI-positive, and notably 17 of the 34 (50%) had a germline *MLH1* or *MSH2* mutation. Altogether, 17 of 18 (94%) mutation-positive patients had at least one of these features; five were less than 50 years old, seven had a previous or synchronous colorectal or endometrial cancer, and 15 had at least one first-degree relative with colorectal or endometrial cancer.

## 5.4. Mathematical analysis (III)

The series of 1044 unselected CRC patients contained 28 *MLH1* or *MSH2* mutation-positive individuals. Using the algorithm of Wijnen and colleagues (1998b), 18% (basic formula) and 46% (alternative formula) of the mutation-positive individuals had a probability of 20 % or higher to be mutation-positive when first-degree pedigrees were utilized. The respective probabilities were 64% and 75% when extended family history was taken into account. In addition, these formulae yielded a probability of 20% or higher for 11 patients in whom no *MLH1* or *MSH2* mutations were detected.

## 5.5. *MBD4* mutation analysis (IV)

Fourteen of 56 (25%) MSI-positive tumors were found to have somatic deletions in the poly-A tracts in the coding region of *MBD4*. The observed frequencies were 26.2%, 22.2%, and 20.0% in colorectal, endometrial, and pancreatic tumors, respectively. Deletions were seen both in CRC patients with *MLH1* germline mutation as well as in sporadic cases. Thirteen deletions occurred in the $(A)_{10}$ tract, and one deletion was observed in the $(A)_6$ tract. One colorectal tumor had somatic deletions in both alleles in the $(A)_{10}$ repeat; a 1-bp deletion in one and a 2-bp deletion in the other allele. In addition, a somatic *MBD4* deletion was detected in one of six MSI-positive cell lines. The control repeat $(A)_8$ tract of the *PMS2* gene showed no deletions, and the $(A)_9$ tracts of the *BLM* and *RECQL* genes showed only 2 (3.6%) deletions. None of the 116 MSS colorectal cancers had a *MBD4* mutation, and neither did any of the eight MSS cell lines. LOH, as evidence of biallelic inactivation, was detected in four of six colorectal tumors.

## 5.6. *MSH6* mutation analysis (unpublished data)

No germline *MSH6* mutations were found in the four sporadic MSI-positive colorectal cancer samples. One of four samples had a somatic 1-bp deletion in the $(G)_8$ of the *MSH6* coding region. Another sample had a somatic 1980T→C (Leu→Pro) change.

## 5.7. *MLH1* expression studies (unpublished data)

All four tumor-RNAs studied showed *MLH1* expression when scrutinized by RT-PCR analysis. Expression was slightly decreased in one tumor. The 446-bp *MSH6* fragment amplified in the same reaction as a control for amplification strength showed strong *MSH6* expression in all tumors but the one showing a somatic 1-bp deletion in mutation studies. In that sample, *MSH6* expression was decreased. Immunohistochemistry revealed loss of expression of *MLH1* in three cases and loss of expression of *MSH2* in one case. Expression of *MLH1* was also absent in that one sporadic sample for which no fresh-frozen tissue was available.

## 5.8. *MLH3* mutation analysis (V)

No *MLH3* germline mutations were found among the 52 CRC patients included in SSCP analysis. Several sequence variants were detected. In exon 1, a missense type of change 1258G→A (Val→Ile) (allele frequency 2.9%) was discovered. The same change was seen in 3 of 56 cancer-free controls (allele frequency 2.7%), confirming its benign nature. In exon 11, a silent change 4377G→A (Gln→Gln) was detected in 28 patients in heterozygous form and in 10 patients in homozygous form, giving an allele frequency of 46%. The same change was seen in 30 of 57 and in 13 of 57 cancer-free controls in heterozygous and homozygous forms, respectively (allele frequency 49%). In addition to these exonic variants, two intronic polymorphisms were detected.

A 1234A→G (Lys→Glu) change was seen in 1 of 13 tumor samples in exon 1 (allele frequency 3.8%). The change was present both in the tumor as well as in the normal DNA of the patient. The same change was seen in 2 of 92 (allele frequency 1.1%) cancer-free controls. The fact that the patient showing this change has a germline *MLH1* mutation favors the benign nature of the change.

Mutation analysis of poly-A repeats in the coding region of *MLH3* revealed somatic deletions in 8 of 93 (8.3%) MSI-positive CRC patients. Seven patients showed a 1 bp deletion in the $(A)_9$ tract in exon 1 (starting at nucleotide 1861 in mRNA). One patient showed a deletion of

38

1-bp in the $(A)_8$ tract in exon 1 (starting at nucleotide 2128). Deletions of 1 bp in these poly-A tracts cause frameshifts and predicted premature stops at codons 609 and 679, respectively. None of these mutations were present in the corresponding normal DNA.

Somatic deletions in the intronic *OBR* $(A)_8$ and $(A)_9$ repeats were seen in 3 of 93 (3.2%), and in 3 of 93 (3.2%) tumors, respectively. None of these mutations were present in the corresponding normal DNA.

# 6. DISCUSSION

## 6.1. MSI analysis (I, II)

MSI analysis is a sensitive method for prescreening for HNPCC with 85-95% of HNPCC patients showing MSI (Aaltonen et al., 1993, 1994; Pedroni et al., 1999). The major drawback is, however, its low specificity, since 10-15% of sporadic colorectal tumors also show MSI (Aaltonen et al., 1993, 1994; Ionov et al., 1993; Pedroni et al., 1999). The frequency of MSI in HNPCC patients and sporadic cases in study I was 100% (18/18) and 9.3% (48/517), respectively, consistent with previous findings.

Of 535 unselected colorectal cancer patients, 66 (12%) had an MSI-positive tumor. MSI detected in colorectal carcinomas predicted germline mutations in *MLH1* or *MSH2* with a moderate specificity, as 18 of 66 (27%) MSI-positive CRC patients were mutation-positive. The sensitivity of MSI analysis, however, seems to be very high since no founder mutations were found among the 469 MSI-negative patients. MSI-negative patients were excluded from thorough mutation analysis. If some of them did in fact carry a germline defect in some cancer-predisposing gene, it most likely would be another, perhaps yet unknown gene, judging from the absence of the mutator phenotype associated with *MLH1* and *MSH2* mutations. In 97% of the tumor specimens used for DNA extraction, the proportion of tumor cells was more than 50%, which is generally sufficient for detection of novel alleles in the tumor.

We confirm the reliable use of BAT26 in MSI analysis, even without the matching normal DNA. However, in studies I and II, the analysis was repeated with the matching normal DNA for all tumor samples showing aberrant alleles to confirm the somatic origin of the changes. In a separate study utilizing both tumor and normal DNA of approximately 500 Finnish CRC patients, we did not encounter a single case of germline polymorphism (unpublished data), in agreement with previous findings (Hoang et al., 1997). Nevertheless, the possibility of germline polymorphisms should be considered, especially when studying individuals of African origin (Pyatt et al., 1999; Samowitz et al., 1999), as germline polymorphisms appear relatively frequently (7.7-12.6%) among the African-American population (Pyatt et al., 1999; Samowitz et al., 1999).

Relying on BAT26 alone in MSI analysis has prompted the question of possible loss of sensitivity and specificity. The proposed panel of five microsatellite markers (Boland et al., 1998) aims at dividing tumors into categories of MSI-H, MSI-L, and MSS, of which only MSI-H tumors are considered as major candidates for HNPCC. When relying on one marker, distinguishing between MSI-H and MSI-L is impossible. Our results, based on a study of 500 tumors utilizing the recommended panel, suggest that BAT26 alone identifies over 95% of MSI-H tumors (unpublished data), and may only fail to identify a subset of MSI-L tumors. As mentioned above, the involvement of *MLH1* or *MSH2* in MSI-L cases is most unlikely.

Using non-colonic tumors for MSI testing could be an option in cases where colorectal adenomas or carcinomas are unavailable. When considering screening for MSI, the issues of sensitivity and specificity need to be acknowledged. It seems that a high percentage of HNPCC patients' extracolonic tumors show MSI (Aarnio et al., 1997; Ichikawa et al., 1999), but so do a considerable proportion of the sporadic tumors, e.g., endometrial (Risinger et al., 1993; Burks et al., 1994; Duggan et al., 1994), gastric (Han et al., 1993; Yamamoto et al., 1997), and ovarian cancers (Orth et al., 1994), and renal cell carcinoma (Uchida et al., 1994). In sporadic endometrial and gastric cancers, as in sporadic CRC, *MLH1* and *MSH2* mutations are infrequent (Kobayashi et al., 1996; Lim et al., 1996; Keller et al., 1996; Wu et al., 1997), and the reason for MSI is hypermethylation of the *MLH1* promoter region (Simpkins et al., 1999; Leung et al., 1999). Large, population-based studies are needed to evaluate the feasibility of MSI analysis of extracolonic tumors in screening for HNPCC.

## 6.2. Germline mutation analysis (I, II, V)

The three founder mutations in *MLH1* accounted for 89% (16/18) of mutations found in a series of 535 unselected colorectal cancer patients. Such a strong founder effect has some practical advantages since it makes predictive testing feasible. Simple PCR-based tests have been developed for each founder mutation (Nyström-Lahti et al., 1995; study II), enabling asymptomatic mutation carriers to be distinguished from non-carriers, and careful surveillance aimed at detecting tumors for early removal can be offered to the former group. At the same time, non-carriers can be released from systematic screening procedures and also be relieved of an excessive fear of cancer. When dealing with gene testing, the ethical

questions should always be taken into consideration. Proper genetic counseling must be a prerequisite for all further procedures.

The sensitivity of the mutation screening method has a definite impact on the accuracy of mutation detection. Direct genomic sequencing, including exons and exon-intron boundaries, seems to be one of the most sensitive methods, although it has its drawbacks. Large genomic deletions, which may account for up to 10% of *MSH2* mutations (Wijnen et al., 1998a), cannot be detected by this method. We must thus acknowledge the possibility of having missed a few mutations.

No germline *MLH3* mutations were found in 46 MSI-positive and six MSI-negative colorectal cancer patients, excluding its frequent role as a gene predisposing to HNPCC. Again, the sensitivity of the mutation detection method, in this case SSCP, should be taken into consideration. SSCP analysis is estimated to have a sensitivity of 70-95% (Castellvi-Bel et al., 1999; Moore et al., 2000), strongly depending on conditions used. Multiple conditions with variable temperatures and ionic concentrations are suggested for optimal sensitivity. When followed by sequencing of all aberrant bands, SSCP analysis should be adequate for detecting mutations. It is an effective and reasonably inexpensive method. However, we cannot overrule the possibility of a lack of sensitivity, and it is indeed possible that we missed some mutations. Despite our negative results, it is plausible that *MLH3* is involved in HNPCC on rare occasions, similar to *PMS1*, *PMS2*, and *TGFbRII*. Furthermore, the patient panel used in this study can perhaps be criticized. Of the 46 MSI-positive patients, 43 had at least one additional first- or second-degree relative with cancer (in 32 cases an HNPCC-associated cancer, and in 11 cases some non-HNPCC cancer), and three patients were selected based on young age (mean 40 years). The germline role of *MLH3* in HNPCC perhaps needs to be further evaluated in a larger panel of samples with strong features of HNPCC.

MSI-negative patients were included in *MLH3* mutation analysis for two reasons. First, the connection between *MLH3* and microsatellite instability is not yet well established. Second, *MLH3* resides in the chromosomal region 14q24.3, and 14q deletions have previously been shown in the tumors of these six patients by comparative genomic hybridization (CGH) (unpublished data). Although 14q deletions are associated with 17.8% of sporadic colon

cancers (Vogelstein et al., 1989), until now potential cancer genes have not been identified in this region.

# 6.3. Frequency of HNPCC (I)

HNPCC is one of the most common hereditary cancer syndromes identified to date. The estimated incidence has ranged from 2% in Finland (Aaltonen et al., 1998) to 13% in the United Kingdom (Houlston et al., 1992). Frequencies of less than 5% have been found in Italy (Ponz de Leon et al., 1993), Northern Ireland (Kee & Collins, 1991), and England (Stephenson et al., 1991). Differences in the frequencies in part reflect difficulties in diagnosis, and can be explained by several reasons. One major reason is that only a few studies are based on the frequency of MMR gene mutations throughout the general population. Some studies may suffer from overestimation due to familial clustering of phenocopies, or underestimation due to variable phenotypic expression of deleterious mutations. In addition, interactions between the genotype and environmental factors may cause regional differences (Modica et al., 1995).

Combining the results obtained from 535 colorectal cancer patients in study I and a previous study including 509 similar Finnish patients (Aaltonen et al., 1998), we ended up with 1044 unselected colorectal cancer patients, 28 (2.7%) of which had a germline *MLH1* or *MSH2* mutation. Thus, the proportion of HNPCC in Finnish colorectal patients seems to be close to 3%. There are, however, several possible reasons for overestimation, or conversely, underestimation of the frequency. The most obvious reasons for overestimation, i.e., bias in favor of young patients, or of proximal tumors, both of which are characteristic of HNPCC, can mostly be overruled by the design of the study calling for accrual of every tumor, whenever possible. However, it is plausible that the existence of founder mutations in Finland increased the overall HNPCC incidence. By excluding the three most common Finnish founder mutations, we end up with only 5 of 1044 (0.5%) mutation-positive patients.

It is reasonable to suggest that 2.7% is an underestimate of HNPCC in the population we studied. MSI can show false negative results either for technical reasons or because the specimen contains an inadequate amount of tumor DNA. The sensitivity of direct genomic sequencing in detecting heterozygous mutations is difficult to evaluate, but it is certainly not

100% (Liu et al., 1995a). Moreover, large deletions that are undetectable by most methods (except Southern hybridization) exist in *MSH2*, and could account for up to 10% of all mutations (Wijnen et al., 1998a). Underestimation can also be due to only CRC patients being screened. If patients with endometrial cancer or other common HNPCC-associated cancers had been screened in the same population at the same time, some additional HNPCC cases would probably have been detected. In addition, the involvement of genes other than *MLH1* and *MSH2*, such as *MSH6* (Akiyama et al., 1997b; Miyaki et al., 1997; Verma et al., 1999), needs to be taken into consideration.

