Molecular classification of familial colorectal and endometrial carcinoma

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ACADEMIC DISSERTATION

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Yesterday is but today's memory, tomorrow is today's dream.

Kahlil Gibran

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ABBREVIATIONS

ABL	*v-abl Abelson murine leukemia viral oncogene homolog 1*
AKT	v-akt murine thymoma viral oncogene homolog
APC	*adenomatous polyposis coli*
BAX	BCL2-associated X protein
BCR	*breakpoint cluster region*
BRAF	*v-raf murine sarcoma viral oncogene homolog*
BRCA1, 2	*breast and ovarian cancer gene 1, 2*
CD44	*CD44 molecule* (Indian blood group)
CDX2	*caudal type homeobox transcription factor 2*
CGH	comparative genomic hybridization
CIN	chromosomal instability
CRC	colorectal carcinoma
C_T	comparative threshold cycle
CTNNB1	*β-catenin*
DAB	3,3' diaminobenzidine (chromogen)
EC	endometrial carcinoma
ERG	*v-ets erythroblastosis virus E26 oncogene homolog*
ETV1	*ets variant gene 1*
FAP	familial adenomatous polyposis coli
GSK3β	*glycogen synthase kinase 3β*
HNPCC	hereditary nonpolyposis colorectal cancer
InSiGHT	International Society for Gastrointestinal Hereditary Tumors
KRAS	*Kirsten rat sarcoma viral oncogene homolog*
LINE-1	long interspersed nuclear element 1
LOH	loss of heterozygosity
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MLPA	multiplex ligation-dependent probe amplification
MLH1, 3	human mutL homolog 1, 3
MMR	mismatch repair
MSH2, 3, 6	human mutS homolog 2, 3, 6
MSI	microsatellite instability
MS-MLPA	methylation specific MLPA
MSS	microsatellite stable
MYC	*v-myc avian myelocytomatosis viral oncogene homolog*
MYH	mutY homolog (E. coli)

Nf1	*neurofibromatosis type 1*
PDK1, 2	pyruvate dehydrogenase kinase, isoenzyme 1, 2
PI3K	phosphatidylinositol 3-kinase
PIK3CA	*phosphatidylinositol 3-kinase catalytic subunit alpha (p110α)*
$PIP_{2,3}$	phosphatidylinositol biphosphate, triphosphate
PMS1, 2	human postmeiotic segregation increased homolog 1, 2
PTEN	phosphatase and tensin homolog
RAS	rat sarcoma viral oncogene homolog
Rb1	*retinoblastoma 1*
SAP	shrimp alkaline phosphatase
SNP	single nucleotide polymorphism
SNuPE	single nucleotide primer extension
TACSTD1	*tumor-associated calcium signal transducer 1*
TCF-4	*transcription factor 4*
TGFβRII	*transforming growth factor receptor II*
TP53	*tumor protein 53*
TMPRSS2	*transmembrane protease, serine 2*
Wnt	wingless-type

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I Abdel-Rahman WM, Ollikainen M, Kariola R, Järvinen HJ, Mecklin J-P, Nyström-Lahti M, Knuutila S, Peltomäki P. Comprehensive characterization of HNPCC-related colorectal cancers reveals striking molecular features in families with no germline mismatch repair gene mutations. *Oncogene* 24:1542-1551, 2005

II Ollikainen M, Abdel-Rahman WM, Moisio A-L, Lindroos A, Kariola R, Järvelä I, Pöyhönen M, Bützow R, Peltomäki P. Molecular analysis of familial endometrial carcinoma: A manifestation of Hereditary nonpolyposis colorectal cancer or a separate syndrome? *Journal of Clinical Oncology* 23:4609-16, 2005

III Ollikainen M, Hannelius U, Lindgren CM, Abdel-Rahman WM, Kere J, Peltomäki P. Mechanisms of inactivation of MLH1 in hereditary nonpolyposis colorectal carcinoma: a novel approach. *Oncogene* 2007 Jan 29; [Epub ahead of print]

IV Ollikainen M*, Gylling A*, Puputti M, Nupponen NN, Abdel-Rahman WM, Bützow R, Peltomäki P. Patterns of *PIK3CA* alterations in familial colorectal and endometrial carcinoma. *Submitted*

* Equal contribution

The publications are referred to in the text by their roman numerals.

ABSTRACT

Colorectal and endometrial cancers (CRC and EC) are among the three most common cancers in Finland, and in other industrial countries, and thus comprise major public health problems. Approximately 25-30% of all colorectal cancers are due to some kind of genetic predisposition. Hereditary nonpolyposis colorectal cancer (HNPCC) is the most common known hereditary cause of CRC. HNPCC is inherited in a dominant manner with predisposing germline mutations in one of the known mismatch repair (MMR) genes: *MLH1*, *MSH2*, *MSH6*, and *PMS2*. Defective MMR leads to an accumulation of mutations especially in repeat tracts such as microsatellites leading to unstable microsatellites, which is the hallmark of HNPCC tumors (and other tumors with deficient MMR). HNPCC is clinically a very heterogeneous disease, which means that the same predisposing mutation may lead to different phenotypes, even within a family. The age at onset varies and the target tissue may vary. HNPCC is actually a cancer syndrome in which predisposed individuals also have an increased risk for developing cancers other than CRC. The lifetime risk for CRC is 75-80%, the risk to develop EC is 50%, and risk for tumors of the stomach, small intestine, liver and biliary tract, pancreas, ovary, ureter and renal pelvis, and brain is below 15%. To help diagnose this heterogeneous disease, diagnostic criteria were developed. Families that fulfill the diagnostic criteria but fail to show any predisposing mutation in MMR genes, however, exist. Studying such families is likely to result in the identification of new genes participating in MMR or in new pathways that may turn out to play important roles in tumorigenesis and inherited cancer susceptibility.

Our aim was to evaluate the genetic background of familial/hereditary CRC and EC in MMR proficient and deficient tumors. First, we performed comprehensive molecular and DNA copy number analyses of CRCs fulfilling the diagnostic criteria for HNPCC. We studied the role of five pathways (MMR, Wnt, p53, CIN, and PI3K/AKT) and divided the tumors into two groups, one with MMR gene germline mutations and the other without. We then compared the observed characteristics between these two groups. We deduced that MMR gene germline mutation negative tumors include two different types of CRCs. The majority were characterized with paucity of common molecular and chromosomal alterations with stable microsatellites and chromosomes, inactive Wnt pathway, and infrequent TP53 mutations. The minority showed classical molecular features representative of tumors with gross chromosomal changes and stable

microsatellites. The majority of MMR mutation negative HNPCC families seem to be due to novel predisposing genes and pathways that differ from both those involved in the development to classical HNPCC tumors and sporadic colorectal tumors.

Second, we investigated the genetic background of familial ECs. We analyzed the role of MMR genes in families with clustered ECs, since part of the familial accumulation of EC shown in both pedigree and population-based studies may represent atypical HNPCC. Familial site-specific endometrial carcinoma is also reported to form a separate entity, but its genetic basis is still unknown. In general, little is known about the molecular background of endometrial tumorigenesis compared to tumor development in the colon. We found 9% of families with familial site-specific EC (in the absence of any associated clustering of other cancers) to be due to germline mutations in the MMR genes. We observed that the simultaneous presence of CRC in the family seems to be a somewhat stronger predictor of MMR gene germline mutations. We studied the role of PI3K/AKT pathway in familial ECs as well and observed that *PIK3CA* amplifications are characteristic of familial site-specific EC without MMR gene germline mutations. Most of the high-level amplifications occurred in tumors with stable microsatellites, suggesting that these tumors are more likely associated with chromosomal rather than microsatellite instability and MMR defect.

Despite advances in deciphering the molecular genetic background of HNPCC, it is poorly understood why certain organs (such as the colorectum and endometrium) are more susceptible than others to cancer development. To clarify whether the mechanisms of MMR gene inactivation play a role in selective organ susceptibility we tested loss of heterozygosity (LOH) and methylation in HNPCC colorectal versus endometrial tumorigenesis. The idea was based on the fact that although a MMR gene mutation is dominant, the phenotype of a mutation carrier is normal in the beginning. A somatic mutation is needed to inactivate the wild type allele before multi-step carcinogenesis can start. We observed that LOH and methylation targeted the wild type allele differently in CRC and EC and the pattern of these somatic events was dependent on the predisposing germline mutation. We conclude that somatic second hits may in part explain the differential tumor susceptibility of different organs in HNPCC.

Precise molecular characterization of families with clustered cancers of the colorectum and endometrium is highly relevant for the proper genetic counseling and clinical management. Furthermore, since the same genes are often involved in familial and sporadic tumorigenesis, our observations in familial CRC and EC are likely to benefit the research done on sporadic cancers as well.

REVIEW OF THE LITERATURE

1. Characteristics of cancer

Cancer is a major public health problem, especially in the western world. In 1953, the Finnish Cancer Registry began registering all suspected or diagnosed cancer cases in Finland and since that time the cancer incidence has increased continuously (The Finnish Cancer Registry, Cancer statistics at www.cancerregistry.fi last updated on Sept 20th 2006). At the moment every fourth Finn is diagnosed with cancer during their lifetime (The Finnish Cancer Registry, Cancer statistics at www.cancerregistry.fi last updated on Sept 20th 2006). Luckily cancer diagnostics and treatments have improved and more than a half of cancer patients can be cured (Finnish Cancer Organisations at www.cancer.fi).

Cancer is not one single disease, but a name for many diseases with uncontrolled cell growth, and invading and immortal cells as common features (Hanahan and Weinberg 2000). Cancer diseases are grouped into different types according to the tissue they arise from. Carcinomas are derived from the epithelia, sarcomas from the mesenchyme, leukemias and lymphomas from blood-forming tissues, and various cancers of the nervous system from the neuroectoderm (Weinberg 2007a) Cancers are typically named after the organ they affect, such as breast cancer, colorectal cancer and endometrial cancer.

Environmental factors, including lifestyle, cause the majority of human cancers (Boffetta 2006). The risk factors associated with the development of cancer are numerous and complex (Shields and Harris 1991). It is often difficult to point out a single cause of a cancer, but certain environmental factors have strong correlation with the occurrence of particular cancers. It has been observed for example that there is a clear correlation between smoking and lung cancer (Brennan *et al.* 2006). In Finland, about 90% of lung cancers are due to smoking (Syöpäjärjestöt, Tietoa syövästä at www.cancer.fi). Most of the cancers arise due to complex interactions between environmental factors and genes (Potter 1999), and the proportion of environmental and genetic effect varies between different cancers. Cancer formation is a multistage process and environmental factors can

affect any stage through genetic and epigenetic mechanisms (Shields and Harris 1991).

Practically every cell contains very similar molecular machinery to control growth, proliferation, differentiation, and death. Tumorigenesis is a process in which this machinery fails to maintain cell integrity and finally leads to cancer. This process includes six essential alterations in cell physiology, including self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg 2000). These six changes can occur in variable order and in parallel.

1.1 Cancer genetics

Cancer is a genetic disease since most of the above mentioned six essential changes are acquired directly or indirectly through changes in the genome. An estimated three to seven mutations, usually in four to five different genes, are needed for cancer formation, depending on cancer type (Vogelstein and Kinzler 2002a). Mutation rate in a tumor cell at the beginning of tumorigenesis is probably the same as in normal cells. The selective conditions in the tumor environment, such as aberrant cell-cell interactions, however, are different from those in the environment of normal cells. The selective advantage in the tumor environment, provided by a mutation in a growth control gene, may give rise to clonal expansion, allowing the tumor cell with the mutation to overtake its sister cells. Cancer can arise by a variety of genetic alterations including point mutations, amplifications, deletions, insertions, and changes in chromosome number and chromosomal translocations (Lengauer *et al.* 1998). Only approximately 5% of such genetic alterations are present in the germline and evidently cause a hereditary cancer (Vogelstein and Kinzler 2002a). Most mutations are somatic, affecting only the neoplastic cells. Thus the expression “cancer is a genetic disease” means that tumorigenesis is driven by altered genes. Most of these alterations are sporadic.

Cancer related genes can be classified into two groups that have opposite effects in normal cell function. Oncogenes normally stimulate cell growth and become hyperactivated in cancer, whereas tumor suppressor genes mainly repress cell

growth in the normal situation but are inactivated in cancer cells. The main differences between oncogenes and tumor suppressor genes are presented in Table 1, on page 16.

1.1.1 Oncogenes

The first cancer genes discovered were derived from cancer-causing viruses. These genes became called oncogenes as they are capable of causing oncogenesis (Vogelstein and Kinzler 2002b). It was observed that viral oncogenes have cellular equivalents, which when mutated cause cancer in dominant manner. At the moment over one hundred cancer related oncogenes are known (Futreal *et al.* 2004). These oncogenes function in normal cells in different ways to increase cell growth, proliferation and differentiation, and to decrease cell mortality. Oncogenes can be classified according to their protein products, which are growth factors, growth factor receptors, transcription factors, signaling proteins, and cell cycle regulators. Majority of altered oncogenes cause sporadic cancers and only few inherited cancer susceptibilities caused by oncogenes are currently known (Table 1).

1.1.2 Tumor suppressor genes

The idea of a recessive cancer gene arose from somatic cell fusion hybridization experiments where mouse A9 cells were capable of suppressing tumorigenesis in malignant cells (Harris *et al.* 1969). These recessive cancer genes are known as tumor suppressors. The normal function of a tumor suppressor gene is to inhibit cell proliferation. Protein products of tumor suppressor genes participate in DNA repair, growth regulation, and differentiation. In 1971, AJ Knudson was the first to show (Knudson 1971) that human tumor suppressor genes exist and since then over 30 tumor suppressor genes with cancer predisposing mutations have been found. Most of the inherited cancer susceptibilities are due to alterations in tumor suppressor genes. The same genes are also altered in the corresponding sporadic cancers (see Table 1).

By studying retinoblastoma Knudson (1971) discovered that familial cases were much more likely to get bilateral disease compared to sporadic cases, and they developed cancer at an earlier age. These observations led to Knudson's two hit

theory (Figure 1): two hits, or mutagenic events, in a tumor suppressor gene are necessary for cancer development. In the hereditary form of cancer one mutation (the first hit) is inherited and a second mutation (the second hit) occurs in somatic cells, while in the non-hereditary form two mutations must occur somatically for cancer development. According to this hypothesis, tumor suppressor genes are recessive at the cellular level since complete loss of function is required to reveal a cancer phenotype. Germline mutations in tumor suppressor genes function dominantly at the organism level, however, predisposing the carrier to early onset of disease by supplying the first hit at birth.

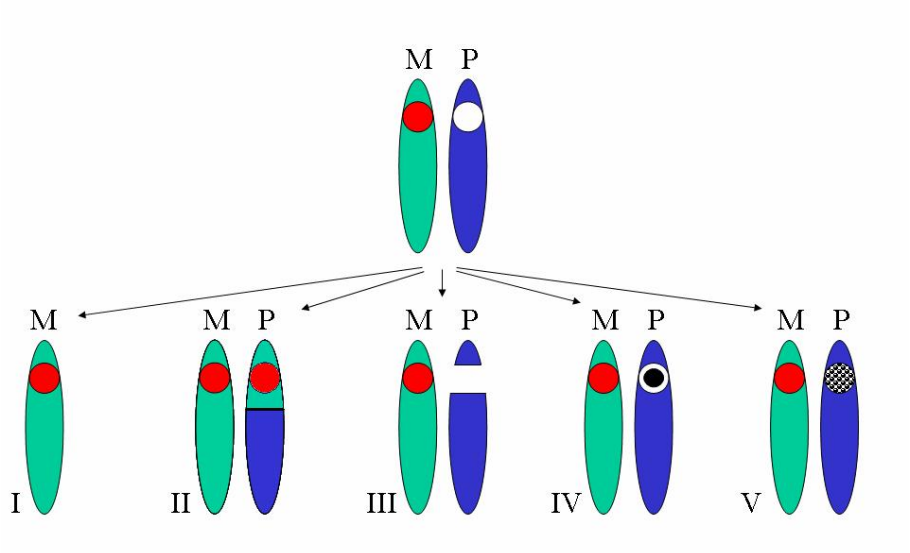

Figure 1 Mechanisms of second hit. M, maternal chromosome with mutant allele; P, paternal chromosome with wild type allele; I, mitotic nondisjuction where the whole P chromosome is lost; II, mitotic recombination (or gene conversion) where the mutated locus replaces the normal locus (or gene); III, deletion of the normal allele; IV, sporadic mutation in paternal allele; V, gene silencing by promoter methylation of the wild type allele. Modified from Aittomaki and Peltomaki (2006).

Table 1. Features of oncogenes and tumor suppressor genes

Features	Oncogene	Tumor suppressor gene
Normal function	Growth stimulation	Growth repression
Type of mutation	Activating (gain-of-function)	Inactivating (loss-of-function)
Dominant or recessive gene mutation	Dominant at cellular level: Activation of one allele is sufficient for transformation Germline mutation in one allele leads to dominantly inherited cancer predisposition in family	Recessive at cellular level: Both alleles need to be inactivated before transformation Germline mutation in one allele leads to dominantly inherited cancer predisposition in family (few exceptions)
Mutation mechanism	Point mutation (*KRAS, CTNNB1, PIK3CA*) Amplification (*PIK3CA*) Chromosomal rearrangement leading to chimeric gene (*BCR-ABL*) or to oncogenic activation (*IGH-MYC*)	Point mutation, both inherited and somatic events (MMR genes, *PTEN*) Large rearrangements leading to gene deletion or duplication, both inherited and somatic events Loss of heterozygosity (MMR genes), somatic events Gene promoter hypermethylation (*MLH1, PTEN*), somatic events
Examples of sporadic and inherited cancers	<u>Altered in multiple sporadic cancers</u> *KRAS* – colorectal and thyroid cancer, acute myeloid leukemia, melanoma *PIK3CA* – colorectal, gastric, ovarian, breast, endometrial cancer *PDGF* – gliomas, sarcomas *ERBB2/HER/NEU* – breast cancer, neuroblastoma *MYC* – promyelocytic leukemia, lung cancer, many carcinomas *JUN* – osteosarcoma *FOS* – osteosarcoma *BCL2* – lymphoma *KIT* - sarcomas *ABL* - chronic myelogenous leukemia <u>Predisposition to only few known cancer syndromes</u> *RET* – multiple endocrine neoplasia type 2A and 2B *MET* – hereditary papillary renal cell carcinoma *CDK4* – familial melanoma	<u>Altered in multiple sporadic cancers*</u> *Rb1* – retinoblastoma, osteosarcoma *BRCA1* – ovarian carcinoma *TP53* – most frequently altered gene in multiple cancers *APC* – colorectal cancer *MLH1* – colorectal and other carcinomas *VHL* – renal cell carcinoma, hemangioblastoma *PTEN* – endometrial and other carcinomas <u>Predisposition to more than 20 cancer syndromes*</u> *Rb1* – hereditary retinoblastoma *BRCA1* – hereditary breast and ovarian cancer *TP53* – Li-Fraumeni syndrome *APC* – familial adenomatous polyposis coli *MLH1* – hereditary nonpolyposis colorectal cancer *VHL* – Von Hippel-Lindau syndrome *PTEN* – Cowden syndrome *FH* – hereditary uterine leiomyomas and renal cell carcinoma

Examples of genes addressed in the present study are in bold. *The same tumor suppressor genes are frequently altered in both sporadic cancers and inherited cancer syndromes. *ABL*, *v-abl Abelson murine*

leukemia viral oncogene homolog 1; *APC*, *adenomatous polyposis coli*; *BCL2*, *B-cell lymphoma gene-2*; *BCR*, *breakpoint cluster region*; *BRCA1*, *breast and ovarian cancer gene 1*; *CDK4*, *cyclin dependent kinase 4*; *CTNNB1*, *β-catenin gene*; *ERBB2/HER/NEU, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2; FH, fumarate hydratase; FOS, v-fos FBJ murine osteosarcoma viral oncogene homolog; JUN, JUN oncogene; IGH, immunoglobulin heavy locus; KIT, v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; KRAS, Kirsten rat sarcoma viral oncogene homolog; MET, proto-oncogene tyrosine kinase; MLH1, human mutL homolog 1;* MMR, mismatch repair*; MSH2, human mutS homolog 2; MSH6, human mutS homolog 6; MYC, v-myc avian myelocytomatosis viral oncogene homolog; PDGF, platelet-derived growth factor; PIK3CA, phosphatidylinositol 3-kinase catalytic subunit alpha gene (p110α); PTEN, phosphatase and tensin homolog; Rb1, retinoblastoma 1; RET, RET oncogene; TP53, tumor protein p53; VHL, von Hippel-Lindau tumor suppressor*. Modified from Aittomaki and Peltomaki (2006).

Tumor suppressor genes are divided into three groups, namely gatekeepers, caretakers (Kinzler and Vogelstein 1997), and landscapers (Kinzler and Vogelstein 1998). The role of gatekeepers is to regulate cell growth by directly inhibiting the growth or by promoting cell death. Since gatekeeping genes are rate-limiting for tumor initiation, mutations in these genes can occur somatically as well as in the germline. The activity of gatekeepers varies between different tissues leading to tissue specificity in cancer predisposition (Kinzler and Vogelstein 1997). For example, mutation in the *Rb1* gene leads to retinoblastoma whereas mutant *NF1* predisposes to cancer of Schwann cells.

Caretakers form a class of genes that, when inactivated, do not directly promote tumors. Instead their inactivation results in genetic instability causing an increased mutation rate affecting all genes, including other tumor suppressor genes and oncogenes, and in that way promote tumor formation. Examples of caretaker genes are the mismatch repair (MMR) genes that predispose to colorectal and endometrial cancer, and the *BRCA1* and *BRCA2* (Hall *et al.* 1990; Narod *et al.* 1991; Wooster *et al.* 1994) in which mutations lead to breast cancer predisposition. Consequently, mutations in caretaker genes as well as in gatekeeper genes lead to very specific tissue distribution of cancer.

Landscaper genes affect tumorigenesis indirectly by changing the microenvironment to support tumor formation. For example in Juvenile polyposis, hamartomatous polyps seem to develop into cancer through the contact of epithelial cells with an abnormal stromal environment (Kinzler and Vogelstein 1998).

1.2 Genetic instability

The normal mutation rate in a somatic cell is approximately $1x10^{-10}$ mutations per base pair per generation (Kunkel and Bebenek 2000). This would provide less than one random mutation in every cell cycle. Such a slow mutation frequency would only generate one mutant gene in each cell of a human being during the lifetime. A variety of experiments show, however, that a cancer cell may contain as many as 10000-100000 mutations (Stoler *et al.* 1999). One explanation for such an accumulation of mutations can be genetic instability. In fact, nearly all solid tumors are genetically unstable (Lengauer *et al.* 1998; Struski *et al.* 2002). In most cancers the instability is observed at the chromosomal level (chromosomal instability, CIN) but in a small subset of cancers the instability is detected at the nucleotide level (microsatellite instability, MSI) (Lengauer *et al.* 1998). In general, an inverse relationship between CIN and MSI exists. Cancers showing MMR deficiency (leading to MSI) are usually diploid and cancers with proficient MMR function are usually aneuploid (Lengauer *et al.* 1997).

1.2.1 Chromosomal instability

Chromosomal instability has been considered to result from losses and gains of whole chromosomes or large portions of them (Michor *et al.* 2005). Recently the term CIN has got a broader meaning. It has been shown that CIN can also occur without changes in copy number (Gaasenbeek *et al.* 2006). It is accompanied by structural abnormalities, such as translocations, and by numeral changes of chromosomes, known as aneuploidy. Aneuploidy is present in almost all epithelial cancers (Struski *et al.* 2002) and results from gains or losses of whole chromosomes. Lost chromosomal regions may contain tumor suppressor genes and gains may alter the gene expression patterns, and together these changes lead to optimal tumor growth. Thiagalingam *et al.* (2001) has proposed a two-step model for aneuploidy. The first step involves defects that lead to abnormalities in chromosome number in the absence of structural changes and the second defect presumably results in a higher frequency of structural changes in chromosomes associated with interchromosomal recombination.

Translocations are structural abnormalities that are present in many different tumors but they can also lead to specific neoplastic diseases, such as leukemias and lymphomas. An example of a specific chromosomal translocation is the

formation of the Philadelphia chromosome, in which part of the *BCR* gene from chromosome 22q11 is fused with part of the *ABL* gene on chromosome 9q34. This oncogenic fusion protein is found in 90% of chronic myelogenous leukemia patients (Kurzrock *et al.* 2003). Such disease specific translocations are very rare events among all cancers, since most cancers show nearly random translocations (Johansson *et al.* 1996). A recent publication showed, however, that recurrent gene fusions of *TMPRSS2* and *ERG* or *ETV1* transcription factor genes occur in prostate cancer (Tomlins *et al.* 2005). Translocations can lead to oncogenic fusion proteins as described above, or a tumor suppressor gene might get inactivated by disruption.

A large number of genes with alterations can give rise to CIN. These genes include those involved in chromosome condensation, sister-chromatin cohesion, kinetochore structure and function, and centromere/microtubule formation and dynamics, as well as cell cycle checkpoint genes (Elledge 1996; Murray 1995; Paulovich *et al.* 1997). This might explain why CIN is a very common feature in many cancer types (Struski *et al.* 2002). Although the molecular background of CIN is not completely known, it may only require a single mutational hit to produce the phenotype (Lengauer *et al.* 1997). So far, however, this notion has been difficult to prove.

Loss of heterozygosity

Chromosomal instability can be detected as loss of heterozygosity (LOH). LOH, that is loss (or reduction of the relative dosage) of one of the two parental alleles (Cavenee *et al.* 1983), can occur either by physical deletion of all copies of the respective allele or by mechanisms that do not affect the net gene dosage (Gaasenbeek *et al.* 2006). LOH is also sometimes called an allelic imbalance since it is more often observed as a decreased dosage of one allele rather that a complete loss of one allele. LOH, like CIN, is a very broadly used term describing the loss of heterozygous alleles producing a tumor with increasing homozygosity. Different mechanisms that can lead to loss of heterozygosity are seen in tumors: deletion of a chromosome segment, mitotic nondisjunction, homologous recombination, nonhomologous recombination leading to translocation, break-induced replication, and gene conversion (Cavenee *et al.* 1983; Gaasenbeek *et al.* 2006; Mei *et al.* 2000; Rowley 2005; Thiagalingam *et al.* 2001). The mechanisms

behind LOH have been shown to be, to some extent at least, chromosome-specific (Gaasenbeek *et al.* 2006; Thiagalingam *et al.* 2001) with some chromosomes having a tendency to show complete loss and others displaying partial losses. In addition, some cancers tend to harbor loss and regain of whole-chromosome or mitotic recombination (Gaasenbeek *et al.* 2006). Overall, LOH is widespread in many tumors (Gaasenbeek *et al.* 2006; Vogelstein *et al.* 1989).