# 6.4. Adenomas in HNPCC patients (II)

HNPCC tumors are likely to arise within adenomas. However, the role of adenomas in the process leading to HNPCC cancer is less clear than in familial adenomatous polyposis (FAP) (Jass, 1989; Lynch et al., 1995; Tsao et al., 1999). Furthermore, in contrast to FAP, where hundreds of polyps appear in the GI tract (Bussey, 1975), HNPCC patients typically develop only one or a few lesions.

In a series of 378 adenoma patients, we found six (1.6%) MSI-positive patients identified by BAT26 deletions. Of MSI-positive lesions, three of seven (43%) carried somatic truncating mutations of the *TGF-bRII* gene. These mutations have also previously been found in adenomas from individuals with HNPCC (Akiyama et al., 1997a), which suggests that *TGF-bRII* inactivation is an early event in HNPCC colon neoplasia. MSI detected in adenomas predicted germline mutations in *MLH1* or *MSH2* with a high specificity, as five of six (83%) MSI-positive adenoma patients were mutation-positive. The one patient with MSI-positive adenoma who had no association with an HNPCC kindred, and in whom no *MLH1* or *MSH2* mutations were detected, was a 66-year-old female. The pedigree of the patient was extended, and data on the relatives was linked to the Finnish Cancer Registry. No cancer had been reported for the patient's brother (aged 69 years) or four children (aged 37 to 40 years). Thus, strong features of HNPCC were not revealed.

The aim of the study was to evaluate MSI analysis in prescreening unselected colorectal adenoma patients for HNPCC. However, during genealogical studies, the series of 378

patients was found to contain 11 patients from known HNPCC families, and in seven of 11 families, the mutation segregating in the family was known. Therefore, the series used was not entirely unselected.

The frequency of MSI in adenomas derived from patients with a germline MMR-mutation was 100% (6/6), whereas the frequency in sporadic adenomas was 0.3% (1/396). This is in accordance with previous reports (Aaltonen et al., 1993, 1994; Young et al., 1993; Samowitz et al., 1999). The small number of mutation-positive individuals prevents the formation of definitive conclusions. Nevertheless, MSI in adenomas appears to be a specific marker for HNPCC. As MSI is very rare in sporadic adenomas, routine screening of such lesions for MSI may not be feasible. However, MSI analysis of adenomas is likely to be useful in cases with features of hereditary predisposition. This aspect is clearly illustrated in study II, in which two adenoma patients were members of known HNPCC families, but had MSI-negative adenomas and were shown not to carry the mutation segregating in the family. The case for utilizing MSI analysis in adenomas is even stronger in colon cancer families with MSI-positive tumors and unidentified mutations. Adenomas are common in the general population, but the detection of an adenoma in colonoscopic screening frequently causes uncertainty and confusion. MSI analysis adds an important parameter to be considered in the interpretation of the patient's cancer risk.

# 6.5. Target genes for MSI (IV, V)

Mismatch repair-deficient cells acquire somatic deletions and insertions in simple repeated sequences throughout the genome (Perucho, 1996). Several genes involved in tumorigenesis harbor mononucleotide repeats in their coding regions and are thus attractive targets for somatic mutations.

Deletions and insertions of a couple of base pairs cause frameshifts and premature stop codons, and are likely to abolish the function of the gene. MMR-genes *MSH3* and *MSH6* frequently show somatic deletions in MSI-positive tumors (Malkhosyan et al., 1996; Akiyama et al., 1997c). Both genes have confirmed roles in human mismatch repair and appear to be selected targets for secondary hits. Accumulation of somatic mutations in

mismatch repair genes, such as *MSH3* and *MSH6*, is believed to further increase genetic instability (Malkhosyan et al., 1996).

Accumulation of somatic mutations in proto-oncogenes and tumor suppressor genes is the cause of cancer in HNPCC patients, as well as in all cancer patients. The increased mutation rate leads to accelerated progression, manifesting in early onset of cancer. Somatic mutations in the poly-A tract in the coding region of *TGF-$\beta$RII* are almost invariably detected in mismatch repair-deficient colorectal tumors (Lu et al., 1995; Markowitz et al., 1995; Parsons et al., 1995; Akiyama et al., 1997a). Truncating mutations in this receptor lead to resistance to inhibitory signals and thus promote cell proliferation.

The recently cloned DNA mismatch repair gene *MBD4* (*MED1*) (Bellacosa et al., 1999) does not have a confirmed role in mismatch repair. However, an association between *MBD4* and human mismatch repair has been proposed based on functional homology to bacterial *MutH* genes. To study whether *MBD4* is involved in MSI-positive colorectal tumorigenesis, we performed somatic mutation analysis of *MBD4* on 52 MSI-positive tumors. In its coding region *MBD4* harbors four potentially hypermutable tracts: one $(A)_{10}$ tract and three $(A)_6$ tracts. Somatic deletions in *MBD4* were detected in 25% of MSI-positive tumors. The lack of frequent deletions in similar control repeats implicates the involvement of *MBD4* as a selected target for MSI. Taken together with a recent study in which *MBD4* somatic deletions were found in 40% of MSI-positive tumors (Bader et al., 1999), the somatic role of *MBD4* in MSI-positive tumorigenesis is confirmed. Further studies are needed to evaluate its possible role as a gene predisposing to HNPCC.

*MBD4* mutations were found both in sporadic tumors as well as in HNPCC tumors. *MLH1* and *MSH2* mutations had previously been excluded in the sporadic cases, and to further confirm the sporadic nature of these tumors, *MSH6* mutation analysis was performed. The limited sensitivity of the mutation detection method used, namely RT-PCR, should be noted. Mutant alleles may differ in their relative expression level, or may have lower stability and are thus often underrepresented. Furthermore, RT-PCR fails to detect splice-site mutations as such, although the result of a splice-site mutation is typically visualized as a shorter PCR product. Thus, it is plausible that we missed some *MSH6* mutations.

Similarly, the role of the recently cloned *MLH3* gene (Lipkin et al., 2000) in mismatch repair is still obscure. An association between *MLH3* and DNA mismatch repair can be proposed based on its homology to bacterial *MutL* genes and since overexpressed dominant negative *MLH3* induces MSI in mammalian cell culture. To study the possible involvement of *MLH3* in HNPCC and MSI-positive colorectal cancer, we performed germline mutation analysis on 46 MSI-positive and six MSI-negative colorectal cancer patients, with negative results, and somatic mutation analysis on 93 MSI-positive colorectal tumors. *MLH3* harbors eight poly-A tracts in its coding region: six discrete $(A)_6$ repeats, one $(A)_8$ repeat, and one $(A)_9$ repeat. Despite the abundant targets for MSI, the frequency of somatic deletions detected in *MLH3* (8.6%) did not significantly differ ($p > 0.05$) from the frequency of deletions detected in similar repeats in the non-coding region (6.5%). Thus, it seems that *MLH3* is not a selected target for MSI, but only accumulates mutations at a more or less equivalent rate to any sequence in the genome with similar nucleotide composition. Possible explanations for the lack of selection for *MLH3* somatic mutations include the possibility that *MLH3* does not have a prominent role in human mismatch repair, and thus inactivating mutations do not contribute to tumor formation. Alternatively, redundant functions of *MLH3* and *PMS2* may prohibit the manifestation of *MLH3* deficiency.

Exonic repeats have typically been used as controls for the background mutation rate. The problem in this approach is the difficulty in estimating the possible selective advantage cells with mutations in these repeats may have. In addition, mutations in some essential genes are not compatible with survival and thus their observed frequency is an underestimate. Our analysis of the intronic repeats gives valuable information on the background mutation rate in MSI-positive tumors. Since these intronic mutations most likely have no impact on tumor development, they are not selected for or against. The observed mutation frequencies were 3.2% in $(A)_9$ and 3.2% in $(A)_8$ repeats. These frequencies can be used as controls in future studies on possible target genes for MSI. Importantly, it is not known whether the background mutation frequencies in different repeats, i.e., $(A)_n$ vs. $(G)_n$, are different, and thus the mutation frequencies observed in a given gene should be compared with the background mutation rate of a similar repeat.

In order to give perspective to the present findings, some target genes in MSI-positive colorectal tumorigenesis are presented in Table 5. More target genes apparently exist and will

likely be revealed in further studies concentrating on genes harboring potentially hypermutable tracts in their coding regions.

| Gene | Function | Repeat | Frequency | Reference |
|---|---|---|---|---|
| MSH6 | MMR-gene | $(C)_8$ | 30% | Malkhosyan et al., 1996 |
| MSH3 | MMR-gene | $(A)_8$ | 39-58% | Malkhosyan et al., 1996; Akiyama et a., 1997c |
| BAX | promotes apoptosis | $(G)_8$ | 51% | Rampino et al., 1997 |
| IGFIIR | growth factor receptor | $(G)_8$ | 6-13% | Souza et al., 1996 Ouyang et al., 1997 |
| TGF-**b**RII | growth factor receptor | $(A)_{10}$ | 88-90% | Parsons et al., 1995; Study I |
| TCF-4 | TF [a] involved in the APC/**b**-cathenin pathway | $(A)_9$ | 34% | Duval et al., 1999 |
| CHK1 | $G_2$ checkpoint kinase | $(A)_9$ | 10% | Bertoni et al., 1999 |
| RIZ | RB-interacting zinc finger protein | $(A)_9, (A)_8$ | 37.5% | Chadwick et al., 2000 |
| MBD4 | MMR-gene (?) | $(A)_{10}, (A)_6$ | 26.2-40% | Bader et al., 1999; Study IV |
| MLH3 | MMR-gene (?) | $(A)_9, (A)_8$ | 8.6% | Study V |
| Intron [b] | not known | $(A)_9, (A)_8$ | 6.5% | Study V |

*Table 5. Frequencies of somatic deletions/insertions in intragenic repeats of several genes in MSI-positive colorectal tumors. [a] transcription factor, [b] intron of the leptin receptor gene OBR.*

# 6.6. Diagnosis of HNPCC (I, II, III)

Characteristic features of HNPCC include frequent synchronous and metachronous tumors as well as an early age of onset (Mecklin & Järvinen, 1986; Vasen et al., 1990; Farrington et al., 1996). Affected individuals are estimated to have a 40% risk for a second colorectal cancer over the next 10 years. Accordingly HNPCC patients need regular surveillance. These facts alone are sufficient to indicate the urgent need for reliable diagnosis of this disease.

Identification of HNPCC patients enables prevention of colorectal cancer by means of colonoscopy and polypectomies. Regular colonoscopy is a powerful tool for the detection of premalignant adenomas and early carcinomas, resulting in improved survival (Vasen et al., 1995, 1998). Colonoscopic screenings at 3-year intervals have been found to decrease the risk for CRC by 50-60%, and prevent cancer deaths in HNPCC patients (Järvinen et al., 2000). Prophylactic subtotal colectomy can be offered as an alternative to lifetime colonoscopic

surveillance for mutation-positive individuals. Unfortunately, the prevention of other HNPCC-associated malignancies is not as straightforward due to the lack of appropriate screening methods.

The means for prevention of colorectal cancer exist. One of the major problems at the moment lies in the difficulties in diagnosing. Traditionally, the diagnosis of HNPCC has been based on a family history of cancer. The so-called Amsterdam criteria were established in an attempt to standardize the diagnosis (Vasen et al., 1991, 1999). These criteria have high specificity, since a considerable proportion of criteria positive patients are found to have a germline MMR-mutation (Nyström-Lahti et al., 1996; Wijnen et al., 1997, 1998b), but are often regarded as overly stringent. By utilizing these criteria, a large proportion of HNPCC patients will be missed, especially if only first-degree pedigrees are available (Rodriguez-Bigas et al., 1997). In a combined series (Aaltonen et al., 1998; study I) of 1044 CRC patients including 28 MMR germline mutation-positive patients, only 3 of 28 (11%) fulfilled these criteria (I) when pedigrees of an average of 8 family members were available. After extending the pedigrees to include an average of 38 family members, 16 of 28 (57%) patients fulfilled the criteria. The low sensitivity of these criteria impedes their use in diagnosis.

In an attempt to improve the diagnosis based on family history, a mathematical algorithm based on 184 Dutch HNPCC kindreds was introduced (Wijnen et al., 1998b). This algorithm is based on simple variables (mean age of CRC diagnosis in the family, fulfillment of Amsterdam criteria, and presence of endometrial cancer in the family) and can be easily used to calculate the probability of finding a deleterious *MLH1* or *MSH2* mutation. In addition, an alternative algorithm to be used in small families was also proposed. A probability of 20% or higher was proposed to justify mutation analyses (Wijnen et al., 1998b). We prospectively evaluated the power of this algorithm in screening patients for HNPCC in our series of 1044 unselected colorectal cancer patients including 28 mutation-positive patients. When first-degree pedigrees were utilized, 18% (basic formula) and 46% (alternative formula) of the mutation-positive individuals had a probability of 20% or higher. The respective probabilities were 64% and 75% when extensive family history was considered. In addition, these formulae yielded a probability of 20% or higher for 11 patients in whom no *MLH1* or *MSH2* mutations were detected. Extensive pedigree data are difficult to obtain in clinical practice. In contrast, first-degree pedigrees are generally easily obtained during patient interview. We must conclude that this algorithm cannot be used for screening for HNPCC due to its low

sensitivity. However, it most likely has some utility in evaluating the probability of HNPCC in individual situations.