1.2.2 Microsatellite instability

Compared to CIN, microsatellite instability (MSI) is a feature of a smaller subset of cancers (Boland *et al.* 1998). The instability is seen at the nucleotide level, including single base substitutions, and insertions and deletions of a few nucleotides (Jiricny 1998). Microsatellites are short tandem repeats of DNA sequence scattered throughout the genome (Weinberg 2007b). The repeated sequence is often simple, consisting of two to four nucleotides (di-, tri-, and tetranucleotide repeats) (Buermeyer *et al.* 1999), and can be repeated 10 to 100 times. Microsatellites owe their variability to an increased rate of mutation compared to other neutral regions of DNA. These high rates of mutation are often caused by slippage of the DNA polymerase during DNA replication (Buermeyer *et al.* 1999; Kolodner and Marsischky 1999; Kunkel 1990). Most of the errors in slippage are corrected, but some mutations can escape repair (Buermeyer *et al.* 1999; Kolodner and Marsischky 1999; Kunkel 1993). Microsatellites are said to be unstable when the germline allele has gained or lost repeat units. Such an alteration can be detected only if many cells are affected by the same change. Thus it can be used as an indicator of clonal expansion typical of a neoplasm (de la Chapelle 2003).

While the molecular background of the CIN phenotype is poorly known, the molecular mechanisms behind MSI are well characterized. MSI is caused by defects in the DNA MMR mechanism responsible for the fidelity of DNA replication (Jiricny 1998). MMR proteins repair base-base mismatches and small insertion-deletion loops generated by the DNA polymerase during replication (Jiricny and Nystrom-Lahti 2000). The key components of MMR are highly conserved from bacteria to mammals, and the knowledge of the human system is mainly based on the observations made in *Saccharomyces cerevisiae* (Jacob and Praz 2002). At least six different MMR proteins are needed in humans to ensure the

fidelity of DNA replication (Peltomaki 2001). MMR proteins form specific heterodimers with each other, depending on the type of the mutation to be corrected (Figure 2). If the mutation to be corrected is a base-base mispair the MSH2 protein functions as a complex with MSH6. The MSH2 protein may co-operate with MSH3, in addition to MSH6, if an insertion-deletion loop is to be repaired (Peltomaki 2003), since MSH3 and MSH6 have partially redundant functions in insertion-deletion loop repair (Das Gupta and Kolodner 2000; Marsischky *et al.* 1996). MLH1 protein forms a heterodimer with PMS2 to coordinate the interplay with the mismatch recognition complex and other proteins necessary for MMR (Peltomaki 2001), primarily in the repair of insertion-deletion loops. MLH1 may also heterodimerize with MLH3 and PMS1. MLH3 contributes to the correction of insertion-deletion loops (Lipkin *et al.* 2000), but the role of PMS1 in MMR is not yet clear (Leung *et al.* 2000).

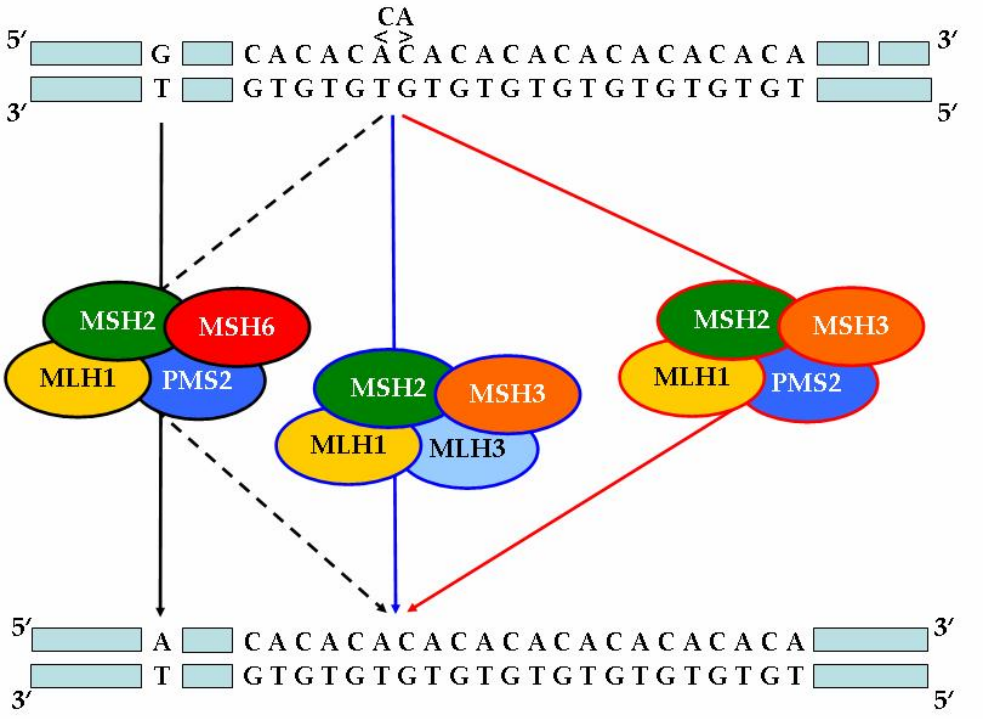

Figure 2 MMR protein complexes during repair. Upper heteroduplex shows two different errors; base/base mispair and two nucleotide insertion loop. Both of these errors can be repaired by the MSH2/MSH6-MLH1/PMS2 complex. Insertion loops can also be repaired by the MSH2/MSH3-MLH1/PMS2 complex and by the MSH2/MSH3-MLH1/MLH3 complex. In all cases the repair process is directed at the primer strand with nicks. Solid arrows denote the main repair pathways and the dashed arrow the secondary pathway. Modified from Jiricny and Nystrom-Lahti (2000).

A defective MMR system results in a mutator phenotype and unstable microsatellites. The mutation rate in MMR deficient tumor cells is 100-1000-fold that of normal cells (Bhattacharyya *et al.* 1994; Parsons *et al.* 1993). Although most microsatellites exist in non-coding DNA, they are also found in a number of human genes. Such genes easily accumulate frameshift mutations during replication and in the presence of defective MMR these mutations remain in the DNA sequence leading to truncated proteins. Genes that are altered in MSI tumors encode proteins involved in signal transduction (TGFβ-RII [Parsons *et al.* 1995], PTEN [Shin *et al.* 2001]), apoptosis (BAX [Rampino *et al.* 1997]), transcription regulation (TCF-4 [Duval *et al.* 2000]) and DNA repair (MSH6, MSH3 [Ohmiya *et al.* 2001]). In addition to MSI, MMR defect leads to genomic instability manifesting as LOH, chromosomal translocations, and gene conversions, since MMR proteins function besides post-replicative repair in several other mechanisms that are highly relevant to tumorigenesis (see chapter 4).

Unstable microsatellites, as a manifestation of a MMR defect, occur in approximately 15% of sporadic colorectal tumors (Boland *et al.* 1998), and in over 90% of HNPCC tumors (Aaltonen *et al.* 1993). A majority of sporadic colon cancers with a high degree of MSI are caused by the inactivation of *MLH1*, which mostly results from biallelic promoter hypermethylation (Veigl *et al.* 1998). In HNPCC, germline mutations in MMR genes accompanied by somatic inactivation of the wild-type allele by different mechanisms lead to a varying degree of MSI in tumors (discussed in chapter 2.1).

1.3 Cancer epigenetics

Gene expression changes that do not involve alterations in DNA sequence are called epigenetic changes. Epigenetic changes are mainly inherited through mitosis and thus passed from parental cell to daughter cells. Epigenetic mechanisms that influence DNA expression include histone modifications and methylation of cytosines located within the CpG dinucleotides (Geiman and Robertson 2002). Approximately 70% of CpG dinucleotides are methylated in the human genome, whereas most of the unmethylated CpGs are located in CpG islands (Bird 1986). CpG islands are present in around 50% of mammalian genes

(Antequera and Bird 1993), usually located close to the transcription start site. Certain CpG islands are normally methylated, including imprinted genes, X-chromosomal genes in women, germline specific genes, and tissue specific genes (Baylin *et al.* 1998).

The aberrant epigenetic profile of a cell can contribute to cancer development. Hypermethylation of a promoter CpG island attracts chromatin inactivating complexes and may prevent transcription factors from binding, thus silencing the respective gene (Lopez-Serra *et al.* 2006; Richardson 2003). A lot of evidence suggests that epigenetic silencing of a tumor suppressor gene is a frequent mechanism of gene inactivation (Costello *et al.* 2000; Esteller *et al.* 2001; Esteller 2002; Herman and Baylin 2003; Myohanen *et al.* 1998). For example, although germline mutations in *MLH1* strongly predispose to HNPCC, in sporadic colorectal cancers *MLH1* silencing by epigenetic mechanisms (methylation) occurs at a much higher rate than mutational inactivation (Cunningham *et al.* 1998; Herman *et al.* 1998; Veigl *et al.* 1998). Table 2 shows some examples of genes found to be hypermethylated in human cancers.

Table 2. Examples of hypermethylated genes in human cancers

Cancer	Methylated genes	Consequence	Reference
Colorectal cancer	*MLH1*	Mutator phenotype	(Herman *et al.* 1998)
Endometrial cancer	*PTEN*	Excessive cell invasion	(Salvesen *et al.* 2001)
Gastric cancer	*MGMT*	Mutator phenotype	(Hong *et al.* 2005)
Ovarian cancer	*p16*	Extended lifespan of a cell	(Wiley *et al.* 2006)
Breast Cancer	*ER*	Transcriptional repression of target genes	(Li *et al.* 2006)
Prostate cancer	*GSTP1*	Increased DNA damage by oxidants and electrophils	(Henrique and Jeronimo 2004)

MLH1, *human MutL homolog 1*; *PTEN*, *phosphatase and tensin homolog*; *MGMT*, *O-6-methylguanine-DNA methyltransferase*; *p16*, *Cyclin-dependent kinase inhibitor-2A*; *ER*, *estrogen receptor*; *GSTP1*, *glutathione-S-transferase 1*

Aberrant methylation in cancer cells includes, besides gene specific hypermethylation, global DNA hypomethylation. A cancer cell can have 20-60% fewer methylated cytosines than a normal cell (Lapeyre and Becker 1979; Lu *et al.* 1983). Hypomethylation contributes to carcinogenesis mainly through repetitive sequences, although growing evidence also exists for gene specific alterations (Table 3). Decreased DNA methylation in a cell can lead to loss of imprinting,

LOH, and aneuploidy, as well as reactivation of transposable elements (Esteller and Herman 2002). For example in colon carcinoma, progressive hypomethylation of LINE-1 correlates with multistage carcinogenesis from normal epithelium to late stage carcinoma (Chalitchagorn *et al.* 2004).

Table 3. Examples of hypomethylated genes in human cancers

Cancer	Hypomethylated genes	Consequence	Reference
Colorectal cancer	*MAGE A1* and *A3*	Promotion of growth	(Kim *et al.* 2006)
Gastric carcinoma	*CCND2*	Continuous proliferation	(Oshimo *et al.* 2003)
Ovarian carcinoma	*SNCG*	Increased proliferation and metastasis	(Gupta *et al.* 2003)
Breast cancer	*uPA*	Promotion of growth and invasion	(Guo *et al.* 2002)
Pancreatic cancer	*maspin*	Increased invasiveness	(Fujisawa *et al.* 2005)
Bladder cancer	*EGR1*	Degradation of extracellular matrix and cell membrane	(Ogishima *et al.* 2005)

MAGE, *melanoma associated antigen*; *CCND2*, *cyclin D2*; *SNCG*, *synuclein* γ; *uPA*, *urokinase-type plasminogen activator*; *maspin*, *protease inhibitor 5*; *EGR1*, *early growth response gene 1*

2. Colorectal cancer

Colorectal cancer is the third most common cancer in Finland, right after prostate and breast cancer. Over 2200 new cases are diagnosed yearly and around 1000 patients die of CRC (The Finnish Cancer Registry, Cancer statistics at www.cancerregistry.fi last updated on Sept 20th 2006). The same trend can be seen in all western countries. Risk factors for CRC include environmental factors such as low-fibre – high-fat diet, sedentary lifestyle, smoking, high alcohol consumption, obesity and diabetes mellitus, and personal or family history of cancer of the small bowel (and large bowel in a family member), endometrium, breast, or ovary (Weitz *et al.* 2005). In general, CRC is a disease of elder people (mean age at onset around 70 years), but hereditary CRCs occur at a much younger age at onset (45 years in HNPCC) (Lynch and de la Chapelle 1999). Approximately 70% of all CRCs are sporadic, occurring by chance or caused by environmental factors, and 25-30% are due to some kind of inherited predisposition (Figure 3). No more than ~5% of such CRCs are inherited as an obvious mendelian trait and are due to highly penetrant mutations in a single

gene, while the rest (~25%) are not associated with clear mendelian families (Rowley 2005). In addition to high penetrance susceptibility genes a number of low penetrance susceptibility genes largely affect the overall risk for CRC (Abdel-Rahman and Peltomaki 2004), whether familial or sporadic. Furthermore, modifier genes and environmental factors bring additional variability to cancer susceptibility (de la Chapelle 2004).