MSI analysis has been successfully used for pre-diagnosing colorectal cancer patients for HNPCC. Its major drawback is low specificity since the MSI-phenomenon is not inclusive for HNPCC. As neither family criteria nor MSI-analysis alone are adequate for diagnosis, perhaps these two could be consolidated. To test this, selection criteria for the molecular diagnosis of HNPCC were retrospectively established on the basis of 509 colorectal cancer patients including 10 mutation-positive patients (Aaltonen et al., 1998). These criteria include (1) age of onset of CRC less than 50 years, or (2) a previous or synchronous CRC or endometrial cancer, or (3) at least one first-degree relative with CRC or endometrial cancer. Criteria-positive patients are subjected to MSI analysis, and MSI-positive patients are then analyzed for *MLH1* and *MSH2* mutations. We prospectively evaluated the combination of these criteria and MSI analysis in 535 colorectal cancer patients to confirm specificity and sensitivity. The MSI and mutation status of these patients was determined before the implementation of these criteria in a procedure where all 66 MSI-positive patients were subjected to mutation analysis.

Utilizing these simple selection criteria, the number of patients in MSI analysis could be reduced from 535 to 117 (reduction 78%), and the number of patients in mutation analysis reduced from 66 to 34 (reduction 48%). Implementation of these criteria increased the specificity of MSI analysis from 27% (18/66 MSI-positive patients had a mutation) to 50%, since 17 of 34 MSI-positive patients had a germline mutation. The sensitivity was only slightly decreased, with 17 of 18 (94%) mutation-positive patients being identified.

When combining the patient material from study I and from Aaltonen and colleagues (1998), we ended up with a series of 1044 unselected colorectal cancer patients including 28 mutation-positive patients. Utilizing this material, it is possible to compare the outcomes of different diagnosing procedures. As concluded above, the Amsterdam criteria as well as the mathematical algorithm rely heavily on extensive family history, and have a questionable value in diagnosing for HNPCC. Figure 5 presents the different flowcharts when utilizing (A) direct MSI analysis followed by mutation analysis and (B) implementation of the selection criteria (Aaltonen et al., 1998) prior to MSI and mutation analyses. By using these simple

selection criteria, the specificity and cost-effectiveness of the screening procedure can be considerably increased.
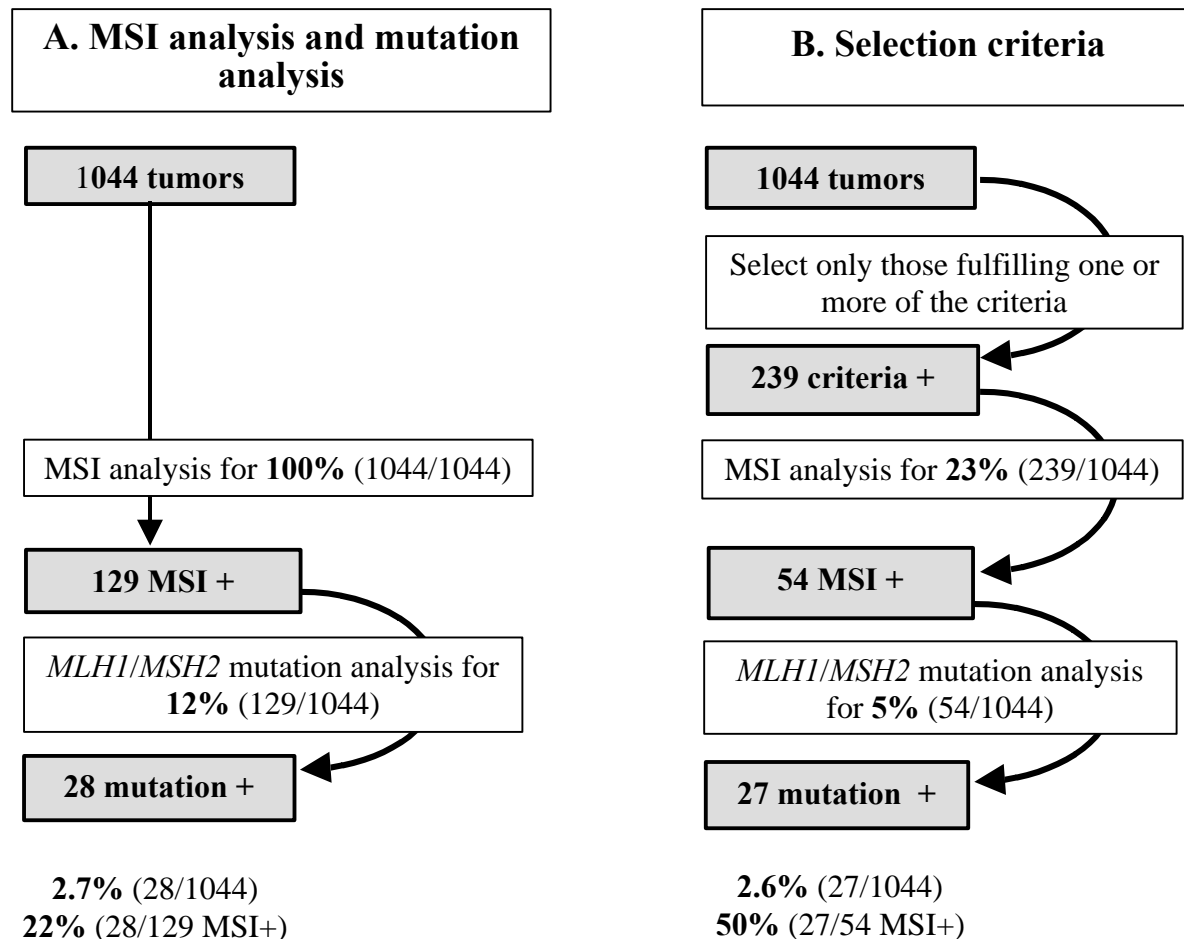


| **A. MSI analysis and mutation analysis** | **B. Selection criteria** |
|---|---|
| **1044 tumors** | **1044 tumors** |
| | Select only those fulfilling one or more of the criteria |
| | **239 criteria +** |
| MSI analysis for **100%** (1044/1044) | MSI analysis for **23%** (239/1044) |
| **129 MSI +** | **54 MSI +** |
| *MLH1*/*MSH2* mutation analysis for **12%** (129/1044) | *MLH1*/*MSH2* mutation analysis for **5%** (54/1044) |
| **28 mutation +** | **27 mutation +** |
| **2.7%** (28/1044) <br> **22%** (28/129 MSI+) | **2.6%** (27/1044) <br> **50%** (27/54 MSI+) |

*Figure 5. Flow chart for the diagnosis of HNPCC using (A) direct MSI analysis followed by mutation analysis, and (B) selection criteria (Aaltonen et al., 1998) prior to MSI analysis and mutation analysis.*

An optimal procedure for diagnosing should maximize both sensitivity (as few false negatives as possible) and specificity (as few false positives as possible). Because extensive pedigree information and verified data on cancer in distant family members cannot always be obtained, the procedure should be based on family data of first-degree relatives only. The use of MSI as a prescreening test in every case is recommended, as it appears to carry only a 5-15% false negative rate (Vasen et al., 1999; Verma et al., 1999). Based on these cornerstones, we propose the following: perform MSI test for patients that fulfill at least one of the three selection criteria proposed by Aaltonen and colleagues (1998), and search for mutations in

those patients whose tumors are MSI-positive. The outcome of the combined series of 1044 unselected colorectal cancer patients (Aaltonen et al., 1998 and study I) resulted in MSI and mutation analyses for only 23% and 5% of the patients, respectively. Utilizing these simple and cost-effective procedures, 96% of HNPCC patients will be identified.

The model we propose relies heavily on the MSI test as a primary screen. A high sensitivity is suggested by the fact that 85-95% of HNPCC tumors have been shown to be MSI-positive (Aaltonen et al., 1994; Lynch & Smyrk, 1998). Our study provided an excellent test of this since we screened all 1044 patients for two founder mutations that account for over half of all mutations detected in Finland so far. Of all 129 MSI-positive patients, 19 were found to have one of these mutations, while of 915 MSI-negative patients, neither of the mutations were found. This indicates that MSI, when appropriately determined, shows a high sensitivity for mutation-positive HNPCC tumors. These data also confirm that BAT26 alone is a sensitive indicator of MSI, as previously suggested (de la Chapelle, 1999; Samowitz et al., 1999; Sutter et al., 1999). However, the specificity of MSI is low mainly because a large proportion of all MSI-positive tumors are due to epigenetic silencing of the *MLH1* gene, a somatic event caused by promoter methylation (Kane et al., 1997). We show here that the specificity can be enhanced to some 50% by selecting patients fulfilling certain simple clinical criteria.

# 7. FUTURE PROSPECTS

Colorectal cancer develops through complicated pathways. Although a model for sequential events has been proposed (Kinzler & Vogelstein, 1996), increasing evidence supporting deviations and modifications of the basic theme is mounting. The identification of all factors in the sequence of events is important. Molecular profiling allows for the identification of novel prognostic markers and possible targets for subsequent gene therapy. As an example, allelic loss of chromosome 18q implicates a poor prognosis (Ogunbiyi et al., 1998). Similarly, loss of imprinting (LOI) might have potential as a diagnostic marker since it has been detected both in cancer tissue and in the normal mucosa and peripheral blood cells of a subset of cancer patients with MSI-positive tumors (Cui et al., 1998). Furthermore, detection of MSI in urine DNA serves as a feasible test for follow-up of patients with transitional cell carcinoma of the bladder (Steiner et al., 1997).

Differences in target genes in different subtypes of colorectal cancer may lead to differential prognosis and survival rates. While it is a well-known fact that MSI-positive tumors have a better prognosis (Sankila et al., 1996), patients with MSI-positive tumors have a high risk for multiple tumors (Cawkwell et al., 1999). Although the underlying mechanisms are somewhat obscure, the importance of determining the MSI status, perhaps in all newly diagnosed cases, is clear. Another important point related to the MSI status is that mismatch repair-deficient cells are highly tolerant of methylating chemotherapeutic drugs such as streptozocin and temazolomide (Claij & Riele, 1999). Interestingly, aspirin appears to suppress the mutator phenotype *in vitro* (Rüschoff et al., 1998), and also has a possible preventive effect *in vivo* (Barnes & Lee, 1998; Gupta & DuBois, 1998).

When considering HNPCC specifically, future prospects include determining the composition of an optimal microsatellite marker panel for MSI analysis, yielding reliable results with a minimum amount of time and resources. The use of immunohistochemistry in evaluating MSI status seems to be a feasible method, but needs to be carefully evaluated. Possible new genes predisposing to HNPCC await identification. For example, the possible germline role of *MBD4* needs to be studied. Furthermore, the possibility of frequent germline *MSH6* mutations should be considered in future studies. The selection criteria for molecular analysis will be further tested. Collection of colorectal cancer samples on the basis of the selection

criteria, followed by MSI and mutation analyses will give a reliable estimation of the specificity of these criteria.

Since colorectal cancer can be effectively controlled by colonoscopic screenings and early removal of lesions, more attention should be directed at extracolonic cancers. Importantly, establishing efficient screening methods for the common extracolonic tumors would further reduce the morbidity and mortality of HNPCC.

# 8. SUMMARY AND CONCLUSIONS

We evaluated molecular diagnosing methods for the most common form of inherited colorectal cancer, i.e., hereditary nonpolyposis colorectal cancer (HNPCC). Early and reliable diagnosis of HNPCC is essential, since cancer deaths can be prevented by early removal of adenomas and carcinomas. As many as 85-95% of HNPCC patients show microsatellite instability (MSI), a phenomenon reflecting defects in DNA mismatch repair genes. Although MSI analysis shows high sensitivity, it is an unspecific marker for HNPCC as some 10-15% of sporadic colorectal tumors are also MSI-positive. However, a germline mutation in one of the DNA mismatch repair genes can confirm the diagnosis.

The combination of microsatellite instability (MSI) analysis and mutation analysis was tested in unselected colorectal carcinoma and adenoma patients (studies I and II). In addition, newly created selection criteria for molecular diagnosis of HNPCC (Aaltonen et al., 1998) and a mathematical model (Wijnen et al., 1998b) were prospectively evaluated (studies I and III). To further study the molecular background of HNPCC and MSI-positive colorectal cancer, the possible involvement of two novel DNA mismatch repair genes, *MBD4* and *MLH3*, was studied (studies IV and V). The present study allows for the following conclusions to be drawn:

1. The frequency of HNPCC in Finnish unselected colorectal cancer patients was 3.4% (18/535). Three founder mutations in *MLH1* accounted for 89% of all cases. The frequency of HNPCC in colorectal adenoma patients was 1.3% (5/378), with founder mutations accounting for 80% of these cases. Probably due to *MLH1* founder mutations, *MSH2* mutations (1/23) are underrepresented in Finnish HNPCC patients.

2. MSI was detected in 12% of unselected colorectal cancer patients. A germline *MLH1* or *MSH2* mutation was identified in 18 of 66 (27%) MSI-positive CRC patients. Thus, the sensitivity of MSI analysis in pre-screening for HNPCC was moderate (27%), but since no founder mutations were found in the 469 MSI-negative patients, the false negative rate is likely very low. Altogether 1.7% (7/402) of adenoma samples showed MSI, and 5 of 6 (83%) MSI-positive adenoma patients had a germline *MLH1* mutation. Therefore, the sensitivity of MSI analysis in screening for HNPCC was very

55

high (83%). Again, the false negative rate is likely to be very low, since no founder mutations were detected among the 372 MSI-negative adenoma patients. In conclusion, MSI analysis is a promising method to pre-screen colorectal carcinoma and adenoma patients for HNPCC.