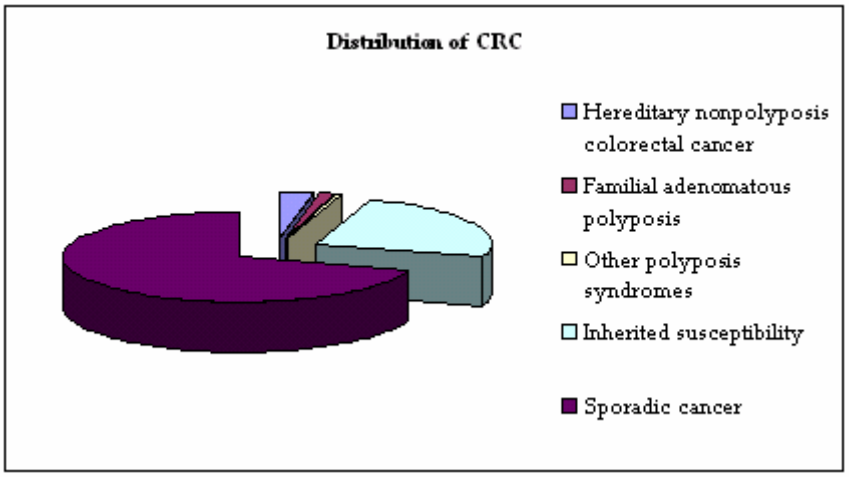

Figure 3 Proportion of inherited susceptibility to colorectal cancer. Hereditary nonpolyposis colorectal cancer accounts for approximately 3-5% (Aaltonen *et al.* 1998; Mecklin 1987; Rodriguez-Bigas *et al.* 1997; Salovaara *et al.* 2000; Scapoli *et al.* 1994) and familial adenomatous polyposis coli for 0.5-1.5% (Bisgaard *et al.* 1994) of all CRCs. Other polyposis syndromes include for example hamartomatous polyposis syndromes, and they account together less than 1% of all CRCs (Burt and Neklason 2005). Around 20-25% of CRCs occur in individuals with a positive family history and can therefore be considered to have some inherited susceptibility (de la Chapelle 2004; Lynch and de la Chapelle 2003). The rest ~70% of CRCs are considered to be sporadic (de la Chapelle 2004; Lynch and de la Chapelle 2003).

Colorectal cancer is probably the most studied cancer type and is used as a model for tumorigenesis of other epithelial cancers. CRC is rather easy to study, since the different stages of CRC are quite easily accessible and there are well-defined inherited susceptibilities to CRC. Studies on hereditary CRC syndromes are valuable for the characterization of the respective sporadic CRCs, since the same genes and pathways are affected in both. The most commonly affected pathway in colorectal carcinogenesis is the Wnt pathway (Huang *et al.* 1996; Morin *et al.* 1997; Rowan *et al.* 2000). Germline mutations in the *APC* gene predispose to

familial adenomatous polyposis (FAP). However, *APC* is also a very important gene in sporadic CRCs (Miyoshi *et al.* 1992; Powell *et al.* 1992). Another well known CRC syndrome, HNPCC, develops through inherited mutations in MMR genes. The same genes are implicated in around 15% of sporadic colorectal cancers (Peltomaki 2003). Other very common alterations in CRC are *TP53* and *KRAS* mutations. *TP53* is genetically altered in over 80% of the cases (Baker *et al.* 1990), and *KRAS* mutations occur in approximately 30% of colorectal cancers (Bos *et al.* 1987). Two major carcinogenetic pathways for colorectal cancer are proposed to exist. The MSI/CIN- tumors have defective MMR and unstable microsatellites, and are usually diploid (CIN-). Tumors developed via the MSS/CIN+ pathway include gross changes in chromosome number (CIN+), but exhibit stable microsatellites (MSS) (Abdel-Rahman *et al.* 2001; Lengauer *et al.* 1997).

Hereditary colorectal cancers can be divided into non-polypotic and polypotic cancers. Non-polypotic syndromes include HNPCC, Muir-Torre syndrome, and Turcot's syndrome (HNPCC variant) (Abdel-Rahman and Peltomaki 2004; Allen and Terdiman 2003). Familial CRC type X (Lindor *et al.* 2005) is also non-polypotic, but its molecular background, to date, is not well characterized. Polypotic syndromes can be further divided into two classes according to whether a large number of adenomatous or hamartomatous polyps are present. Adenomatous polypotic syndromes include FAP, attenuated FAP, Turcot's syndrome (APC variant), and MYH-associated polyposis (Abdel-Rahman and Peltomaki 2004; Allen and Terdiman 2003). Juvenile polyposis, Peutz-Jeghers, and Cowden syndrome belong to hamartomatous polypotic syndromes (Abdel-Rahman and Peltomaki 2004; Allen and Terdiman 2003). Two groups of familial CRCs (HNPCC and Familial CRC type X) are discussed in more detail in the next two paragraphs.

2.1 Hereditary nonpolyposis colorectal cancer

HNPCC is a well-characterized hereditary cancer syndrome. It is the most common form of hereditary colorectal cancers, comprising around 4% of all CRCs (Figure 3). The average age at onset for CRC among HNPCC patients is 45 years (Lynch and de la Chapelle 1999), which is around 20 years earlier than in sporadic colorectal cancer. Predisposed individuals have a 75-80% lifetime risk of developing CRC (Aarnio *et al.* 1999; Lynch and de la Chapelle 1999), but they are

also at an increased risk for extracolonic carcinomas. Lifetime risk for endometrial carcinoma (EC) is 50% in predisposed females, and risk for cancer of the stomach, small intestine, ovary, pancreas, ureter, and renal pelvis is less than 15% in individuals with HNPCC predisposition (Aarnio *et al.* 1995; Watson and Lynch 1993).

HNPCC colorectal cancers are predominantly associated with the proximal colon, are poorly differentiated or show mucinous or signet-ring differentiation, and are relatively non-aggressive (Jass *et al.* 1994). Since no clear pathologic features (like hundreds of adenomas as in FAP) by which HNPCC could easily be identified exist, diagnosis was mainly based on individual patient and family history of cancer, until the criteria for HNPCC diagnostics were established. These criteria, called Amsterdam criteria I, (Table 4) were first defined in 1990 by the International Collaborative Group on HNPCC, and they only accept CRC as a manifestation of HNPCC (Vasen *et al.* 1991). The criteria were then revised in 1998 as the Amsterdam criteria II (Vasen *et al.* 1999), which also include extracolonic HNPCC associated cancers. In addition to these two criteria, the Bethesda guidelines (Rodriguez-Bigas *et al.* 1997) were developed to help clinicians by describing all clinical conditions in which there is suspicion of HNPCC, even if the respective family does not fulfill Amsterdam criteria. Bethesda guidelines have also been revised (Table 4) to better suit present day analyses that are very useful in HNPCC diagnostics (Umar *et al.* 2004).

Predisposition to HNPCC comes through inherited germline mutations in the MMR genes: *MSH2* (Fishel *et al.* 1993; Leach *et al.* 1993; Peltomaki *et al.* 1993), *MLH1* (Bronner *et al.* 1994; Lindblom *et al.* 1993; Papadopoulos *et al.* 1994; Tannergard *et al.* 1994), *MSH6* (Akiyama *et al.* 1997; Miyaki *et al.* 1997a), and *PMS2* (Nicolaides *et al.* 1994). Most of the known 500 HNPCC associated mutations (The International Society for Gastrointestinal Hereditary Tumors, Database for mutations at www.insight-group.org) occur in *MLH1*, *MSH2*, and *MSH6*, whereas mutations in *PMS2* are rare and are mainly associated with Turcot's syndrome (De Rosa *et al.* 2000; Hamilton *et al.* 1995; Miyaki *et al.* 1997b; Truninger *et al.* 2005). With a few exceptions (mainly associated with *PMS2* and Turcot's syndrome) HNPCC is a dominantly inherited cancer syndrome, even though at the cellular level, both copies of the respective MMR gene need to be inactivated before the multistep tumorigenesis can start.

Table 4. Diagnostic criteria and guidelines for HNPCC

Amsterdam criteria I and II	Bethesda guidelines
1. At least three relatives with HNPCC associated cancer (in colorectum, endometrium, small bowel, ureter, renal pelvis)	1. CRC diagnosed in a patient before age 50
2. One should be a first degree relative of the other two	2. Presence of synchronous, metachronous CRC or other HNPCC associated tumors, regardless of age
3. At least two affected generations	3. CRC with the MSI-high histology diagnosed in a patient before age 60
4. At least one member diagnosed before age 50	4. CRC diagnosed in one or more first-degree relatives with an HNPCC associated tumor, with one of the tumors diagnosed before age 50
5. FAP should be excluded	
6. Tumors should be verified by pathological examination	5. CRC diagnosed in two or more first- or second-degree relatives, with HNPCC associated tumors, regardless of age

MMR deficiency in HNPCC tumors (as well as in sporadic tumors) leads to unstable microsatellites. This is detected by testing the tumors for five microsatellite markers comprising the Bethesda panel (Boland *et al.* 1998). MSI is scored to be high (MSI-high) if more than two of the Bethesda panel markers are unstable. MSI-low is a definition for tumors with no more than one unstable microsatellite marker (Boland *et al.* 1998). Most of the HNPCC cases with germline mutation in *MLH1* or *MSH2* show MSI-high phenotype and fulfill the Amsterdam criteria I, and are referred to as classical HNPCC families (Liu *et al.* 1996; Nystrom-Lahti *et al.* 1996). Whereas germline mutations in *MSH6* can lead to MSI-high or MSI-low, and such tumors may even show stable microsatellites (Berends *et al.* 2002; Hendriks *et al.* 2004; Wu *et al.* 1999). These families are usually less typical HNPCC families, with late onset and frequent endometrial carcinomas. Findings show that germline mutations in *PMS2* can lead to autosomal dominant HNPCC with MSI-high phenotype in families fulfilling Amsterdam criteria II (Hendriks *et al.* 2006; Worthley *et al.* 2005). Germline mutations in *MLH1* and *MSH2* lead to a more severe HNPCC phenotype than mutations in other MMR genes, since MLH1 and MSH2 are necessary in all MMR complexes and the absence of these proteins results in the instability of their interaction partners (Peltomaki 2001) (see also Figure 2).

Germline mutations in MMR genes drive the tumorigenic pathway by causing accumulation of genetic alterations in the genome. The main target genes are those containing repetitive tracts within coding sequence, such as *TGFβRII*, which is mutated in up to 90% of MSI CRCs (Parsons *et al.* 1995). These target genes may, in addition to tumor progression, correspond to tissue specificity. For example, frameshift mutations in *TGFβRII* are characteristic of MSI CRC, but most endometrial carcinomas with MSI lack these mutations, suggesting a different route to MSI in EC (Kuismanen *et al.* 2002). Such tissue specific selection may provide one possible explanation for tumor spectrum of HNPCC since growth advantage may be acquired by different genes depending on a tissue type.

Molecular testing for HNPCC is started by analyzing microsatellite markers and MMR protein expression according to the Bethesda guidelines (Umar *et al.* 2004). When the MSI-high phenotype is observed or if one of the MMR proteins lacks expression, mutations in *MLH1* and *MSH2*, or in the gene pinpointed by protein expression analysis, are searched from the germline. By following these guidelines, approximately 30-90% of HNPCC families, depending on the population, can be identified (de la Chapelle 2005; Liu *et al.* 1996; Nystrom-Lahti *et al.* 1996; Renkonen *et al.* 2003; Salovaara *et al.* 2000). However, up to 50% of families fulfilling the Amsterdam criteria fail to show any mutations in MMR genes by conventional methods (Abdel-Rahman *et al.* 2006). Among such families, testing for hidden mutations, large deletions, and splicing errors may lead to identification of additional HNPCC families (Renkonen *et al.* 2003). Still around 30-50% of families with the HNPCC phenotype remain truly mutation negative. These families will be discussed in the next chapter.

2.2 Familial CRC type X and other MMR proficient colorectal cancers

The literature describing families with nonpolypotic CRC is conflicting. A large proportion of families with nonpolypotic CRC meet the diagnostic criteria for HNPCC, and are therefore erroneously classified as HNPCC despite the lack of MMR gene germline mutations. Renkonen *et al.* (2003) showed that some of the CRC families fulfilling either the Amsterdam or Bethesda criteria (originally considered to be HNPCC families) and lacking evidence of MMR gene involvement seem to constitute a clinically and molecularly separate entity. In

these families the average age at CRC onset is significantly higher and tumors are more often located in the distal colon than in HNPCC individuals with MMR gene germline mutations. Moreover, such tumors frequently show MSS phenotype (Lindor *et al.* 2005; Llor *et al.* 2005; Mueller-Koch *et al.* 2005; Renkonen *et al.* 2003). Such families are also reported to show increased incidence for CRC (Lindor *et al.* 2005; Llor *et al.* 2005; Mueller-Koch *et al.* 2005; Renkonen *et al.* 2003) but not for extracolonic carcinomas (Lindor *et al.* 2005), which is a common feature of HNPCC families. Moreover, relatives in such families have a lower incidence of CRC (Lindor *et al.* 2005; Llor *et al.* 2005). Lindor et al (2005) hypothesized that families fulfilling the strict Amsterdam criteria with no MMR defects include cancers that occur by chance, are related to shared lifestyle, or are due to genetic defects that need to be defined. This heterogeneous group of familial CRCs is nowadays known as familial CRC type X (Lindor *et al.* 2005), describing the lack of understanding of the etiology behind these families, which are not necessarily displaying hereditary CRC.

3. Endometrial cancer

Endometrial cancer (EC) is the third most common cancer, after breast and colorectal cancer, in Finnish females. Approximately 800 new cases are diagnosed every year (The Finnish Cancer Registry, Cancer statistics at www.cancerregistry.fi last updated on Sept 20th 2006), but the curability is mostly very high. Endometrial cancer affects mainly perimenopausal or postmenopausal women, hence 95% of ECs are diagnosed in females over 50 years of age and 58% in females over 65 (The Finnish Cancer Registry, Cancer statistics at www.cancerregistry.fi last updated on Sept 20th 2006). The incidence of EC is increasing slightly, mainly due to population aging. Familial syndromes with a much lower age at onset of EC, however, also occur (discussed in the next paragraphs). An estimated 5% of patients with EC diagnosed younger than 55 have a family history of this cancer (Gruber and Thompson 1996). Known risk factors for EC include estrogen replacement therapy, early menarche, late menopause, nulliparity, obesity, hypertension, and diabetes mellitus (Henderson and Feigelson 2000; Hinkula *et al.* 2002; Lynch *et al.* 1994; McPherson *et al.* 1996). In addition, positive personal or family history of breast, ovarian, or colorectal cancer is a risk factor for EC (Gruber and Thompson 1996; Hemminki *et al.* 2004; Suomi *et al.* 1995).

The change from a normal endometrium to carcinoma is thought to involve a stepwise accumulation of alterations in regulatory pathways leading to abnormal cell growth (Enomoto *et al.* 1991), as it has been described for CRC. Unlike in CRC, however, the molecular basis of EC remains poorly known. Endometrial carcinomas can be classified into two broad categories. Type I consisting of ECs with endometrioid histology, and Type II including uterine serous carcinomas (Bokhman 1983). In addition to pathological characteristics, these two types differ from each other by genetic alterations. Type I tumors are commonly associated with abnormalities in the MMR genes (Risinger *et al.* 1997; Salvesen *et al.* 2000), *KRAS* (Enomoto *et al.* 1991; Lax *et al.* 2000), *PTEN* (Mutter *et al.* 2000; Risinger *et al.* 1997), and *CTNNB1* (Kariola *et al.* 2005; Machin *et al.* 2002). Type II tumors mainly show alterations in *TP53* (Kounelis *et al.* 2000; Zheng *et al.* 1996).

3.1 Endometrial cancer as a part of known syndromes

Approximately 90% of endometrial cancers occur sporadically and only 10% are considered familial (Boltenberg *et al.* 1990). The majority of endometrial carcinomas recognized as inherited occur in affected women belonging to HNPCC families. Notably, EC is the most common extracolonic cancer in HNPCC families (Watson and Lynch 1993) and the risk for EC equals or exceeds the risk for CRC in females (Aarnio *et al.* 1999; Dunlop *et al.* 1997). Females with HNPCC have a ten-fold increased lifetime risk of EC compared to the general population (Dunlop *et al.* 1997). HNPCC is due to germline mutations in the MMR genes, mainly in *MLH1*, *MSH2*, and *MSH6* (see chapter 2.1). Risk for EC among females in such families varies according to the predisposing mutation in the family, *MSH6* mutation carriers having the highest risk. Wijnen *et al.* (1999) and Hendriks *et al.* (2004) have reported about an excess of EC in female carriers of *MSH6* mutations, the frequency and the cumulative risk for EC by age of 70 being around 70%, exceeding the 30% risk for CRC. The average age of onset for EC in affected HNPCC females is 50 years (Peltomaki *et al.* 2001) which is significantly lower than in sporadic cases (66 [Ylikoski and Komulainen 1999]). Almost all HNPCC associated ECs show endometrioid histology. When compared to sporadic ECs, HNPCC associated ECs more frequently show poor differentiation, Crohn-like lymphoid reaction, lymphangioinvasive growth, and more tumor-

infiltrating lymphocytes (van den Bos *et al.* 2004). The spectrum of ECs in women with HNPCC is wide, however, including tumors of all grades and histologies.

HNPCC associated ECs are diagnosed, like CRCs, according to the Amsterdam and Bethesda criteria followed by testing tumors for microsatellite instability and MMR protein expression (see chapter 2.1 for further details). MSI is demonstrated in HNPCC ECs, but in a lower proportion than in HNPCC associated CRCs, and the MSI pattern is more heterogeneous (Kuismanen *et al.* 2002). Unlike in HNPCC associated CRCs with unstable microsatellites, *TGFβRII* seems not to be a target loci in endometrial tumorigenesis, even though ECs show MSI. Instead, frameshift mutations in *PTEN* associate with MSI ECs (Kuismanen *et al.* 2002). This implies that the genesis of ECs occurs by a route distinct from CRCs, even if driven by MMR defect.

Germline mutations in *PTEN* predispose to hamartoma tumor syndrome, namely Cowden syndrome. Cowden syndrome mainly affects breast, thyroid, uterus, brain, and mucocutaneus tissues (Starink *et al.* 1986). Risk for EC is around 5-10% (Eng 2000) among affected females with Cowden syndrome.

3.2 Familial site-specific endometrial carcinoma

In addition to known hereditary cancer syndromes with endometrial carcinoma associated with other cancers, occasional families show clustering of EC alone. Familial clustering of EC only, without clustering of any other cancers, is called familial site-specific endometrial carcinoma (Sandles *et al.* 1992). It is unclear whether such a syndrome truly exists on a molecular level or if it is a manifestation of other syndrome(s) with EC as a component.

4. MMR pathway in cancer

The main role of the MMR system is in post-replication repair of single-base substitutions and small insertion-deletion loops made by DNA polymerase that have escaped the proofreading. The "mutator phenotype" and MSI resulting from the failure of this repair function have been discussed above. In addition to post-replicative repair, MMR proteins have several other functions that are highly relevant to carcinogenesis. During homologous recombination the MMR system prevents homeologous recombination between diverged sequences (Chen and Jinks-Robertson 1999) and thus evades chromosomal translocations, amplifications, deletions, or insertions. MMR proteins are also involved in double-strand break repair together with NER enzymes Rad1-Rad10 (Sugawara *et al.* 1997). Some MMR components participate in the recognition of DNA adducts and damaged bases in the DNA structure. This leads to initiation of the signal pathway that can activate cell-cycle check points and trigger apoptosis (Hickman and Samson 1999; Li 1999). It is easy to understand that since MMR proteins are involved in such a large number of cellular mechanisms contributing to DNA integrity and apoptosis, the defects in MMR system are extremely harmful to normal cell function.

5. Wnt pathway in cancer

The Wnt signaling pathway is very ancient, dating back to at least 650 million years (Teo *et al.* 2006). The Wnt pathway is a key component controlling patterning and organogenesis in the developing embryo by determining cell fate and axis formation in all metazoan organisms (Willert and Jones 2006). Overactivation of the Wnt pathway results in expression of specific target genes leading to cell growth and proliferation, and finally to carcinogenesis. In the absence of an activating signal (see Figure 4), adenomatous polyposis coli (APC) forms a degradation complex with axin and glycogen synthase kinase-3β (GSK3β) which directs β–catenin to ubiquitination and degradation (Orford *et al.* 1997). When the Wnt pathway is activated by a Wnt ligand via the Frizzled receptor, or by activating mutations, the degradation complex is destabilized and β–catenin can enter the nucleus where it operates as a co-activator of transcription factors (Morin *et al.* 1997). Target genes, such as *c-MYC* (He *et al.* 1998), *CD44* (Wielenga *et*

al. 1999) and *cyclin D1* (Tetsu and McCormick 1999), function mainly in determining cell fate and proliferation, as well as promoting CIN.

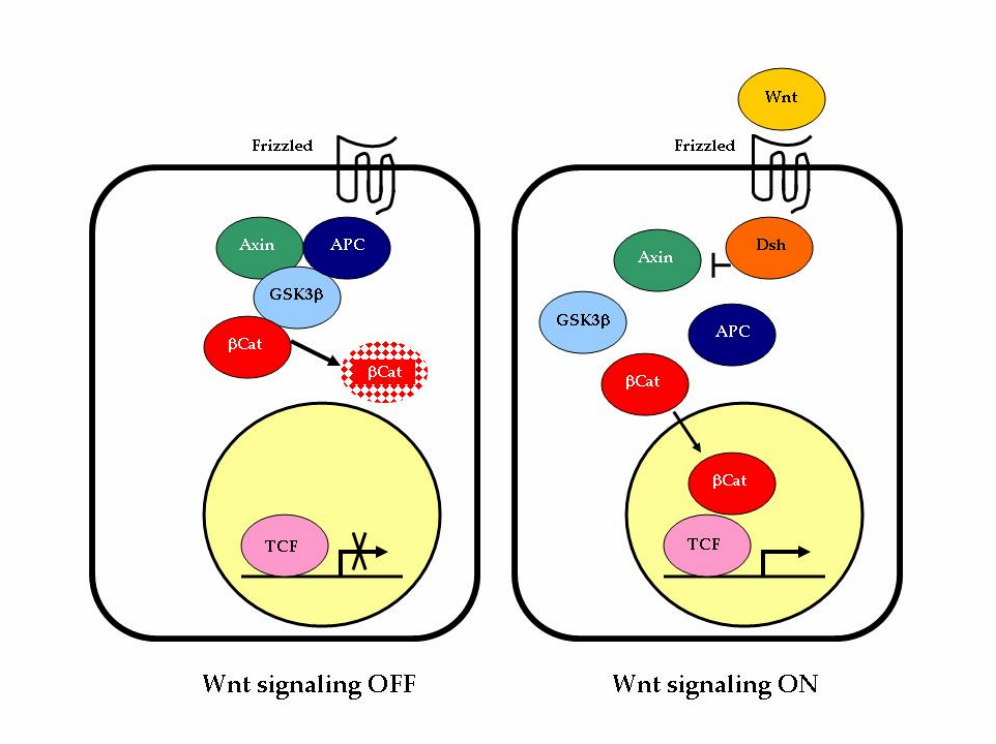

Figure 4 Activation and key components of the Wnt pathway. In the absence of Wnts, β-catenin is phosphorylated by GSK3β in multiprotein complex in which Axin and APC are also included. Wnt signaling leads to stabilization of β-catenin that enters the nucleus and co-activates target gene transcription. APC, adenomatous polyposis coli; βCat, β-catenin; Dsh, dishevelled; GSK3β, glycogen synthase 3β; TCF, T-cell factor; Wnt, Wnt-ligand.

The variety of target genes activated by the Wnt pathway explains its important role in the initiation of tumorigenesis. In the adenoma-carcinoma sequence of colorectal carcinogenesis (Kinzler and Vogelstein 1996), loss of APC function is one of the earliest events in the transformation, leading to formation of adenomas (Jen *et al.* 1994). Mutations in *APC* are not sufficient for progression to carcinomas, however, but provide a growth advantage by the activation of certain critical target genes. Further mutations in tumor suppressor genes and in different pathways are required (Vogelstein and Kinzler 1993; Vogelstein and Kinzler 2004). This can be accomplished by chromosomal instability, as is the case in most CRCs.

6. PI3K/AKT pathway in cancer

The phosphatidylinositol 3-kinase (PI3K)/AKT pathway is, in addition to the Wnt pathway and MMR pathway, an important regulator of mammalian cell proliferation and survival. A number of genes such as *PIK3CA* encoding p110α (Samuels *et al.* 2004), *PTEN* (Yokoyama *et al.* 2000), and *AKT* (Ringel *et al.* 2001) in the PI3K/AKT pathway are dysregulated in a wide variety of human cancers.

PI3Ks constitute a large family of heterodimeric proteins with separate regulatory (*e.g.* p85) and catalytic (*e.g.* p110) subunits. The PI3K family is divided into three classes (I-III) differing in structure, substrate specificity, mechanism of activity, and function (Vanhaesebroeck and Waterfield 1999; Wymann *et al.* 2003). The most important and best known class involved in tumorigenesis is the class IA proteins, which consist of regulatory p85 and catalytic p110α (Domin and Waterfield 1997; Walker *et al.* 1999). Figure 5 illustrates the key effects of the active PI3K/AKT pathway. In the absence of an activating signal from the cell membrane receptor tyrosine kinase, p85 binds to p110α and inactivates its kinase activity. When a growth factor binds to a receptor tyrosine kinase, p85 docks to the activated receptor, relieves the inhibition of p110α, and mediates the recruitment of the catalytic subunit to the plasma membrane (Yu *et al.* 1998). Activated p110α phosphorylates phosphatidylinositol 4,5-biphosphate (PIP_2) to become the tumor promoting second messenger phosphatidylinositol 3,4,5-triphosphate (PIP_3). PIP_3 recruits proteins containing a pleckstrin homology domain such as AKT, PDK1, and PDK2 to the cellular membrane (Osaki *et al.* 2004), where PDKs phosphorylate AKT. Activated AKT is the predominant and essential mediator of survival, growth, and proliferation stimuli leading to carcinogenesis acquired by PI3K.