3.  Fulfillment of the following selection criteria for molecular analysis were evaluated among unselected CRC patients: (1) age of onset less than 50 years, or (2) previous/synchronous CRC or endometrial cancer, or (3) at least one first-degree relative with CRC or endometrial cancer. By selecting only criteria-positive patients for further analyses, the sensitivity of MSI analysis can be increased. Of 535 CRC patients, 117 (22%) were-criteria positive and 34 of these 117 (29%) were MSI-positive; 17 of the 18 (94%) mutation-positive individuals were among these 34. These simple criteria can be used routinely in clinics for identifying patients for more detailed diagnostic analyses (i.e. MSI and mutation analyses). Implementation of these selection criteria assures a significantly higher specificity (50%), with only a minor reduction in sensitivity.

    In a series of 1044 colorectal cancer patients, the mathematical algorithm (Wijnen et al., 1998b) utilizing family history was able to identify 18% and 64% of mutation-positive patients when first-degree pedigrees (on average, 8 family members) and extended family histories (on average, 38 family members), respectively, were used. The corresponding proportions for an alternative formula designed for small families were 46% and 75%. Thus, the mathematical algorithm (Wijnen et al., 1998b) cannot be relied on when screening for HNPCC.

4.  The involvement of recently cloned human mismatch repair genes *MBD4* (Bellacosa et al., 1999) and *MLH3* (Lipkin et al., 2000) was evaluated in MSI-positive tumors. Both genes harbor simple mononucleotide repeats in their coding regions which makes them attractive targets for the mutator phenotype. Somatic deletions of *MBD4* were observed in 25% of MSI-positive tumors, indicating a selective advantage in tumorigenesis. Possible germline involvement was not studied. Somatic deletions in *MLH3* were observed in 8.6% of MSI-positive colorectal tumors. A frequency of 6.5% was detected in similar repeats in noncoding regions, indicating that the *MLH3* deletions are merely a consequence of the MSI phenotype, rather than selected events

56

driving tumorigenesis. No germline *MLH3* mutations were found, which excluded the possibility of it being a frequent HNPCC gene.

MSI analysis is recommended for colorectal adenoma patients with features of hereditary predisposition. Especially patients in families with MSI-positive tumors and either identified or unidentified mutations benefit from MSI analysis. A negative MSI result in these cases favors the sporadic nature of the adenoma, whereas a positive result strongly suggests the presence of HNPCC.

As a final conclusion, we propose the following procedure for diagnosing HNPCC. All colorectal cancer patients should be evaluated for the selection criteria. All criteria-positive patients should be included in MSI analysis utilizing at least the BAT26 mononucleotide marker, or, if feasible, utilizing immunohistochemistry for MLH1, MSH2, and possibly MSH6. All MSI-positive patients should be analyzed for *MLH1* and *MSH2* germline mutations. In addition, the possible involvement of *MSH6* mutations should be considered. This scenario implicates doing MSI and mutation analyses for approximately 23% and 5% of patients, respectively. By utilizing this cost-effective procedure, 96% of HNPCC patients will be identified.

# 9. ACKNOWLEDGMENTS

Espoo, July 2000

Anu-Maria Loukola

# 10. REFERENCES

Aaltonen LA, Peltomäki P, Leach F, Sistonen P, Pylkkänen L, Mecklin J-P, Järvinen H, Powell S, Jen J, Hamilton SR, Petersen GM, Kinzler KW, Vogelstein B, and de la Chapelle A. Clues to the pathogenesis of familial colorectal cancer. *Science* 260: 812-816, 1993

Aaltonen LA, Peltomäki P, Mecklin J-P, Järvinen H, Jass JR, Green JS, Lynch HT, Watson P, Tallqvist G, Juhola M, Sistonen P, Hamilton SR, Kinzler KW, Vogelstein B, and de la Chapelle A. Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. *Cancer Research* 54: 1645-1648, 1994

Aaltonen LA, Salovaara R, Kristo P, Canzian F, Hemminki A, Peltomäki P, Chadwick RB, Percesepe A, Kääriäinen H, Ahtola H, Eskelinen M, Härkönen N, Julkunen R, Kangas E, Ojala S, Tulikoura J, Valkamo E, Järvinen HJ, Mecklin J-P, and de la Chapelle A. Incidence of hereditary nonpolyposis colorectal cancer, and molecular screening for the disease. *New England Journal of Medicine* 1338:1481-1487, 1998

Aarnio M, Mecklin J-P, Aaltonen LA, Nyström-Lahti M, and Järvinen HJ. Life-time risk of different cancers in hereditary nonpolyposis colorectal cancer (HNPCC) syndrome. *International Journal of Cancer* 64: 430-433, 1995

Aarnio M, Salovaara R, Aaltonen LA, Mecklin J-P, and Järvinen H. Features of gastric cancer in hereditary non-polyposis colorectal cancer syndrome. *International Journal of Cancer* 74: 551-555, 1997

Ahn J, Ludecke HJ, Lindow S, Horton WA, Lee B, Wagner MJ, Horsthemke B, and Wells DE. Cloning of the putative tumour suppressor gene for hereditary multiple exostoses (*EXT1*). *Nature Genetics* 11: 137-143, 1995

Akiyama Y, Iwanaga R, Kiyoshi S, Shiba K, Ushio K, Ikeda E, Iwama T, Nomizu T, and Yasuhito Y. *Transforming growth factor b type II receptor* gene mutations in adenomas from hereditary nonpolyposis colorectal cancer. *Gastroenterology* 112: 33-39, 1997a

Akiyama Y, Sato H, Yamada T, Nagasaki H, Tsuchiya A, Abe R, and Yuasa Y. Germ-line mutation of the *hMSH6/GTBP* gene in an atypical hereditary nonpolyposis colorectal cancer kindred. *Cancer Research* 57: 3920-3923, 1997b

Akiyama Y, Tsubouchi N, and Yuasa Y. Frequent somatic mutations of *hMSH3* with reference to microsatellite instability in hereditary nonpolyposis colorectal cancers. *Biochemical and Biophysical Research Communications* 236: 248-252, 1997c

Bader S, Walker M, Hendrich B, Bird A, Bird C, Hooper M, and Wyllie A. Somatic frameshift mutations in the *MBD4* gene of sporadic colon cancers with mismatch repair deficiency. *Oncogene* 18: 8044-8047, 1999

Baker SJ, Fearon E, Nigro J, Hamilton S, Preisinger A, Jessup M, van Tuinen P, Ledbetter D, Barker D, Nakamura Y, White R, and Vogelstein B. Chromosome 17 deletions and *p53* mutations in colorectal carcinomas. *Science* 244: 217-221, 1989

Baker SJ, Preisinger AC, Jessup JM, Paraskeva C, Markowitz S, Wilson JK, Hamilton S, and Vogelstein B. *P53* gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Research* 50: 7717-7722, 1990

Barnes CJ, and Lee M. Chemoprevention of spontaneous intestinal adenomas in the adenomatous polyposis coli *Min* mouse model with aspirin. *Gastroenterology* 114: 873-877, 1998

Baylin SB, Herman JG, Graff JR, Vertino PM, and Issa J-P. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Advances in Cancer Research* 72: 141-196, 1998

Bellacosa A, Cicchillitti L, Schepis F, Riccio A, Yeung AT, Matsumoto Y, Golemis EA, Genuardi M, and Neri G. MED1, a novel human methyl-CpG-binding endonuclease, interacts with DNA mismatch repair protein MLH1. *Proceedings of the National Academy of Science USA* 96: 3969-3974, 1999

Bertoni F, Codegoni AM, Furlan D, Tibiletti MG, Capella C, and Broggini M. *CHK1* frameshift mutations in genetically unstable colorectal and endometrial cancers. *Genes, Chromosomes & Cancer* 26: 176-180, 1999

Boland CR, Thibodeau SN, Hamilton SR, Sudransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, and Srivastava S. A National Cancer Institute Workshop on Microsatellite Instability for Cancer Detection and Familial Predisposition: Development of International Criteria for the Determination of Microsatellite Instability in Colorectal Cancer. *Cancer Research* 58: 5248-5257, 1998

Bradshaw PS, Hamoudi R, Min T, Catovsky D, Houlston RS, and Yuille MR. Fluorescent BAT-25 and BAT-26 analysis of T cell promyelocytic leukaemia. *Leukemia* 13: 2104-2106, 1999

Bronner CE, Baker SM, Morrison PT, Warren G, Smith LG, Lescoe MK, Kane M, Earabino C, Lipford J, Lindblom A, Tannergård P, Bollag RJ, Godwin AR, Ward DC, Nordenskjöld M, Fishel R, Kolodner R, and Liskay RM. Mutation in the DNA mismatch repair gene homologue *hMLH1* is associated with hereditary non-polyposis colon cancer. *Nature* 368: 258-61, 1994

Burks RT, Kessis TD, Cho KR, and Hedrick L. Microsatellite instability in endometrial carcinoma. *Oncogene* 9: 1163-1166, 1994

Burt RW, Bishop DT, Lynch HT, Rozen P, and Winawer SJ. Risk and surveillance of individuals with heritable factors for colorectal cancer. *WHO Bulletin* 68: 655-664, 1993

Bussey HJR. Family studies, histopathology, differentail diagnosis, and results of treatment. In: *Familial polyposis coli*. The Johns Hopkins University Press, Baltimore, USA, 1975

Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Kral A, Yeger H, Lewis WH, Jones C, and Housman DE. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell* 60: 509-520, 1990

Cannon-Albright LA, Skolnick MH, Bishop DT, Lee RG, and Burt RW. Common inheritance of susceptibility to colonic adenomatous polyps and associated colorectal cancers. *New England Journal of Medicine* 319: 533-537, 1988

Castellvi-Bel S, Sheikhavandi S, Telatar M, Tai LQ, Hwang M, Wang Z, Yang Z, Cheng R, and Gatti RA. New mutations, polymorphisms, and rare variants in the *ATM* gene detected by a novel SSCP strategy. *Human Mutations* 14: 156-162, 1999

Cawkwell L, Gray S, Murgatroyd H, Sutherland F, Haine L, Longfellow M, O'Loughlin S, Cross D, Kronborg O, Fenger C, Mapstone N, Dixon M, and Quirke P. Choice of management strategy for colorectal cancer based on a diagnostic immunohistochemical test for defective mismatch repair. *Gut* 45: 409-415, 1999

Chadwick RB, Jiang G-L, Bennington GA, Yuan B, Johnson CK, Stevens MW, Niemann TH, Peltomäki P, Huang S, and de la Chapelle A. Candidate tumor suppressor *RIZ* is frequently involved in colorectal carcinogenesis. *Proceedings of the National Academy of Science* 97: 2662-2667, 2000

Chung DC, and Rustgi AK. DNA mismatch repair and cancer. *Gastroenterology* 109: 1685-1699, 1995

Claij N, and te Riele H. Microsatellite instability in human cancer: a prognostic marker for chemotherapy? *Experimental Cell Research* 246: 1-10, 1999

Cravo M, Lage P, Albuquerque C, Chaves P, Claro I, Gomes T, Gaspar C, Fidalgo P, Soares J, and Nobre-Leitao C. BAT-26 identifies sporadic colorectal cancers with mutator phenotype: a correlative study with clinico-pathological features and mutations in mismatch repair genes. *Journal of Pathology* 188: 252-257, 1999

Cui H, Horon IL, Ohlsson R, Hamilton SR, and Feinberg AP. Loss of imprinting in normal tissue of colorectal cancer patients with microsatellite instability. *Nature Medicine* 4: 1276-1280, 1998

Cunningham JM, Christensen AR, Tester DJ, Kim C-Y, Roche PC, Burgart LJ, and Thibodeau SN. Hypermethylation of the *MLH1* promoter in colon cancer with microsatellite instability. *Cancer Research* 58: 3455-3460, 1998

de Boer J, and Hoeijmakers JHJ. Nucleotide excision repair and human syndromes. *Cancer Biology* 21: 453-460, 2000

de la Chapelle A, and Peltomäki P. Genetics of hereditary colon cancer. *Annual Review of Genetics* 29: 329-348, 1995

de la Chapelle A, and Wright FA. Linkage disequilibrium mapping in isolated populations: the example of Finland revisited. *Proceedings of the National Academy of Science USA* 95: 12416-12423, 1998

de la Chapelle A. Testing tumors for microsatellite instability. *European Journal of Human Genetics* 7: 407-408, 1999

de Lange T. Activation of telomerase in a human tumor. *Proceedings of the National Academy of Science USA* 91: 2882-2885, 1994

de Rosa M, Fasano C, Panariello L, Scarano MI, Belli G, Iannelli A, Ciciliano F, and Izzo P. Evidence for a recessive inheritance of Turcot's syndrome caused by compound heterozygous mutations within the *PMS2* gene. *Oncogene* 19: 1719-1723, 2000

Depniak T, Kurzawski G, Gorski B, Kladny J, Domagala W, and Lubinski J. Value of pedigree/clinical data, immunohistochemistry and microsatellite instability analyses in reducing the cost of determining *hMLH1* and *hMSH2* gene mutations in patients with colorectal cancer. *European Journal of Cancer* 36: 49-54, 2000

Duggan BD, Felix JC, Muderspach LI, Tourgeman D, Zheng J, and Shibata D. Microsatellite instability in sporadic endometrial carcinoma. *Journal of the National Cancer Institute* 86: 1216-1221, 1994

Dunlop MG. Screening for large bowel neoplasms in individuals with a family history of colorectal cancer. *British Journal of Surg*ery 79: 488-494, 1992

Duval A, Iacopetta B, Ranzani GN, Lothe RA, Thomas G, and Hamelin R. Variable mutation frequencies in coding repeats of *TCF-4* and other target genes in colon, gastric, and endometrial carcinoma showing microsatellite instability. *Oncogene* 18: 6806-6809, 1999

Edelmann W, Umar A, Yang K, Heyer J, Kucherlapati M, Lia M, Kneitz B, Avdievich E, Fan K, Wong E, Crouse G, Kunkel T, Lipkin M, Kolodner RD, and Kucherlapati R. The DNA mismatch repair genes *Msh3* and *Msh6* cooperate in intestinal tumor suppression. *Cancer Research* 60: 803-807, 2000

Ellis NA, Groden J, Ye TZ, Straughen J, Lennon DJ, Ciocci S, Proytcheva M, and German J. The Bloom's syndrome gene product is homologous to RecQ helicases. *Cell* 83: 655-666, 1995

Eng C. Cowden syndrome. *Journal of Genetic Counseling* 6: 181-191, 1997

Eng C. Commentary. Will the real Cowden syndrome please stand up: revised diagnostic criteria. *Journal of Medical Genetics* (in press).