The catalytic subunit of PI3K is encoded by a *PIK3CA* gene (Samuels *et al.* 2005). *PIK3CA* is mutated in 25-40% of sporadic cancers of the colon, rectum and stomach (Samuels *et al.* 2004), endometrium (Oda *et al.* 2005), and breast (Campbell *et al.* 2004). *PIK3CA* acts as an oncogene, since virtually all observed mutations are missense mutations clustered in functionally important regions (kinase and helical domains) that are highly conserved through evolution (Samuels *et al.* 2004). Functional studies show that *PIK3CA* mutations increase the activity of PI3K (Ikenoue *et al.* 2005; Kang *et al.* 2005; Samuels *et al.* 2005). In

addition, amplification of the *PIK3CA* gene associated with increased PI3K activity occurs at least in ovarian cancer (Byun *et al.* 2003; Shayesteh *et al.* 1999).

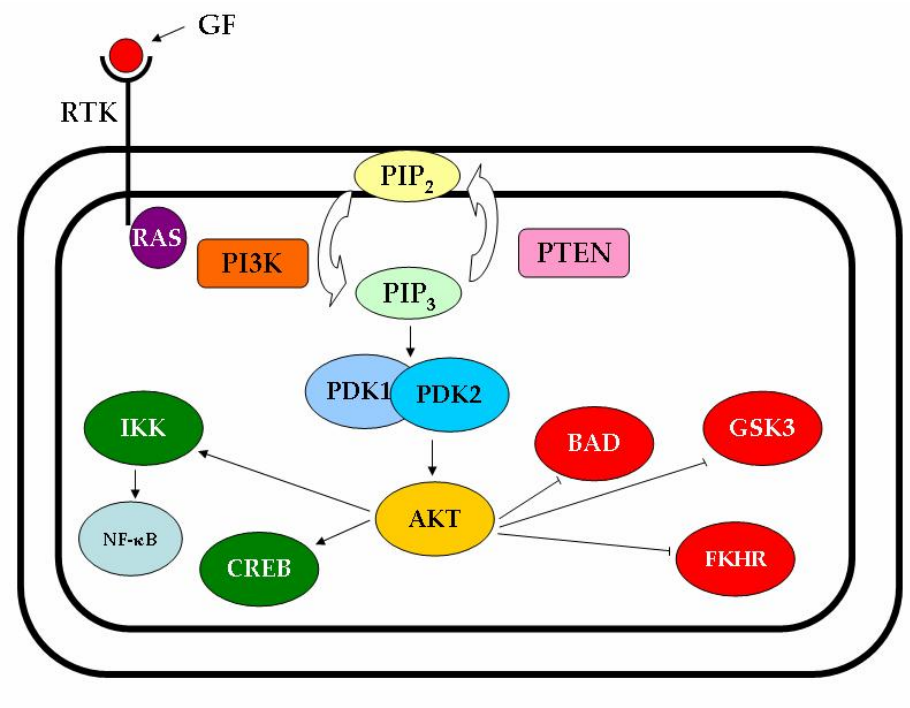

Figure 5 PI3K/AKT pathway activation. Binding of a growth factor to a receptor tyrosine kinase initiates the signaling leading to activation of AKT. AKT regulates a great variety of target proteins by phosphorylation. Molecules shown in green are prosurvival molecules that are activated by AKT and molecules in red are proapoptotic molecules that are inhibited by AKT. AKT, v-akt murine thymoma viral oncogene homolog; BAD, member of the Bcl-2 protein family; CREB, cAMP response element binding protein; FKHR, forkhead; GF, growth factor; GSK3, glycogen synthase kinase 3; IKK, inhibitory κB kinase; NF- κB, nuclear factor κB; PDK, pyruvate dehydrogenase kinase; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol biphosphate; PIP_3, phosphatidylinositol biphosphate; PTEN, phosphatase and tensin homolog; Ras, rat sarcoma viral oncogene homolog.

The tumor suppressor gene *PTEN* is an important PI3K/AKT pathway member that negatively regulates PI3K signaling by dephosphorylating PIP_3 to PIP_2 (Li *et al.* 1997; Maehama and Dixon 1998; Steck *et al.* 1997). Loss of PTEN function strongly correlates with the activation of AKT in cancer cells (Sun *et al.* 1999). Activation of the PI3K/AKT pathway may thus result from activating mutations in PI3-kinase genes or inactivating mutations in the tumor suppressor gene *PTEN*.

AIMS OF THE STUDY

This study was conducted to elucidate the genetic background of familial/hereditary cancers of the colorectum and endometrium in MMR proficient and deficient cases. Specific aims were:

1. To define the pathways leading to familial colorectal carcinomas by evaluating the role of selected target genes of tumorigenesis and the role of genomic instability (I, IV)

2. To define the genetic basis of familial site-specific endometrial carcinoma through studies of germline and tumor alterations (II, IV)

3. To examine the nature and role of the "second hit" in HNPCC colorectal versus endometrial tumorigenesis by studying the role of loss of heterozygosity and *MLH1* promoter methylation as inactivating somatic events (III)

MATERIALS AND METHODS

1. Patients and samples

This study was based on Finnish familial and hereditary colorectal and endometrial tumors and corresponding normal samples. Colorectal tumors were from well-characterized families from the Hereditary Colon Cancer Registry of Finland. All families fulfilled either Amsterdam or Bethesda criteria for HNPCC and had been included in mutation analyses based on clinical and family criteria. The families were divided into two groups based on whether a germline mutation in a MMR gene (*MLH1*, *MSH2*, *MSH6*, or *PMS2*) was present (n=48) or absent (n=24) after thorough mutation screening (Holmberg *et al.* 1998; Huang *et al.* 2001; Liu *et al.* 2001; Renkonen *et al.* 2003).

All HNPCC associated cancers of the endometrium (n=60) were derived from well-characterized HNPCC families with MMR gene germline mutations as described above for CRCs. Collection of familial site-specific ECs (defined by the presence of EC in at least one first-degree relative of the index patient, n=30) was based on a consecutive series of patients treated for EC at the Department of Obstetrics and Gynecology, Helsinki University Central Hospital, in 1986 – 1997. All patients were interviewed for the family history of cancer and the information received was confirmed through the church registries and the Finnish Cancer Registry. The genetic background of these families was completely unknown.

Sample cohort for studying second hits in *MLH1* (III) consisted of 47 HNPCC patients with 25 CRCs and 32 ECs. Each patient carried one of the three Finnish founder mutations in *MLH1*: Mut1 (3.5 kb in-frame deletion of codons 578-632 of exon 16 and flanking sequences), Mut2 (frameshift mutation g>a at 454-1 splice acceptor of exon 6), or Mut3 (missense mutation T>G at 320, I107R, in exon 4).

Fresh frozen and/or paraffin derived specimens of tumor and corresponding normal tissues were collected from pathology departments of different hospitals in Finland and used for immunohistochemical analysis and DNA extraction. Areas with pure normal or high tumor percentages, with minimal intervening stroma or inflammatory cells, were selected and verified histologically and

subsequently dissected for DNA preparation. Tumor percentages ranged from 50% to 95%. In some cases blood was also available for genomic DNA extraction.

2. Mutation and gene amplification analyses

All mutation detection methods used were based on PCR amplification. PCR was performed by a standard method (unless reported otherwise) with initial heating at 94°C for 1 min, followed by an additional 35 cycles with 1 min denaturation at 94°C, 1 min annealing at primer specific temperature, and 2 min elongation at 72°C. Final extension was achieved by 3 min incubation at 72°C.

2.1 Single-strand conformational polymorphism analysis (I, IV)

Single-strand conformational polymorphism (SSCP) analysis was used to screen hotspot mutations in exon 15 of *BRAF*, exon 2 of *KRAS*, exon 3 of *CTNNB1* (β-catenin), and in exons 1, 9 and 20 of *PIK3CA*. Samples were PCR amplified and separated on polyacrylamide gels with 1 x MDE Gel Solution (Cambrex Bio Science Rockland Inc.). SSCP gels were silver stained after the run. Changes observed in SSCP were confirmed by direct sequencing. Primer sequences for detecting the hotspot mutations are published as follows: *BRAF* in publication I, *KRAS* in Deng *et al.* (2004), *CTNNB1* in Kitaeva *et al.* (1997), and *PIK3CA* exon 9 in Li *et al.* (2005). Exons 1 and 20 of *PIK3CA* were divided into two overlapping fragments to improve PCR amplification and are listed in publication IV.

2.2 Direct sequencing (I-II, IV)

Genes showing alterations by SSCP in studies I and IV were sequenced for possible mutations in *BRAF*, *KRAS*, *CTNNB1* and *PIK3CA* genes. PCR reactions were performed with conditions and primers as described above. Immunohistochemistry was used to pinpoint affected MMR genes in study II and the individual exons of *MLH1*, *MSH2*, and *MSH6* were thereafter sequenced with primers described in Chadwick *et al.* (2001). Additionally, screening for two *MSH6* mutations previously found in the Finnish population was performed with primers for fragment “4k” (Wu *et al.* 1999), for 3052delCT (Huang *et al.* 2001), and

with primers described in our publication II for E995X (Vahteristo *et al.* 2001). Furthermore, the possible presence of a frameshift mutation affecting the *MSH6*-C_8 repeat was evaluated using primers described in Malkhosyan *et al.* (1996). The PCR conditions were as previously described.

The Finnish founder mutation, Mut1 (a large genomic deletion, described in Nystrom-Lahti *et al.* [1995]) was searched for by a direct test in a PCR assay using two forward primers. One primer preceding and the other one located within the deleted fragment were used together with a common reverse primer, as this mutation is not identifiable by exon-specific sequencing.

2.3 Multiplex Ligation-dependent Probe Amplification (II-IV)

Multiplex ligation-dependent probe amplification (MLPA) was used to detect large genomic deletions and amplifications in *MLH1*, *MSH2*, and *MSH6* (II, III) using SALSA P003 and SALSA P008 MLPA kits, and in *PIK3CA* (IV) using SALSA Gain probe mix P173 MLPA kit (MRC Holland). HNPCC samples from our collection known to carry large genomic deletions in MMR genes were used as positive controls when studying the respective genes. Normal DNA specimens derived from lymphocytes of healthy controls were included in every assay. The probe mixture SALSA P003 contains probes for all *MLH1* and *MSH2* exons. In addition, seven control probes for other genes from different chromosomes are included. The SALSA P008 probe mixture contains probes for all *MSH6* exons and a complete set of probes for *PMS2*. This kit also contains two probes for *MLH1* exon 1, one for *MSH2* exon 1, and six for *MLH3* and *MSH3* exons. Additional target genes for other probes are *TACSTD1*, *MYH*, and *APC*. The SALSA Gain probe mix P173 contains 43 probe pairs from 28 tumorigenesis related genes that have been reported to have a higher copy number in some tumors, including three probes for the *PIK3CA* gene; one for the p85 binding domain (exon 1), one for the C2 domain (exon 6), and one for the kinase domain (exon 18).

MLPA method is based on the usage of specific probes consisting two parts that anneal adjacent to each other on the same DNA strand. After hybridization to the target site these two oligos are ligated to form one single probe (ligation is possible only when both of the oligos are annealed) that can be PCR amplified. For each MLPA reaction 100-150 ng of paraffin-derived DNA was denatured and

subsequently hybridized with the MLPA probes. Ligase-65 enzyme was used to ligate the annealed probe pairs. The ligation products were amplified by PCR using a fluorescently labeled primer. The PCR products were separated by capillary electrophoresis (on ABI 3730 Automatic DNA sequencer, Applied Biosystems) and analyzed using Genemapper v3.0 or v4.0 (Applied Biosystems). Relative peak values were calculated by dividing each peak area by the sum of all peak areas of that sample. The resulting relative peak value was then divided by the mean relative peak value of normal DNAs from healthy controls, to obtain a dosage ratio (formulas below).

$$D = (P_x/P_{tot})_T \,/\, \text{mean of } (P_x/P_{tot})_N$$

where *D* is the dosage ratio, P_x is the peak area of a given probe, P_{tot} is the sum of all peak areas of all probes. T denotes tumor and N normal sample. For sequences present in two copies/diploid genome, a dosage ratio of 1 is expected. Dosage ratio of $\leq$0.6 was considered a deletion and > 1.7 an amplification.

2.4. Quantitative real-time PCR (IV)

Primers and Taqman probes were designed for *PIK3CA* exon 21 and *glucokinase* gene (*GCK*) exon 2 by using Assays-by-Design File Builder software (Applied Biosystems). All primers and detailed PCR conditions are presented in publication IV. All samples were subjected to PicoGreen (Invitrogen) measurement prior to real-time PCR analysis to obtain equal DNA concentrations in all samples. In each assay (96 wells), a no-template background control, and a positive control were included. A normal colorectal tissue specimen was also included as a calibrator sample. The PCR was performed in the ABI 7500 Sequence Detection System (Applied Biosystems). A validation experiment was carried out according to guidelines "Real-time PCR Systems Chemistry Guide" (Applied Biosystems). The practically identical slopes of the target gene and reference gene demonstrated equal efficiencies of amplification over a range of DNA concentrations, making it possible to calculate *PIK3CA* copy number using the comparative threshold cycle (C_T) method (Applied Biosystems).

3. Loss of heterozygosity analyses (I-III)

LOH at the *MLH1* locus was studied by two quantitative methods, the Matrix Assisted Laser Desorption Ionization – Time-of-Flight (MALDI-TOF) and Single Nucleotide Primer Extension (SNuPE). LOH was also studied by analyzing a set of microsatellite markers. For all different applications for LOH detection, the ratio of allelic peak areas or frequencies was calculated as follows:

$$L = (A1_T/A2_T) / (A1_N/A2_N)$$

where L is the LOH ratio, $A1_T$ is the area of allele 1 in the tumor, $A2_T$ is the area of allele 2 in the tumor. $A1_N$ is the area of allele 1 in the normal sample, and $A2_N$ is the area of allele 2 in the normal sample. A sample was scored as showing LOH if $L \leq 0.6$ or $L \geq 1.67$ (indicating that one of the alleles have decreased by 40% or more), and was scored as showing putative LOH or allelic imbalance if $0.6 < L < 0.8$ or $1.25 < L < 1.67$ (indicating a decrease of 21-39% for one allele).

3.1 Matrix-Assisted Laser Desorption/Ionization – Time-of-Flight (III)

MALDI-TOF was used to determine LOH at the *MLH1* locus using two single nucleotide polymorphisms (SNP) (promoter G>A rs1800734 and exon 8 G>A rs1799977) and two mutations (Mut2, g>a at 454-1 splice acceptor of exon 6, and Mut3, T>G at nucleotide 320 in exon 4) in the *MLH1* gene. The assays were calibrated using DNA samples with known genotypes (determined by sequencing). Based on titration experiments using the PicoGreen assay (Molecular Probes), samples of 5 ng of DNA isolated from normal and tumor tissue of the patients were then subjected to the MassEXTEND assay (Sequenom Inc.). Amplification was performed with standard PCR conditions, primers are listed in publication III. The PCR product was purified from uncorporated dNTP's with shrimp alkaline phosphatase (SAP) (GE Healthcare) by incubating the samples at 37°C for 20 min. The SAP enzyme was heat inactivated for 5 min at 85°C.

After purification, the primer extension reaction was carried out with primers that matched the target sequence adjacent to the studied SNP. Each reaction

contained 0.58 units of Thermosequenase enzyme (GE healthcare) 1x termination mix (dNTPs/ddNTPs) and 5 µM of test specific MASSEXTEND primer (Metabion). After the primer extension cycles, the products were treated with ion exchange resin (Sequenom Inc.) to remove salts. Each reaction was then spotted to a Maldimatrix-containing Advanced SpectroCHIPS (Sequenom Inc.) and subjected to MALDI-TOF mass spectrometry. The SpectroCHIPS were analyzed by an Autoflex MassARRAY mass spectrometer (Bruker Daltonics). All samples were assayed at least in triplicate and two people performed the analysis independently. The degree of LOH was calculated as previously described. An example of a tumor sample showing LOH is illustrated in Figure 6.

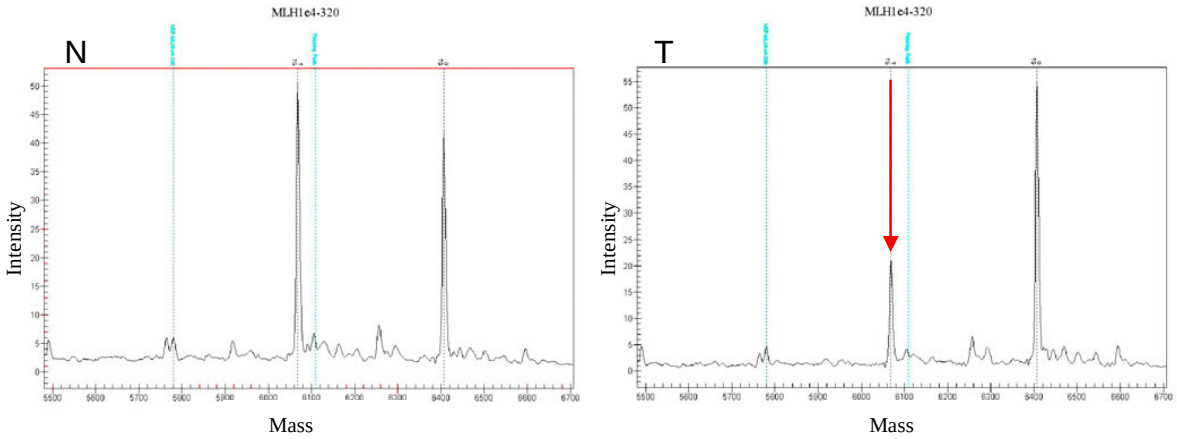

Figure 6 Loss of *MLH1* Mut3 (T>G at 320 in exon 4, MLH1e4-320) wild type allele. Arrow denotes loss of T allele in the tumor sample. N, normal sample; T, tumor sample of the same patient with CRC.

3.2 Single Nucleotide Primer Extension (III)

Single nucleotide primer extension (SNuPE) was used to verify the MALDI-TOF method of LOH detection, by analyzing the *MLH1* exon 8 polymorphism (G>A rs1799977). SNuPE has previously been used in our laboratory to quantify the relative expression of mRNA transcripts and the method is described in Renkonen *et al.* (2003). Genomic DNA was PCR amplified and used as a template for the primer extension reaction, where the primer extension continues until the terminator ddATP is incorporated into alleles having A at the polymorphic site. For alleles with a G at the polymorphic site the strand elongation continues until the next A is reached and ddATP is incorporated into the elongated fragment. The primer extension products were separated by capillary electrophoresis

(on ABI 3730 Automatic DNA sequencer, Applied Biosystems) and analyzed using Genemapper v4.0 (Applied Biosystems). Allelic dosages were determined from the peak areas of the fragments and their ratios in the tumor DNA relative to normal DNA were calculated as previously described.

3.3 Analysis of microsatellite markers to detect LOH (I-III)

Loss of heterozygosity can also be analyzed by studying microsatellite markers. We used microsatellite markers inside and around *MLH1* (D3S1612, D3S3512, D3S3718, D3S1611, D3S1298, and D3S3521) to study the width of LOH in study III. In studies I and II we used the Bethesda panel of five markers (BAT25, BAT26, D5S346, D2S123, and D17S250) to study LOH. In study II additional markers flanking *MLH1* (BAT21, D3S1611, and D3S1298), and *MSH2* and *MSH6* (D2S2378 and CA7) were used, and in study I another nine markers (D1S244, D1S228, D1S2667, D4S2962, D8S507, D9S167, D10S219, D13S263, and D18S474) were studied for LOH. Primers and PCR conditions for microsatellite markers are available at The GDB Human Genome Database (http://gdbwww.gdb.org/) and for BAT21, BAT25, and BAT26 at Alvino *et al.* (2002). As the forward primers were fluorescently labeled the PCR products were run on an automated DNA genotyper (ABI3730, Applied Biosystems) and the data was analyzed with the Genotyper 2.0 or with GeneMapper v3 software (Applied Biosystems). Calculations for LOH status were done as previously described.

4. Instability analyses

4.1 Detection of microsatellite instability (I-III)

Tumor samples were screened for MSI using the Bethesda panel of five microsatellite markers (BAT25, BAT26, D2S123, D5S346 and D17S250) as recommended by the International Workshop (Boland *et al.* 1998). All markers were run on an ABI3730 genotyper (Applied Biosystems) and analyzed by the Genotyper 2.0 or GeneMapper v3 software (Applied Biosystems).

4.2 Detection of chromosomal instability (I)

Comparative genomic hybridization (CGH) was used to detect chromosomal instability. Chromosomal CGH was performed according to a previously described method (el-Rifai *et al.* 1997) using a mixture of fluorescein-isothiocyanate (FITC)-conjugated dCTP and dUTP (Dupont) in tumor DNA labeling by nick translation. Reference DNA was conjugated with Texas Red (Dupont) and labeled as described for tumor DNA. The use of a fluorochrome-dCTP and -dUTP mixture in labeling produces fewer artifacts, yields higher quality results, and more representative labeling of the genome (el-Rifai *et al.* 1997). The results were analyzed by ISIS digital image analysis system (MetaSystems GmbH) with an integrated high-sensitivity monochrome charge-coupled device camera and automated CGH analysis software.

5. Methylation analyses

5.1 HpaII/MspI assay (II)

The *MLH1* promoter methylation status was analyzed by HpaII/MspI assay. This relies on the inability of HpaII to cut methylated CCGG sequences. Four HpaII target sites occur in the *MLH1* promoter region (-567, -527, -347, and -341) (Kuismanen *et al.* 1999). We investigated them in three separate reactions. Each sample was analyzed in three settings: (1) the sample was digested by HpaII to reveal methylation in the case the internal cytosine at the target site was methylated, (2) the sample was digested with MspI, which is insensitive to methylation but shares the digestion site with HpaII, to verify the presence of the target site, (3) undigested DNA was used as a positive control in the PCR. These three parallel analyses were then PCR amplified and ran on an agarose gel to detect for methylation. If the studied CCGG sequence was methylated a band was present in the HpaII treated sample and in the undigested sample while no band was present in the MspI treated sample. Detailed protocols and PCR primers are described in Kuismanen *et al.* (1999).

5.2 Methylation specific MLPA (III)

Methylation of the *MLH1* promoter was studied as a second hit by a methylation specific (MS) -MLPA method using the SALSA MS-MLPA ME001 kit (MRC Holland). The kit contains probe pairs for 24 tumor suppressor genes including two probes for the *MLH1* gene and 15 control probes lacking the HhaI site. The MS-MLPA method relies on the inability of the HhaI restriction enzyme to digest methylated target sequences, producing a signal for methylation after PCR amplification. If the target site is unmethylated, the genomic DNA/MS-MLPA probe complex will be digested, which prevents PCR amplification, and no signal will be generated. All reactions were carried out according to the manufacturer's instructions. Normal DNA specimens derived from the lymphocytes of healthy controls were included in every assay. For each MLPA reaction 100 ng of paraffin-derived DNA was denatured and subsequently hybridized with the MLPA probes. After hybridization, the hybrids were divided into two halves, one for ligation reaction and the other one for both ligation and digestion reactions. Ligase-65 enzyme was used to ligate the annealed probe pairs and HhaI restriction enzyme was used to digest (unmethylated) ligation products. The rest of the analysis was done as previously described in section 2.3. Figure 7 illustrates a tumor sample showing methylation at *MLH1* locus. Dosage ratio was obtained by a following calculation:

$$D_m = (P_x/P_{ctrl})_{Dig} \,/\, (P_x/P_{ctrl})_{Undig}$$

where D_m is the methylation dosage ratio, P_x is the peak area of a given probe, P_{ctrl} is the sum of all peak areas of control probes. Dig denotes HhaI digested sample and Undig undigested sample. Based on our titration experiments with cell lines known to have full methylation (RKO) or a complete lack of methylation of *MLH1* (HCT116), as well as on studies in which we correlated the methylation level and MLH1 protein expression by immunohistochemical analysis, a dosage ratio of 0.10 or higher at the CpG island adjacent to the translation start site of *MLH1* (corresponding to 10% of methylated DNA) was regarded to indicate promoter methylation.

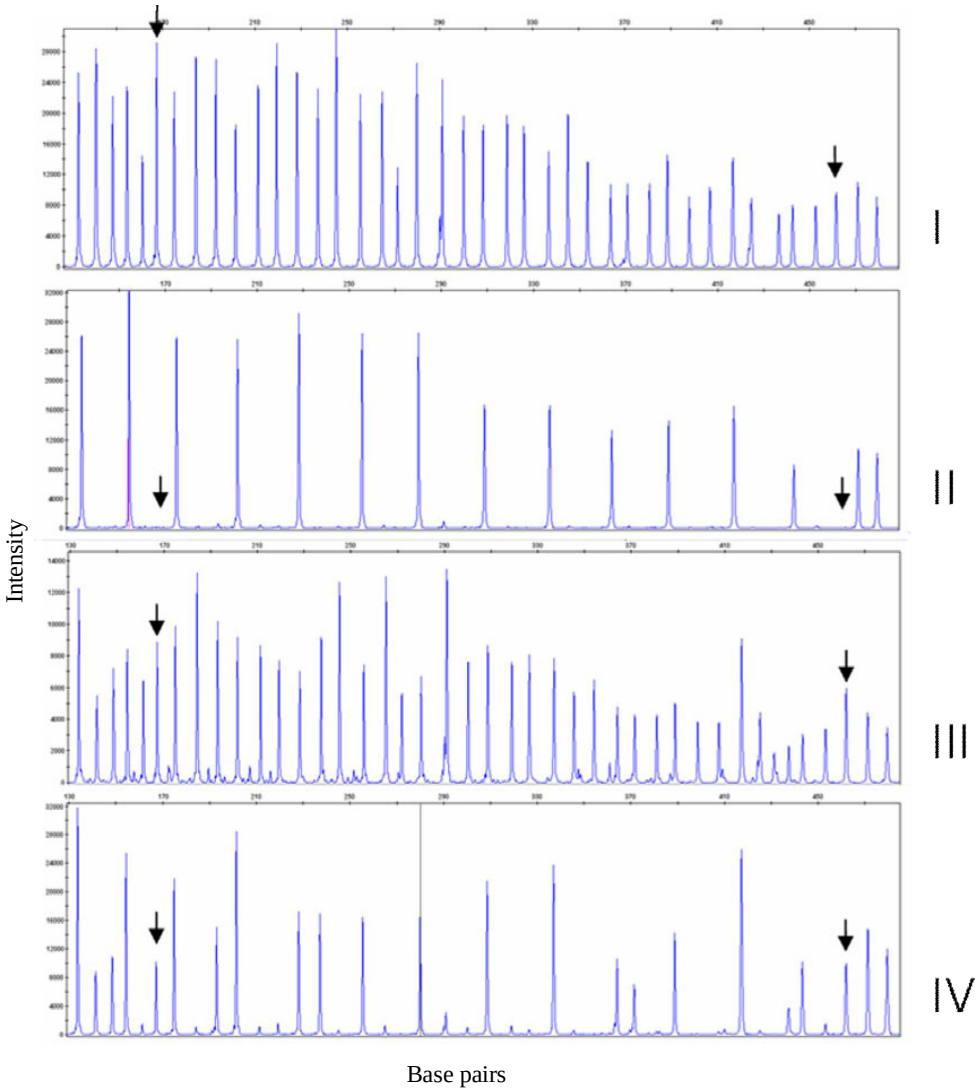

Figure 7 Methylation of *MLH1* promoter detected by MS-MLPA. The chromatogram shows altogether 41 peaks of which, 26 peaks denote methylation sites in 24 tumor suppressor genes and an additional 15 peaks control genes. Control genes always produce a peak after PCR and target genes only when methylated. I, undigested normal sample; II HhaI digested normal sample; III, undigested tumor sample; IV, HhaI digested tumor sample showing methylation at *MLH1* promoter and some other sites. Arrows denote *MLH1* gene.