Eppert K, Scherer SW, Ozcelic H, Pirone R, Hoodless P, Kim H, Tsui LC, Bapat B, Gallinger S, Andrulis IL, Thomsen GH, Wrana JL, and Attisano L. *MADR2* maps to 18q21 and encodes a TGFβ-regulated MAD-related protein that is fuctionally mutated in colorectal carcinoma. *Cell* 86: 543-552, 1996

Esteller M, Silva JM, Dominiguez G, Bonilla F, Matias-Guiu X, Lerma E, Bussaglia E, Prat J, Harkes IC, Repasky EA, Gabrielson E, Schutte M, Baylin SB, and Herman JG. Promoter hypermethylation and *BRCA1* inactivation in sporadic breast and ovarian tumors. *Journal of the National Cancer Institute* 92: 564-569, 2000

European Chromosome 16 Tuberous Sclerosis Consortium. Identification and characterization of the tuberous sclerosis gene on chromosome 16. *Cell* 75: 1305-1315, 1993

Fan Y-S, Davis LM, and Shows TB. Mapping of small DNA sequences by fluorescence *in situ* hybridization directly on banded metaphase chromosomes. *Proceedings of the National Academy of Science* 87: 6223-6227, 1990

Farrington SM, Lin-Goerke J, Ling J, Wang Y, Burczak JD, Robbins DJ, and Dunlop MG. Systematic analysis of *hMSH2* and *hMLH1* in young colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Research* 57: 808-811, 1996

Fearon E, Cho K, Nigro J, Kern S, Simons J, Ruppert J, Hamilton S, Preisinger A, Thomas G, Kinzler K, and Vogelstein B. Identification of chromosome 18q genes that is altered in colorectal cancers. *Science* 247: 49-56, 1990

Fearon ER, and Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 61: 759-767, 1990

Feinberg AP. Genomic imprinting and cancer. In: *The Genetic Basis of Human Cancer*. Eds. Vogelstein B, and Kinzler KW. McGraw-Hill, New York, 95-107, 1998

Ferguson LR, and Harris PJ. Studies on the role of specific dietary fibers in protection against colon cancer. *Mutation Research and Fundemamental Molecular Mechanisms of Mutagenesis* 350: 173-184, 1996

Finnish Cancer Registry. Cancer Incidence in Finland 1996 and 1997. Cancer Statistics of the National Research and Development Center for Welfare and Health, 2000

Fishel R, Lescoe MK, Rao MRS, Copeland NG, Jenkins NA, Garber J, Kane M, and Kolodner R. The human mutator gene homolog *MSH2* and its association with hereditary nonpolyposis colon cancer. *Cell* 75: 1027-1038, 1993

Folkman J. Tumor angiogenesis: a possible control point in tumor growth. *Annals of International Medicine* 82: 96-100, 1975

Gessler M, Poutska A, Cavenee W, Neve RL, Orkin SH, and Bruns GA. Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. *Nature* 343: 774-778, 1990

Giardiello FM, Welsh SB, Hamilton SR, Offerhaus GJ, Gittelsohn AM, Booker SV, Krush AJ, Yardley JH, and Luk GD. Increased risk of cancer in the Peutz-Jeghers syndrome. *New England Journal of Medicine* 316: 1511-1514, 1987

Giovannucci E, and Willett WC. Dietary factors and risk of colon cancer. *Annals of Medicine* 26: 443-452, 1994

Gonzalez-Zulueta M, Ruppert JM, Tokino K, Tsai YC, Spruck III CH, Miyao N, Nichols PW, Hermann GG, Horn T, Steven K, Summerhayes IC, Sidransky D, and Jones PA. Microsatellite instability in bladder cancer. *Cancer Research* 53: 5620-5623, 1993

Graham DY. Helicobacter pylori infection is the primary cause of gastric cancer. *Gastroenterology* 35 Suppl 12: 90-97, 2000

Greenblatt MS, Bennett WP, Hollstein M, and Harris CC. Mutations in the *p53* tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Research* 54: 4855-4878, 1994

Groden J, Thliveris A, Samowitz W, Carlson M, Gelbert L, Albertsen H, Joslyn G, Stevens J, Spirio L, Robertson M, Sargeant L, Krapcho K, Wolff E, Burt R, Hughes JP, Warrington J, McPherson J, Wasmuth J, LePaslier D, Abderrahim H, Cohen D, Leppert M, and White R. Identification and characterization of the familial *adenomatous polyposis coli* gene. *Cell* 66: 589-600, 1991

Guilford P, Hopkins J, Harraway J, McLeod M, McLeod N, Harawira P, Taite H, Scoular R, Miller A, and Reeve AE. *E-cadherin* germline mutations in familial gastric cancer. *Nature* 392: 402-405, 1998

Gupta RA, and DuBois RN. Aspirin, NSAIDs, and colon cancer prevention: mechanisms? *Gastroenterology* 114: 1095-1100, 1998

Hadziyannis SJ. Primary liver cancer and its relationship to chronic infection with the hepatitis B virus. *Springer Seminar of Immunopathology* 3: 473-485, 1981

Hahn H, Wickling C, Zaphiropoulos PG, Gailani MR, Shanley S, Chidambaram A, Vorechovsky I, Holmberg E, Unden AB, Gillies S, Negus K, Smyth I, Pressman C, Leffell DJ, Gerrard B, Goldstein AM, Dean M, Toftgard R, Chenevix-Trench G, Wainwright B, and Bale AE. Mutations of the human homolog of Drosophila *patched* in the nevoid basal cell syndrome. *Cell* 85: 841-851, 1996a

Hahn SA, Schutte M, Shamsul Hoque ATM, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CI, Fischer A, Yeo CJ, Hruban RH, and Kern SE. *DPC4*, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 271: 350-353, 1996b

Hamilton SR, Liu B, Parsons RE, Papadopoulos N, Jen J, Powell SM, Krush AJ, Berk T, Cohen Z, Tetu B, Burger PC, Wood PA, Taqi F, Booker SV, Peterson GM, Offerhaus GJA, Tersmette AC, Giardiello FM, Vogelstein B, and Kinzler KW. The molecular basis of Turcot's syndrome. *New England Journal of Medicine* 332: 839-847, 1995

Han HJ, Yanagisawa A, Kato Y, Park JG, and Nakamura Y. Genetic instability in pancreatic cancer and poorly differentiated type of gastric cancer. *Cancer Research* 53: 5087-5089, 1993

Hemminki A, Markie D, Tomlinson I, Avizienyte E, Roth S, Loukola A, Bignell G, Warren W, Aminoff M, Höglund P, Järvinen H, Kristo P, Pelin K, Ridanpää M, Salovaara R, Toro T, Bodmer W, Olschwang S, Olsen AS, Stratton MR, de la Chapelle A, and Aaltonen LA. A serine/threonine kinase gene defective in Peutz-Jeghers Syndrome. *Nature* 391: 184-187, 1998

Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, Markowitz S, Willson JK, Hamilton SR, Kinzler KW, Kane MF, Kolodner RD, Vogelstein B, Kunkel TA, and Baylin SB. Incidence and functional consequences of *hMLH1* promoter hypermethylation in colorectal carcinoma. *Proceedings of the National Academy of Science USA* 95: 6870-6875, 1998

Higuchi R. A simple and rapid preparation of samples for PCR. In: *Principles and Applications for DNA Amplification*. Ed. Erlich HA. New York, Stockton Press, 31-38, 1989

Hoang J-M, Cottu PH, Thuille B, Salmon RJ, Thomas G, and Hamelin R. BAT-26, an indicator of the replication error phenotype in colorectal cancers and cell lines. *Cancer Research* 57: 300-303, 1997

Houlston RS, Collins A, Slack J, and Norton NE. Dominant genes for colorectal cancer are not rare. *Annals of Human Genetics* 56: 99-103, 1992

Howe JR, Roth S, Ringold JC, Summers RW, Järvinen HJ, Sistonen P, Tomlinson IPM, Houlson RS, Bevan S, Mitros FA, Stone EM, and Aaltonen LA. Mutations in the *SMAD4/DPC4* gene in juvenile polyposis. *Science* 289: 1086-1088, 1998

Iacopetta B, and Hamelin R. Rapid and nonisotopic SSCP-based analysis of the BAT-26 mononucleotide repeat for identification of the replication error phenotype in human cancers. *Human Mutation* 12: 355-360, 1998

Ichikawa Y, Lemon SJ, Wang S, Franklin B, Watson P, Knezetic JA, Bewtra C, and Lynch HT. Microsatellite instability and expression of *MLH1* and *MSH2* in normal and malignant endometrial and ovarian epithelium in hereditary nonpolyposis colorectal cancer family members. *Cancer Genetics and Cytogenetics* 112: 2-8, 1999

Ionov Y, Peinado MA, Malkhosyan S, Shibata D, and Perucho M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 363: 558-561, 1993

Irby RB, Mao W, Coppola D, Kang J, Loubeau JM, Trudeau W, Karl R, Fujita DJ, Jove R, and Yeatman TJ. Activating *SRC* mutation in a subset of advanced human colon cancers. *Nature Genetics* 21: 187-190, 1999

Issa J-P, Ottaviano YL, Celano P, Hamilton SR, Davidson NE, and Baylin SB. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. *Nature Genetics* 7: 536-540, 1994

Jass JR. Do all colorectal carcinomas arise in preexisting adenomas? *World Journal of Surgery* 13: 45-51, 1989

Jass JR, and Stewart SM. Evolution of hereditary nonpolyposis colorectal cancer. *Gut* 33: 783-786, 1992

Jass JR, Stewart SM, Stewart J, and Lane MR. Hereditary nonpolyposis colorectal cancer - morphologies, genes and mutations. *Mutation Research* 310:125-133, 1994

Jass JR. Towards a molecular classification of colorectal cancer. *International Journal of Colorectal Diseases* 14: 194-200, 1999

Jass JR, Biden KG, Cummings M, Simms LA, Walsh M, Schoch E, Meltzer S, Wright C, Searle J, Young J, and Leggett BA. Characterization of a subtype of colorectal cancers combining features of the suppressor and mild mutator pathways. *Journal of Clinical Pathology* 52: 455-460, 1999

Jegers H, McCusick VA, and Katz KH. Generalized intestinal polyposis and melanin spots of the oral mucosa, lips and digits: a syndrome of diagnostic significance. *New England Journal of Medicine* 241: 1031-1036, 1949

Jen J, Powell SM, Papadopoulos N, Smith KJ, Hamilton SR, Vogelstein B, and Kinzler KW. Molecular determinants of dysplasia in colorectal lesions. *Cancer Research* 54: 5523-5526, 1994

Jiricny J. Mediating mismatch repair. *Nature Genetics* 24: 6-8, 2000

Jones PA. DNA methylation errors and cancer. *Cancer Research* 56: 2463-2467, 1996

Jones PA, and Laird PW. Cancer epigenetics comes of age. *Nature Genetics* 21: 163-167, 1999

Jäger AC, Bisgaard M-L, Myrhoj T, Bernstein I, Rehfeld JF, and Nielsen FC. Reduced frequency of extracolonic cancers in hereditary nonpolyposis colorectal cancer families with monoallelic *hMLH1* expression. *American Journal of Human Genetics* 61: 129-138, 1997

Järvinen HJ. Epidemiology of familial adenomatous polyposis in Finland: Impact of family screening on the colorectal cancer rate and survival. *Gut* 33: 357-360, 1992

Järvinen HJ. Juvenile gastrointestinal polyposis. *Problems in General Surgery* 10: 749-757, 1993

Järvinen HJ, Mecklin J-P, and Sistonen P. Screening reduces colorectal cancer rate in families with hereditary nonpolyposis colorectal cancer. *Gastroenterology* 108: 1405-1411, 1995

Järvinen HJ, Aarnio M, Mustonen H, Aktan-Collan K, Aaltonen LA, Peltomäki P, de la Chapelle A, and Mecklin J-P. Controlled 10-year trial on screening for colorectal cancer in families with hereditary nonpolyposis colorectal cancer. *Gastroenterology* 118: 829-834, 2000

Kane MF, Loda M, Gaida GM, Lipman J, Mishra R, Goldman H, Jessup JM, and Kolodner R. Methylation of the *hMLH1* promoter correlates with lack of expression of *hMLH1* in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Reseach* 57: 808-811, 1997

Kastan MB, Onyekwere O, Sidransky D, Vogelstein B, and Craig RW. Participation of p53 protein in the cellular response to DNA damage. *Cancer Research* 51: 6304-6311, 1991

Kee F, and Collins BJ. How prevalent is cancer family syndrome? *Gut* 32: 509-512, 1991

Keller G, Grimm V, Vogelsang H, Bischoff P, Mueller J, Siewert JR, and Hofler H. Analysis for microsatellite instability and mutations of the DNA mismatch repair gene *hMLH1* in familial gastric cancer. *International Journal of Cancer* 68: 571-576, 1996

Kim H, Jen J, Vogelstein B, and Hamilton SR. Clinical and pathological characteristics of sporadic colorectal carcinomas with DNA replication errors in microsatellite sequences. *American Journal of Pathology* 145: 148-156, 1994

Kinzler KW, and Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 87: 159-170, 1996

Kinzler KW, and Vogelstein B. Landscaping the cancer terrain. *Science* 280: 1036-1037, 1998

Knudson AG. Mutation and cancer: Statistical study of retinoblastoma. *Proceedings of the National Academy of Science USA* 68: 820-823, 1971

Kobayashi K, Matsushima M, Koi S, Saito H, Sagae S, Kudo R, and Nakamura Y. Mutational analysis of mismatch repair genes, *hMLH1* and *hMSH2*, in sporadic endometrial carcinomas with microsatellite instability. *Japanese Journal of Cancer Research* 87: 141-145, 1996

Kokal W, Sheibani K, Terz J, and Harada JR. Tumor DNA content in the prognosis of colorectal carcinoma. *Journal of American Medical Association* 255: 3123-3127, 1986

Kolodner RD. Mismatch repair: mechanisms and relationship to cancer susceptibility. *Trends in Biochemistry* 20: 397-402, 1995

Kolodner R. Biochemistry and genetics of eukaryotic mismatch repair. *Genes & Development* 10: 1433-1443, 1996

Konishi M, Kikuchi-Yanoshita R, Tanaka K, Muraoka M, Onda A, Okumura Y, Kishi N, Iwama T, Mori T, Koike M, Ushio K, Chiba M, Nomizu S, Konishi F, Utsunomiya J, and Miyaki M. Molecular nature of colon tumors in hereditary nonpolyposis colon cancer, familial polyposis, and sporadic colon cancer. *Gastroenterology* 111, 307-317, 1996

Kouri M, Laasonen A, Mecklin J-P, Järvinen H, Franssila K, and Pyrhönen S. Diploid predominance in hereditary nonpolyposis colorectal carcinoma evaluated by flow cytometry. *Cancer* 65: 1825-1829, 1990

Kuerbitz SJ, Plunkett BS, Walsh WV, and Kastan MB. Wild type *p53* is a cell cycle checkpoint determinant following irradiation. *Proceedings of the National Academy of Science* 93: 4816-4820, 1992

Lahiri DK, and Nürnberger JI. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. *Nucleic Acid Research* 19: 5444, 1991

Lane DP. A death in the life of *p53*. *Nature* 362: 786-787, 1993

Larsen F, Gundersen G, Lopez R, and Prydz H. CpG islands are markers in the human genome. *Genomics* 13: 1095-1107, 1992

Larson AA, Kern S, Sommers RL, Yokota J, Cavenee WK, and Hampton GM. Analysis of replication error (RER[+]) phenotypes in cervical carcinoma. *Cancer Research* 56: 1426-1431, 1996

Latif F, Tory K, Gnarra J, Yao M, Duh F-M, Orcutt ML, Stackhouse T, Kuzmin I, Modi W, Geil L, Schmidt L, Zhou F, Li H, Wei MH, Chen F, Glenn G, Choyke P, Walther MM, Weng Y, Duan D-SR, Dean M, Glavac D, Richards FM, Crossey PA, Ferguson-Smith MA, Le

Paslier D, Chumakov I, Cohen D, Chinault AC, Maher ER, Linehan WM, Zbar B, and Lerman MI. Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* 260: 1317-1320, 1993

Le Marchand L, Wilkens LR, Kolonel LN, Hankin JH, and Lyu L-C. Associations of sedentary lifestyle, obesity, smoking, alcohol use, and diabetes with the risk of colorectal cancer. *Cancer Research* 57: 4787-4794, 1997

Leach FS, Nicolaides NC, Papadopoulos N, Liu B, Jen J, Parsons R, Peltomäki P, Sistonen P, Aaltonen LA, Nyström-Lahti M, Guan X-Y, Zhang J, Meltzer PS, Yu J-W, Kao F-T, Chen DJ, Cerosaletti KM, Fournier REK, Todd S, Lewis T, Leach RJ, Naylor SL, Weissenbach J, Mecklin J-P, Järvinen H, Petersen GM, Hamilton SR, Green J, Jass J, Watson P, Lynch HT, Trent JM, de la Chapelle A, Kinzler KW, and Vogelstein B. Mutations of a *mutS* homolog in hereditary nonpolyposis colorectal cancer. *Cell* 75: 1215-25, 1993

Lemmens I, van der Ven WJM, Kas K, Zhang CX, Giraud S, Wautot V, Buisson N, De Witte K, Salandre J, Lenoir G, Pugeat M, Calender A, Parente F, Quincey D, Gaudray P, De Wit MJ, Lips CJM, Höppener JWM, Khodaei S, Grant AL, Weber G, Kytölä S, The BT, Farnebo F, Phelan C, Hayward N, Larsson C, Pannett AAJ, Forbes SA, Bassett JHD, and Thakker RV. Identification of the multiple endocrine neoplasia type 1 (*MEN1*) gene. *Human Molecular Genetics* 6: 1177-1183, 1997

Leung SY, Yuen ST, Chung LP, Chu KM, Chan AS, and Ho JC. *hMLH1* promoter methylation and lack of *hMLH1* expression in sporadic gastric carcinomas with high-frequency microsatellite instability. *Cancer Research* 59: 159-164, 1999

Levinson G, and Gutman GA. High frequencies of short frameshifts in poly-CA/TG tandem repeats borne by bacteriophage M13 in *Escherichia coli* K-12. *Nucleic Acid Research* 15: 5323-5338, 1987

Li GM, and Modrich P. Restoration of mismatch repair to nuclear extracts of H6 colorectal tumor cells by a heterodimer of human *MutL* homologs. *Proceedings of the National Academy of Science* 92: 1950-1954, 1995

Liaw D, Marsh DJ, Li J, Dahia PL, Wang SI, Zheng Z, Bose S, Call KM, Tsou HC, Peacocke M, Eng C, and Parsons R. Germline mutations of the *PTEN* gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nature Genetics* 16: 64-67, 1997

Lim PC, Tester D, Cliby W, Ziesmer SC, Roche PC, Hartmann L, Thibodeau SN, Podratz KC, and Jenkins RB. Absence of mutations in DNA mismatch repair genes in sporadic endometrial tumors with microsatellite instability. *Clinical Cancer Research* 2: 1907-1911, 1996

Lin KM, Shashidharan M, Ternent CA, Thorson AG, Blatchford GJ, Christensen MA, Lanspa SJ, Lemon SJ, Watson P, and Lynch HT. Colorectal end extracolonic cancer variations in *MLH1*/*MSH2* hereditary nonpolyposis colorectal cancer kindreds and the general population. *Diseases of the Colon & Rectum* 41: 428-433, 1998

Lindahl T. Instability and decay of the primary structure of DNA. *Nature* 362: 709-715, 1993

Lindblom A, Tannergård P, Werelius B, and Nordenskjöld M. Genetic mapping of a second locus predisposing to hereditary non-polyposis colorectal cancer. *Nature Genetics* 5: 279-288, 1993

Lipkin SM, Wang V, Jacoby R, Banerjee-Basu S, Baxevanis AD, Lynch HT, Elliot RM, and Collins FS. *MLH3*: a DNA mismatch repair gene associated with mammalian microsatellite instability. *Nature Genetics* 24: 27-35, 2000

Liu B, Nicolaides NC, Markowitz S, Wilson JKW, Parsons R, Jen J, Papadopoulos N, Peltomäki P, de la Chapelle A, Hamilton S, Kinzler KW, and Vogelstein B. Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability. *Nature Genetics* 9: 48-55, 1995a

Liu L, Lassam NJ, Slingerland JM, Bailey D, Cole D, Jenkins R, and Hogg D. Germline *p16*(*INK4A*) mutation and protein dysfunction in a family with inherited melanoma. *Oncogene* 11: 405-412, 1995b

Liu B, Parsons R, Papadopoulos N, Nicolaides NC, Lynch HT, Watson P, Jass JR, Dunlop M, Wyllie A, Peltomäki P, de la Chapelle A, Hamilton SR, Vogelstein B, and Kinzler KW. Analysis of mismatch repair genes in hereditary nonpolyposis colorectal cancer patients. *Nature Medicine* 2: 169-174, 1996

Lloyd KM, and Dennis M. Cowden's disease: a possible new symptom complex with multiple system involvement. *Annals of International Medicine* 58: 136-142, 1963

Lu S-L, Akiyama Y, Nagasaki H, Saitoh K, and Yuasa Y. Mutations of the *transforming growth factor-b type II receptor* gene and genomic instability in hereditary nonpolyposis colorectal cancer. *Biochemical and Biophysical Research Communications* 216: 452-457, 1995

Lu S-L, Kawabata M, Imamura T, Akiyama Y, Nomizu T, Miyazono K, and Yuasa Y. HNPCC associated with germline mutation in the *TGF-b type II receptor* gene. *Nature Genetics* 19: 17-18, 1998

Lynch HT, Watson P, Kriegler M, Lynch JF, Lanspa SJ, Marcus J, Smyrk T, Fitzgibbons RJ Jr, and Cristofaro G. Differential diagnosis of hereditary nonpolyposis colorectal cancer (Lynch syndrome I and Lynch syndrome II). *Diseases of the Colon & Rectum* 31: 372-377, 1988

Lynch HT, Smyrk T, and Jass JR. Hereditary nonpolyposis colorectal cancer and colonic adenomas: aggressive adenomas? *Seminars in Surgical Oncology* 11: 406-410, 1995

Lynch HT, and Smyrk TC. Identifying hereditary nonpolyposis colorectal cancer. *New England Journal of Medicine* 338: 1537-1538, 1998

Lynch HT, and de la Chapelle A. Genetic susceptibility to non-polyposis colorectal cancer. *Journal of Medical Genetics* 36: 801-818, 1999

Malkhosyan S, Rampino N, Yamoto H, and Perucho M. Frameshift mutator mutations. *Nature* 382: 499-500, 1996

Malkin D, Li FP, Strong LC, Fraumeni JF Jr, Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA, and Friend SH. Germ line *p53* mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science* 250: 1233-1238, 1990

Marcus VA, Madlensky L, Gryfe R, Kim H, So K, Millar A, Temple LK, Hsieh E, Hiruki T, Narod S, Bapat BV, Gallinger S, and Redston M. Immunohistochemistry for *hMLH1* and *hMSH2*: a practical test for DNA mismatch repair-deficient tumors. *American Journal of Surgical Pathology* 23: 1248-1255, 1999

Markowitz S, Wang J, Myeroff L, Parsons R, Sun LZ, Lutterbugh J, Fan RS, Zborowska E, Vogelstein B, Brattain M, and Willson JKV. Inactivation of the *type II TGF-b receptor* in colon cancer cells with microsatellite instability. *Science* 268: 1336-1338, 1995

Marshall CJ. Tumor suppressor genes. *Cell* 64: 313-326, 1991

Marsischky GT, Filosi N, Kane MF, and Kolodner R. Redundancy of Saccharomyces cerevisiae MSH3 and MSH6 in MSH2-dependent mismatch repair. *Genes & Development* 10: 407-420, 1996

Marwick C. Helicobacter: new name, new hypothesis involving type of gastric cancer. *The Journal of the American Medical Association* 264: 2724-2727, 1990

Marx JL. Human papilloma virus and cervical cancer. *Science* 231: 920, 1986

Massague J. TGF-beta signal transduction. *Annual Revision of Biochemistry* 67: 753-791, 1998

Mecklin J-P, and Järvinen HJ. Clinical features of colorectal carcinoma in cancer family syndrome. *Diseases of the Colon & Rectum* 29: 160-164, 1986

Mecklin J-P. Frequency of hereditary colorectal carcinoma. *Gastroenterology* 93: 1021-1025, 1987

Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W, Bell R, Rosenthal J, Hussey C, Tran T, McClure M, Frye C, Hattier T, Phelps R, Haugen-Strano A, Katcher H, Yakumu K, Gholami Z, Shaffer D, Stone S, Bayer S, Wray C, Bogden R, Dayananth P, Ward J, Tonin P, Narod S, Bristow PK, Norris FH, Helvering L, Morrison P, Rosteck P, Lai M, Barret JC, Lewis C, Neuhausen S, Cannon-Albright L, Goldgar D, Wiseman R, Kamb A, and Skolnick MH. A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. *Science* 266: 417-419, 1994

Miyaki M, Konishi M, Tanaka K, Kikuchi-Yanoshita R, Muraoka M, Yasuno M, Igari T, Koike M, Chiba M, and Mori T. Germline mutation of *MSH6* as the cause of hereditary nonpolyposis colorectal cancer. *Nature Genetics* 17: 271-272, 1997

Miyaki M. Imprinting and colorectal cancer. *Nature Medicine* 4: 1236-1237, 1998

Modica S, Roncucci L, Benatti P, Gafa L, Tamassia MG, Dardanoni L, and Ponz de Leon M. Familial aggregation of tumors and detection of hereditary non-polyposis colorectal cancer in 3-year experience of 2 population-based colorectal cancer registries. *International Journal of Cancer* 62: 685-690, 1995

Modrich P. Strand-specific mismatch repair in mammalian cells. *Journal of Biological Chemistry* 272: 24727-24730, 1997

Moore L, Godfrey T, Eng C, Smith A, Ho R, and Waldman FM. Validation of fluorescent SSCP analysis for sensitive detection of *p53* mutations. *Biotechniques* 28: 986-992, 2000

Mulligan LM, Kwok JB, Healey CS, Elsdon MJ, Eng C, Gardner E, Love DR, Mole SE, Moore JK, Papi L, Ponder MA, Telenius H, Tunnacliffe A, and Ponder BAJ. Germ-line mutations of the *RET* proto-oncogene in multiple endocrine neoplasia type 2A. *Nature* 363: 458-460, 1993

Mulvihill JJ. The frequency of hereditary large bowel cancer. In: *Prevention of Hereditary Large Bowel Cancer*. Eds. Ingall JR, and Mastromarino AJ. New York, Alan R Liss, 61-75, 1983

Muto T, Bussey HJR, and Morson BC. The evolution of the cancer of the rectum. *Cancer* 36: 2251-2276, 1975

Myöhänen SK, Baylin SB, and Herman JG. Hypermethylation can selectively silence individual *p16*ink4a alleles in neoplasia. *Cancer Research* 58: 591-593, 1998

Nicolaides NC, Papadopoulos N, Liu B, Wei YF, Carter KC, Ruben SM, Rosen CA, Haseltine WA, Fleischmann RD, Fraser CM, Adams MD, Venter JC, Dunlop MG, Hamilton SR, Petersen GM, de la Chapelle A, Vogelstein B, and Kinzler KW. Mutations of two *PMS* homologues in hereditary nonpolyposis colon cancer. *Nature* 371: 75-80, 1994

Nishisho I, Nakamura Y, Miyoshi Y, Miki Y, Ando H, Koyama K, Utsunomiya J, Baba S, Hedge P, Markham A, Krush AJ, Peterson G, Hamilton SR, Nilbert MC, Levy DB, Bryan TM, Preisinger AC, Smith KJ, Su LK, Kinzler KW, and Vogelstein B. Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. *Science* 253: 665-669, 1991

Nixon DW. Diet and chemoprevention of polyps and colorectal cancer. *Seminars in Surgical Oncology* 11: 411-414, 1995

Nyström-Lahti M, Kristo P, Nicolaides NC, Sheng-Yung C, Aaltonen LA, Moisio A-L, Järvinen HJ, Mecklin J-P, Kinzler KW, Vogelstein B, de la Chapelle A, and Peltomäki P. Founding mutations and Alu-mediated recombination in hereditary colon cancer. *Nature Medicine* 1: 1203-1206, 1995

Nyström-Lahti M, Wu Y, Moisio A-L, Hofstra RMW, Osinga J, Mecklin J-P, Järvinen H, Leisti J, Buys CHCM, de la Chapelle A, and Peltomäki P. DNA mismatch repair gene mutations in 55 kindreds with verified or putative hereditary nonpolyposis colorectal cancer. *Human Molecular Genetics* 5: 763-769, 1996

Ogunbiyi OA, Goodfellow PJ, Herfarth K, Gagliardi G, Swanson PE, Birnbaum EH, Read TE, Fleshman JW, Kodner IJ, and Moley JF. Confirmation that chromosome 18q allelic loss in colon cancer is a prognostic indicator. *Journal of Clinical Oncology* 16: 427-433, 1998

Orth K, Hung J, Gazdar A, Bowcock A, Methis M, and Sambrook J. Genetic instability in human ovarian cancer cell lines. *Proceedings of the National Academy of Science USA* 91: 9495-9499, 1994

Ouyang H, Shiwaku HO, Hagiwara H, Miura K, Abe T, Kato Y, Ohtani H, Shiiba K, Souza RF, Meltzer SJ, and Horii A. The *insulin-like growth factor II receptor* gene is mutated in genetically unstable cancers of the endometrium, stomach, and colorectum. *Cancer Research* 57: 1851-1854, 1997

Papadopoulos N, Nicolaides NC, Wei YF, Ruben SM, Carter KC, Rosen CA, Haseltine WA, Fleischmann RD, Fraser CM, Adams MD, Venter JC, Hamilton SR, Petersen GM, Watson P, Lynch HT, Peltomäki P, Mecklin J-P, de la Chapelle A, Kinzler KW, and Vogelstein B. Mutation of a *MutL* homolog in hereditary colon cancer. *Science* 263:1625-1629, 1994

Park M. Oncogenes. In: *The Genetic Basis of Human Cancer*. Eds. Vogelstein B, and Kinzler KW. McGraw-Hill Companies Inc., USA, 205-228, 1998

Parsons R, Myeroff LL, Liu B, Willson JK, Markowitz SD, Kinzler KW, and Vogelstein B. Microsatellite instability and mutations of the *transforming growth factor beta type II receptor* gene in colorectal cancer. *Cancer Research* 55: 5548-5550, 1995

Patel U, Grundfest BS, Gupta M, and Banerjee S. Microsatellite instabilities at five chromosomes in primary breast tumors. *Oncogene* 9: 29-32, 1994

Pedroni M, Tamassia MG, Percesepe A, Roncucci L, Benatti P, Lanza G Jr, Gafa R, Di Gregorio C, Fante R, Losi L, Gallinari L, Scorcioni F, Vaccina F, Rossi G, Cesinaro AM, and Ponz de Leon M. Microsatellite instability in multiple colorectal tumors. *International Journal of Cancer* 81: 1-5, 1999

Peltomäki P, Aaltonen LA, Sistonen P, Pylkkänen L, Mecklin J-P, Järvinen H, Green J, Jass JR, Weber JL, Leach FS, Petersn GM, Hamilton SR, de la Chapelle A, and Vogelstein B. Genetic mapping of a locus predisposing to human colorectal cancer. *Science* 260: 810-816, 1993

Peltomäki P, and Vasen HF. Mutations predisposing to hereditary nonpolyposis colorectal cancer: database and results of a collaborative study. The International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer. *Gastroenterology* 113: 1146-1158, 1997

Perucho M. Microsatellite instability: the mutator that mutates the other mutator. *Nature Medicine* 2: 630-631, 1996

Peutz JLA. On a very remarkable case of familial polyposis of the mucous membrane of the intestinal tract and nasopharynx accompanied by peculiar pigmentations of the skin and mucous membrane. *Nederlandisch Tijdshrift voor Geneeskunde Jaar* 10: 136-146, 1921

Ponz de Leon M, Sassatelli R, Benatti P, and Roncucci L. Identification of hereditary nonpolyposis colorectal cancer in the general population: the 6-year experience of a population-based registry. *Cancer* 71: 3493-3501, 1993

Ponz de Leon M. Prevalence of hereditary nonpolyposis colorectal carcinoma (HNPCC). *Annals of Medicine* 26: 209-214, 1994

Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, Vogelstein B, and Kinzler KW. *APC* mutations occur early during colorectal tumorigenesis. *Nature* 359: 235-237, 1992

Pretlow TP, Brasitus TA, Fulton NC, Cheyer C, and Kaplan EL. *K-ras* mutations in putative preneoplastic lesions in human colon. *Journal of the National Cancer Institute* 85: 2004-2007, 1993

Prolla TA, Pang Q, Alani E, Kolodner RD, and Liskay RM. MLH1, PMS1, and MSH2 interactions during the initiation of DNA mismatch repair in yeast. *Science* 265: 1091-1093, 1994

Prolla TA, Baker SM, Harris AC, Tsao JL, Yao X, Bronner CE, Zheng B, Gordon M, Reneker J, Arnheim N, Shibata D, Bradley A, and Liskay RM. Tumor susceptibility and spontaneous mutation in mice deficient in *Mlh1*, *Pms1* and *Pms2* DNA mismatch repair. *Nature Genetics* 18: 276-279, 1998

Pyatt R, Chadwick RB, Johnson CK, Adebamowo C, de la Chapelle A, and Prior TW. Polymorphic variation at the BAT-25 and BAT-26 loci in individuals of African origin. *The American Journal of Pathology* 155: 349-353, 1999

Rampino N, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC, and Perucho M. Somatic frameshift mutations in the *BAX* gene in colon cancers of the microsatellite mutator phenotype. *Science* 275: 967-969, 1997

Rideout WM, Coetzee GA, Olumi AF, and Jones PA. 5-methylcytosine as an endogenous mutagen in the human LDL receptor and p53 genes. *Science* 249: 1288-1290, 1990

Risinger JI, Berchuck A, Kohler MF, Watson P, Lynch HT, and Boyd J. Genetic instability of microsatellites in endometrial carcinoma. *Cancer Research* 53: 5100-5103, 1993

Rodriguez-Bigas MA, Boland CR, Hamilton SR, Henson DE, Jass JR, Khan PM, Lynch H, Perucho M, Smyrk T, Sobin L, and Srivastava S. A National Cancer Institute Workshop on Hereditary Nonpolyposis Colorectal Cancer Syndrome: Meeting Highlights and Bethesda Guidelines. *Journal of the National Cancer Institute* 89: 1758-1762, 1997

Rouleau GA, Merel P, Lutchman M, Sanson M, Zucman J, Marineau C, Hoang-Xuan K, Demczuk S, Desmaze C, Plougastel B, Pulst SM, Lenoir G, Bijlsma E, Fashold R, Dumanski J, de Jong P, Parry D, Eldrige R, Aurias A, Delattre O, and Thomas G. Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosis type 2. *Nature* 363: 515-521, 1993

Rudin CM, and Thompson CB. Apoptosis and cancer. In: *The Genetic Basis of Human Cancer*. Eds. Vogelstein B, and Kinzler KW. McGraw-Hill Companies Inc., USA, 193-204, 1998

Rüschoff J, Wallinger S, Dietmaier W, Bocker T, Brockhoff G, Hofstädter F, and Fishel R. Aspirin suppresses the mutator phenotype associated with hereditary nonpolyposis colorectal cancer by genetic selection. *Proceedings of the National Academy of Science USA* 95: 11301-11306, 1998

Samowitz WS, Slattery ML, Potter JD, and Leppert MF. BAT-26 and BAT-40 instability in colorectal adenomas and carcinomas and germline polymorphisms. *American Journal of Pathology* 154: 1637-1641, 1999

Sankila R, Aaltonen LA, Järvinen HJ, and Mecklin J-P. Better survival rates in patients with *MLH1*-assoviated hereditary nonpolyposis colorectal cancer. *Gastroenterology* 110: 682-687, 1996

Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T, Sfez S, Ashkenazi M, Pecker I, Frydman M, Harnik R, Patanjali SR, Simmons A, Clines GA, Sartiel A, Gatti RA, Chessa L, Sanal O, Lavin MF, Jaspers NGJ, Taylor MR, Arlett CF, Miki T, Weissman SM, Lovett M, Collins FS, and Shiloh Y. A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science* 268: 1749-1753, 1995

Schmidt L, Duh F-M, Chen F, Kishida T, Glenn G, Choyke P, Scherer SW, Zhuang Z, Lubensky I, Dean M, Allikmets R, Chimdambaram A, Bergerheim UR, Feltis JT, Casadevall C, Zamarron A, Bernues M, Richard S, Lips CJ, Walther MM, Tsui LC, Geil L, Orcutt ML, Stackhouse T, Lipan J, Slife L, Brauch H, Decker J, Niehans G, Hughson MD, Moch H, Storkel S, Lerman MI, Linehan WM, and Zbar B. Germline and somatic mutations in the tyrosine kinase domain of the *MET* proto-oncogene in papillary renal carcinomas. *Nature Genetics* 16: 68-73, 1997

Shepherd NA, and Bussey HJR. Polyposis syndromes – an update. *Current Topics of Pathology* 81: 323-351, 1990

Shibata D, Schaeffer J, Li ZH, Capella G, and Perucho M. Genetic heterogeneity of the *c-K-ras* locus in colorectal adenomas but not in adenocarcinomas. *Journal of the National Cancer Institute* 85: 1058-1063, 1993

Shibata M, Peinado MA, Ionov Y, Malkhosyan S, and Perucho M. Genomic instability in repeated sequences is an early somatic event in colorectal tumorigenesis that persists after transformation. *Nature Genetics* 6: 273-281, 1994

Shibata D, Navidi W, Salovaara R, Li Z-H, and Aaltonen LA. Somatic microsatellite mutations as molecular tumor clocks. *Nature Medicine* 2: 676-681, 1996

Shimodaira H, Filosi N, Shibata H, Suzuki T, Radice P, Kanamaru R, Friend SH, Kolodner RD, and Ishioka C. Functional analysis of human *MLH1* mutations in *Saccharomyces cerevisiae*. *Nature Genetics* 19: 384-389, 1998

Simpkins SB, Bocker T, Swisher EM, Mutch DG, Gersell DJ, Kovatich AJ, Palazzo JP, Fishel R, and Goodfellow PJ. *MLH1* promoter methylation and gene silencing is the primary cause of microsatellite onstability in sporadic endometrial cancers. *Human Molecular Genetics* 8: 661-666, 1999

Smith AJ, Stern HS, Penner M, Hay K, Miri A, Bapat BV, and Gallinger S. Somatic *APC* and *k-ras* codon 12 mutations in aberrant crypt foci from human colons. *Cancer Research* 54: 5527-5530, 1994

Souza RF, Appel R, Yin J, Wang S, Smolinski KN, Abraham JM, Zou TT, Shi YQ, Lei J, Cottrell J, Cymes K, Biden K, Simms L, Leggett B, Lynch PM, Frazier M, Powell SM, Harpaz N, Sugimura H, Young J, and Melzer SJ. Microsatellite instability in the *insulin-like growth factor II receptor* gene in gastrointestinal tumours. *Nature Genetics* 14: 255-257, 1996

Spigelman AD, Murday V, and Phillips RKS. Cancer and the Peutz-Jeghers syndrome. *Gut* 30: 1588-1590, 1989

Spigelman AD, and Phillips RKS. In: *Peutz-Jeghers syndrome, Familial Adenomatous Polyposis and other Polyposis Syndromes.* Eds. Phillips RKS, Spigelman AD, and Thompson JPS. Edward Arnold, London, 189-202, 1994

Starink TM, van der Veen JPW, Arwert F, de Waal LP, de Lange GG, Gille JJP, and Eriksson AW. The Cowden syndrome: a clinical study in 21 patients. *Clinical Genetics* 29: 222-233, 1986

Steiner G, Schoenberg MP, Linn JF, Mao L, and Sidransky D. Detection of bladder cancer recurrence by microsatellite analysis of urine. *Nature Medicine* 3: 621-624, 1997

Stephenson BM, Finan JP, Gascoyne J, Garbett F, Murday VA, and Bishop DT. Frequency of familial colorectal cancer. *British Journal of Surgery* 78: 1162-1166, 1991

Sternfeld B. Cancer and the protective effect of physical activity: the epidemiological evidence. *Medical Science in Sports and Exercise* 1195-1209, 1992

Stickens D, Clines G, Burbee D, Ramos P, Thomas S, Hogue D, Hecht JT, Lovett M, and Evans GA. The *EXT2* multiple exostoses gene defines a family of putative tumour suppressor genes. *Nature Genetics* 14: 25-32, 1996

Strachan T, and Read AP. Organization of the human genome. In: *Human Molecular Genetics.* BIOS Scientific Publishers Ltd., Oxford, UK, 139-168, 1999

Strand M, Prolla TA, Liskay RM, and Petes TD. Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair genes. *Nature* 365: 274-276, 1993

Strickler JG, Zheng J, Shu Q, Burgart LJ, Alberts SR, and Shibata D. *p53* mutations and microsatellite instability in sporadic gastric cancer: when guardians fail. *Cancer Research* 54: 4750-4755, 1994

Sutter C, Gebert J, Bischoff P, Herfarth C, and von Knebel Doeberitz M. Molecular screening of potential HNPCC patients using a multiplex microsatellite PCR system. *Molecular and Cellular Probes* 13: 157-65, 1999

Suzuki H, Komiya A, Aida S, Akimoto S, Shiraishi T, Yatani R, Igarashi T, and Shimazaki J. Microsatellite instability and other molecular abnormalities in human prostate cancer. *Japanese Journal of Cancer research* 86: 956-961, 1995

Syvänen A-C, Ikonen E, Manninen T, Bengström M, Söderlund H, Aula P, and Peltonen L. Convenient and quantitative determination of the frequency of a mutant allele using solid-phase minisequencing: application to aspartylglucosaminuria in Finland. *Genomics* 12: 590-595, 1992

Taunton J. How to starve a tumor. *Chemical Biology* 4: 493-496, 1997

Thiagalingam S, Lengauer C, Leach FC, Schutte M, Hahn SA, Overhauser J, Willson JKV, Markowitz S, Hamilton SR, Kern SE, Kinzler WK, and Vogelstein B. Evaluation of candidate tumor suppressor genes on chromosome 18 in colorectal cancers. *Nature Genetics* 13: 343-346, 1996

Thibodeau SN, French AJ, Cunningham JM, Tesyer D, Burgart LJ, Roche PC, McDonnell SK, Schaid DJ, Vockley CW, Michels VV, Farr GH, and O'Connell MJ. Microsatellite instability in colorectal cancer: Different mutator phenotypes and the principle involvement of *hMLH1*. *Cancer Research* 58: 1713-1718, 1998

Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, and Issa J-PJ. CpG island methylator phenotype in colorectal cancer. *Proceedings of the National Academy of Science USA* 96: 8681-8686, 1999

Tsao J-L, Tavare S, Salovaara R, Jass JR, Aaltonen LA, and Shibata D. Colorectal adenoma and cancer divergence: evidence of multilineage progression. *American Journal of Pathology* 154: 1825-1830, 1999

Tsao J-L, Yatabe Y, Salovaara R, Järvinen HJ, Mecklin J-P, Aaltonen LA, Tavaré S, and Shibata D. Genetic reconstruction of individual colorectal tumor histories. *Proceedings of the National Academy of Science* 97: 1236-1241, 2000

Uchida T, Wada C, Wang C, Egawa S, Ohtani H, and Koshiba K. Genomic instability of microsatellite repeats and mutations of *H-*, *K-*, and *N-ras* and *p53* genes in renal cell carcinoma. *Cancer Research* 54: 3682-3685, 1994

Umar A, Risinger JI, Glaab WE, Tindall KR, Barrett JC, and Kunkel TA. Functional overlap in mismatch repair by human *MSH3* and *MSH6*. *Genetics* 148: 1637-1646, 1998

Wallace MR, Marchuk DA, Andersen LB, Letcher R, Odeh HM, Saulino AM, Fountain JW, Brereton A, Nicholson J, Mitchell AL, Brownstein BH, and Collins FS. Type 1 Neurofibromatosis gene: identification of a large transcript disrupted in three NF1 patients. *Science* 249: 181-186, 1990

Van Slegtenhorst M, de Hoogt R, Hermans C, Nellist M, Janssen B, Verhoef S, Lindhout D, van den Ouweland A, Halley D, Young J, Burley M, Jeremiah S, Woodward K, Nahmias J, Fox M, Ekong R, Osborne J, Wolfe J, Povey S, Snell RG, Cheadle JP, Jones AC, Tachataki M, Ravine D, Sampson JR, Reeve MP, Richardson P, Wilmer F, Munro C, Hawkins TL, Sepp T, Ali JBM, Ward S, Green AJ, Tates JRW, Kwiatkowska J, Henske EP, Short MP, Haines JH, Jozwiak S, and Kwiatkowski DJ. Identification of the Tuberous Sclerosis Gene *TSC1* in chromosome 9q34. *Science* 277: 805-808, 1997

Wargovich MJ, and Levin B. Grist for the mill: role of cereal fiber and calcium in prevention of colon cancer. *Journal of the National Cancer Institute* 88: 67-69, 1996

Warthin AS. Heredity with reference to carcinoma. *Archives of Internal Medicine*. 12: 546-555, 1913

Vasen HFA, Johan G, Offerhaus A, Hartog Jager FC, Menko FH, Nagengast FM, Griffioen G, Hogezand RB, and Heinz AP. The tumor spectrum in hereditary non-polyposis colorectal

cancer: a study of 24 kindreds in the Netherlands. *International Journal of Cancer* 46: 31-34, 1990

Vasen HFA, Mecklin J-P, Meera Khan P, and Lynch HT. The International Collaborative Group on hereditary non-polyposis colorectal cancer (ICG-HNPCC). *Diseases of the Colon & Rectum* 34: 424-425, 1991

Vasen HFA, Taal BG, Nagengast FM, Griffioen G, Menko FH, Kleibeuker JH, Offenhaus GJA, and Meera Khan P. Hereditary nonpolyposis colorectal cancer: results of long-term surveillance in 50 families. *European Journal of Cancer* 31A: 1145-1148, 1995

Vasen HFA, Wijnen J, Menko FH, Kleibeuker JH, Taal BG, Griffioen G, Nagengast FM, Meijers-Heijboer EH, Bertario L, Varesco L, Bisgaard M-L, Mohr J, Fodde R, and Meera Khan P. Cancer risk in families with hereditary nonpolyposis colorectal cancer diagnosed by mutation analysis. *Gastroenterology* 110: 1020-1027, 1996

Vasen HFA, van Ballegooijen M, Buskens E, Kleibeuker JK, Taal BG, Griffioen G, Nagengast FM, Menko FH, and Meera Khan P. A cost-effectiveness analysis of colorectal screening of hereditary nonpolyposis colorectal carcinoma gene carriers. *Cancer* 82: 1632-1637, 1998

Vasen HFA, Watson P, Mecklin J-P, Lynch HT, and the ICG-HNPCC. New Clinical Criteria for Hereditary Nonpolyposis Colorectal Cancer (HNPCC, Lynch Syndrome) Proposed by the International Collaborative Group on HNPCC. *Gastroenterology* 116: 1453-1456, 1999

Watson P, and Lynch HT. Extracolonic cancer in hereditary nonpolyposis colorectal cancer. *Cancer* 71: 677-685, 1993

Weinberg RA. Tumor suppressor genes. *Science* 254: 1138-1145, 1991

Verma L, Kane MF, Brassett C, Schmeits J, Gareth R Evans D, Kolodner RD, and Maher ER Mononucleotide microsatellite instability and germline *MSH6* mutation analysis in early onset colorectal cancer. *Journal of Medical Genetics* 36: 678-682, 1999

Wheeler JMD, Beck NE, Kim HC, Tomlinson IPM, McC Mortensen NJ, and Bodmer WF. Mechanisms of inactivation of mismatch repair genes in human colorectal cancer cell lines: the predominant role of *hMLH1*. *Proceedings of the National Academy of Science* 96: 10296-10301, 1999

Wijnen J, Meera Khan P, Vase H, van der Klift H, Mulder A, van Leeuwen-Cornelisse I, Bakker B, Losekoot M, Moller P, and Fodde R. Hereditary nonpolyposis colorectal cancer families not complying with the Amsterdam criteria show extremely low frequency of mismatch-repair-gene mutations. *American Journal of Human Genetics* 61: 329-335, 1997

Wijnen J, van der Klift H, Vasen H, Meera Khan P, Menko F, Tops C, Meijers Heijboer H, Lindhout D, Moller, and Fodde R. *MSH2* genomic deletions are a frequent cause of HNPCC. *Nature Genetics* 20: 326-328, 1998a

Wijnen JT, Vasen HFA, Khan PM, Zwinderman AH, van der Klift H, Mulder S, Tops C, Moller P, and Fodde R. Clinical findings with implications for genetic testing in families with clustering of colorectal cancer. *New England Journal of Medicine* 339: 511-518, 1998b

Winawer SJ, O'Brien MJ, Waye JD, Kronborg O, Bond J, Frühmorgen P, Sobin LH, and the WHO Collaborating Center for the Prevention of Colorectal Cancer. Risk and surveillance of individuals with colorectal polyps. *WHO Bulletin*. 68: 789-795, 1990

Winawer SJ, Zauber AG, Ho MN, O'Brien MJ, Gottlieb LS, Sternberg SS, Waye JD, Schapiro M, Bond, JH, Panish JF, Ackroyd F, Shike M, Kurtz RC, Hornsby-Lewis L, Gerdes H, and Stewart ET. Prevention of colorectal cancer by colonoscopic polypectomy. The National Polyp Study Workgroup. *New England Journal of Medicine* 329: 1977-81, 1993

Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AMM, and Bos JL. Genetic alterations during colorectal tumor development. *New England Journal of Medicine* 319: 525-532, 1988

Vogelstein B, Fearon ER, Kern SE, Hamilton SR, Preisinger AC, Nakamura Y, and White R. Allelotype of colorectal carcinomas. *Science* 244: 207-211, 1989

Vogelstein B, and Kinzler KW. The multistep nature of cancer. *Trends in Genetics* 9: 138-141, 1993

Wooster R, Cleton-Jansen A-M, Collins N, Mangion J, Cornelis RS, Cooper CS, Gusterson BA, Ponder BAJ, von Deimling A, Wiestler OD, Cornelisse CJ, Devilee P, and Stratton MR. Instability of short tandem repeats (microsatellites) in human cancers. *Nature Genetics* 6, 152-156, 1994

Wooster R, Bignell G, Lancaster J, Swift S, Seal S, Mangion J, Collins N, Gregory S, Gumbs C, Micklem G, Barfoot R, Hamoudl R, Patel S, Rice C, Biggs P, Hashim Y, Smith A, Connor F, Arason A, Gudmundsson J, Ficenec D, Kelsell D, Ford D, Tonin P, Bishop DT, Spurr NK, Ponder BAJ, Eeles R, Peto J, Devilee P, Cornelisse C, Lynch H, Narod S, Leinor G, Egilsson V, Barkardottir RB, Easton DF, Bentley DR, Futreal PA, Ashworth A, and Stratton MR. Identification of the breast cancer susceptibility gene *BRCA2*. *Nature* 378: 789-792, 1995

Voskuil DW, Vasen HFA, Kampman E, van't Veer P, the National Collaborative Group on HNPCC. Colorectal cancer risk in HNPCC families: development during lifetime and in successive generations. *International Journal of Cancer* 72: 205-209, 1997

Wu MS, Sheu JC, Shun CT, Lee WJ, Wang JT, Wang TH, Cheng AL, and Lin JT. Infrequent *hMSH2* mutations in sporadic gastric adenocarcinoma with microsatellite instability. *Cancer Letter* 112: 161-166, 1997

Yamamoto H, Sawai H, and Perucho M. Frameshift somatic mutations in gastrointestinal cancer of the microsatellite mutator phenotype. *Cancer Research* 57: 4420-4426, 1997

Yee CJ, Roodi N, Verrier CS, and Parl FF. Microsatellite instability and loss of heterozygosity in breast cancer. *Cancer Research* 54: 1641-1644, 1994

Young, J, Leggett B, Gustafson C, Ward M, Searle J, Thomas L, Buttenshaw R, and Chenevix-Trench G. Genomic instability occurs in colorectal carcinomas but not in adenomas. *Human Mutation* 2: 351-354, 1993

Zhang Y, Musci T, and Derynck R. The tumor suppressor Smad4/Dpc4 as a central mediator of Smad function. *Current Biology* 7: 270-276, 1997

Zhou XP, Hoang JM, Li YJ, Seruca R, Carneiro F, Sobrinho-Simoes M, Lothe RA, Gleeson CM, Russell SE, Muzeau F, Flejou JF, Hoang-Xuan K, Liderau R, Thomas G, and Hamelin R. Determination of the replication error phenotype in human tumors without the recuirement for matching normal DNA by analysis of mononucleotide repeat microsatellites. *Genes, Chromosomes & Cancer* 21: 101-107, 1998

Zuckerman AJ. Role of the hepatitis B virus in primary liver cancer. *Journal of Toxicology and Environmental Health* 5: 275-280, 1979

Zuo L, Weger J, Yang Q, Goldstein AM, Tucker MA, Walker GJ, Hayward N, and Dracopoli NC. Germline mutations in the p16ink4a binding domain of *CDK4* in familial melanoma. *Nature Genetics* 12: 97-99, 1996