6. Protein expression analysis

6.1 Immunohistochemical analysis (I, II, IV)

Protein expression was studied using immunohistochemistry on 3-5µm thick formalin-fixed paraffin-embedded tissue sections mounted on silanized slides (Dako). Most of the slides included tumor and corresponding normal tissues from the same section. The normal tissue was used as an internal reference for the evaluation of the staining results. Dewaxing and rehydration was performed with xylene and EtOH series. The antigen-retrieval step was performed by microwave boiling as shown in Table 5. After cooling the slides were washed in Tris-buffered saline, pH 7.2, and the subsequent steps were performed using the EnVision+ System-HRP (DAB) (Dako) according to manufacturer's instructions. In addition, prior to primary antibody incubation, the slides were incubated with 10% nonimmune goat serum (Dako) for 30 min. The mouse primary antibodies are presented in Table 5. Nonimmune mouse IgG1 (Dako) was used as a negative control for mouse primary antibodies. Finally, Mayer's Hematoxylin was used for counterstaining.

Table 5. Antibodies and antigen retrieval step used in immunohistochemistry

Protein	Primary antibody	Buffer	Microwave treatment
MLH1	anti-MLH1 clone G168-15; Pharmingen	EDTA buffer, pH 8.0	10 min at 750W
MSH2	anti-MSH2 clone FE-11; Calbiochem/Oncogene Research	EDTA buffer, pH 8.0	10 min at 750W
MSH6	anti-MSH6 clone 44; Transduction Laboratories	EDTA buffer, pH 8.0	10 min at 750W
PTEN	anti-PTEN clone 6H2.1; Cascade Biosciences	10mM citrate buffer pH 6.0	20 min at 750W
β-catenin	anti-β-catenin clone 14; BD Transduction Laboratories	10mM citrate buffer pH 6.0	5 min at 750W followed by 5 min at 450W
CDX2	anti-CDX2 clone CDX2-88; BioGenex	10mM citrate buffer pH 6.0	5 min at 750W followed by 5 min at 450W
p53	anti-p53 clone DO7; DakoCytomation	10mM citrate buffer pH 6.0	5 min at 750W followed by 5 min at 450W

7. Statistical analyses

Statistical significance for differences between study groups was evaluated with the Fisher's exact test, χ^2, or *t*-test (VassarStats at http://faculty.vassar.edu/lowry/VassarStats.html), as appropriate. All reported p-values were two-sided and values below 0.05 were interpreted as statistically significant.

RESULTS

1. Molecular classification of familial CRC (I, IV)

This study was based on our previous observation that 58% of HNPCC families that meet the diagnostic criteria for HNPCC but lack MMR gene germline mutation (Holmberg *et al.* 1998; Huang *et al.* 2001; Liu *et al.* 2001; Nystrom-Lahti *et al.* 1996) still remain MMR gene mutation negative after comprehensive mutation screening by advanced methods (Renkonen *et al.* 2003). We studied the molecular background of HNPCC mutation positive and negative CRCs to find out if molecular differences exist between these two groups.

1.1 HNPCC mutation positive CRC

All 48 HNPCC colorectal tumors with predisposing MMR gene germline mutations showed loss of the respective protein by immunohistochemistry and most of the tumors had MSI-high phenotype (one MSI-low, one MSS). The Wnt signaling pathway was studied in 31 tumors and it was deregulated due to aberrant β-catenin in 25/31 (81%) (Kariola *et al.* 2005), of which 9/25 (36%) had an activating mutation in *CTNNB1* exon 3. Immunohistochemical studies on CDX2 revealed complete or partial expression loss in 2/31 (6%) tumors, while normal colonic epithelia showed strong nuclear staining. We found no mutations in *CDX2*. Stabilization of the p53 protein was observed with immunohistochemistry in only 4/31 (13%) tumors and it was found to be due to a pathogenic mutation in *TP53*. The RAS and PI3K/AKT pathways were studied in all 48 HNPCC associated CRCs. RAS pathway members *KRAS* and *BRAF* were sequenced for mutations in known hot spots. Altogether 15/48 (31%) tumors showed *KRAS* mutations that were distributed equally in codons 12 and 13. We found no *BRAF* mutations. The PI3K/AKT pathway was altered in 27/48 (56%) tumors. The key regulator of the PI3K/AKT pathway, *PIK3CA*, showed alterations in 8/48 (17%) tumors, of which five were mutations and four amplifications (one tumor had both alterations). In the same tumors PTEN, the counter-effector for PIK3CA, showed decreased or lost expression in 16/44 (36%). *PIK3CA* and PTEN alterations occurred simultaneously in three tumors.

1.2 HNPCC mutation negative CRC

All but two of the 24 colorectal tumors negative for MMR gene germline mutations were MSS and showed normal expression of MLH1, MSH2, and MSH6 by immunohistochemistry. Two tumors had unstable microsatellites and absent or weak MLH1 expression possibly due to detected *MLH1* promoter methylation. All immunohistochemical studies could be performed on 18 tumors, based on availability of tissue sections. Abnormal Wnt signaling was present in 7/18 (39%) tumors indicated by nuclear β-catenin, but in contrast to mutation positive HNPCC CRCs, no *CTNNB1* mutations were identified. Meanwhile, in 6/7 (86%) tumors the nuclear β-catenin was coupled with the lost APC expression. Furthermore, four of these tumors showed chromosomal loss of the *APC* locus by CGH or LOH analysis. Tumor specific CDX2 expression changes were observed in 2/18 (11%) MMR mutation negative tumors and none of the tumors had mutations in *CDX2* exon 3. Altogether 8/18 (44%) MMR gene mutation negative tumors showed p53 stabilization and among those, pathogenic mutations were identified in 5/8 (63%). Sequencing of *KRAS* and *BRAF* revealed mutations in both genes, although only one tumor harbored *BRAF* V600E mutation. All four *KRAS* mutations affected codon 12, in contrast with tumors harboring MMR gene mutations. The PI3K/AKT pathway was altered in 13/22 (59%) tumors. Only two tumors (10%) showed *PIK3CA* alterations, of which one was a mutation and the other one an amplification. PTEN, the repressor of PI3K/AKT pathway, was altered in 8/18 (44%) tumors. None of the tumors showed concomitant *PIK3CA* and PTEN alterations.

We observed certain molecular distinctions between MMR gene mutation positive and mutation negative CRCs. First, Wnt pathway was affected by an abnormal β-catenin far more frequently in MMR mutation positive tumors than mutation negative tumors (81% vs. 36%, p=0.005). Second, the frequency of p53 stabilization was significantly higher in the MMR gene mutation negative tumors compared to mutation positive tumors (p=0.04).

MMR gene mutation negative tumors could be further divided into two groups, according to CGH results. A chromosomally stable group (CIN-) consisted of 9/16 (56%) of all tumors studied and they all showed less than five (mean 1) chromosome arm gains or losses. The rest of the tumors were classified as the unstable group (CIN+) with chromosome arm gains or losses ranging from 7 to 18

(mean 10.6). The biggest molecular differences between CIN- and CIN+ groups were localization of β-catenin, in the frequency of altered PI3K/AKT pathway and LOH. Membranous β-catenin and alterations in PI3K/AKT pathway were associated with CIN- carcinomas (6/7 in CIN- group vs. 0/6 in CIN+ group, p=0.005), and LOH with CIN+ carcinomas (Table 6).

Table 6. Colorectal tumors classified according to MMR defect and CIN

	MMR gene mutation positive CRC	MMR gene mutation negative CRC*	
Genetic instability	MSI (n=48)	MSS/CIN- (n=9)	MSS/CIN+ (n=7)
Mean age at onset	45.2	53.7	58.6
Main location of CRC	right-sided	right-sided	left-sided
Localization of β-catenin	nuclear	membranous	nuclear
TP53 mutations	frequent	infrequent	frequent
LOH frequency**	ND	36%	48%
Altered PI3K/AKT pathway†	27/48 (56%)	5/7 (71%)	3/9 (33%)
Altered RAS pathway‡	15/48 (31%)	0/7 (0%)	2/9 (22%)

ND, not determined; *Including only CRCs with known CIN status; **Average degree of LOH per tumor when 11 markers were studied; †Mutation or amplification of *PIK3CA* and altered PTEN protein expression are included; ‡ Mutation in *BRAF* and *KRAS* are included.

2. Molecular classification of familial EC (II, IV)

Familial clustering of endometrial cancer may occur as a part of HNPCC with MMR gene germline mutations, or it may constitute a separate entity termed familial site-specific endometrial carcinoma. The criteria we used for the familial site-specific EC was that the EC needed to be present in at least one first-degree relative of the index patient and any associated clustering of other cancers non existent. We have molecularly characterized both HNPCC associated and non-associated ECs to clarify the genetic basis of familial endometrial cancers.

2.1 EC as a part of HNPCC tumor spectrum

MMR gene germline mutations were found in 11/519 (2%) consecutive EC patients diagnosed at the Department of Obstetrics and Gynecology, Helsinki

University Central Hospital in ten years time. Nine of these patients belonged to families with CRC and could therefore be diagnosed with HNPCC at the outset. Six of the patients with HNPCC associated EC carried germline mutation in *MLH1* and three in *MSH2*. Two additional EC patients, both from families with clustering of EC only, showed germline mutations in MMR genes. A truncating germline mutation in *MSH6* (3261insC) was identified in one family and a missense mutation in *MSH2* (D603N) was identified in another. Both of the families displayed clustering of EC only without any other cancers. Our findings demonstrate that EC may be the only manifestation of HNPCC, although HNPCC families presenting with EC only merely constitute a small fraction of families with site-specific EC.

These 11 ECs, with MMR gene germline mutations, were included into our existing HNPCC EC panel to make a total of 60. All showed loss of the respective protein by immunohistochemistry. In contrast to studied HNPCC associated CRCs in which all but two were MSI, 22/60 (37%) of the HNPCC associated ECs demonstrated MSS.

We studied the role of the PI3K/AKT pathway in EC as well as in CRC tumorigenesis. *PIK3CA* was altered in 10/60 (17%) of HNPCC associated ECs and 37/53 (70%) showed PTEN expression changes. Most (7/10, 70%) of the *PIK3CA* alterations were mutations in this group of ECs. *KRAS* was relatively seldom affected since only 6/60 (10%) of tumors showed *KRAS* mutation.

2.2 Familial site-specific EC

Most (20/22, 91%) of the families with familial site-specific EC could not be shown to be associated with MMR gene germline mutations. Still, immunohistochemical analyses of 29 available tumors revealed that the expression of MLH1 was lost in seven tumors, MSH2 coupled with MSH6 in two tumors, and MSH6 alone in three tumors. MSI status was determined for all 30 ECs and 4/30 (13%) displayed MSI-high, 5/30 (17%) MSI-low, and 21/30 (70%) MSS phenotype. Only 4/30 (13 %) of ECs displayed MSI-high and showed lost MMR protein expression. All five tumors with loss of MSH2 and/or MSH6 expression showed LOH or putative LOH at one of the flanking markers. Only 2/7 ECs with lost MLH1 expression

displayed LOH at the intragenic D3S1611 marker and 1/7 tumors showed *MLH1* promoter methylation.

The MMR gene mutation negative ECs showed *PIK3CA* alterations in 9/30 (30%) tumors. *PIK3CA* amplifications were the predominant alteration observed in *PIK3CA* among these ECs, occurring more frequently when compared to HNPCC associated ECs (7/29 vs. 3/57, p=0.015). Moreover, the degree of amplification was higher in the former vs. latter group of ECs (average copy number ratio 5.0 vs. 3.5). PTEN was expressed in an abnormal manner in half (14/28, 50%) of the studied tumors, and as in HNPCC associated ECs, *KRAS* was mutated in only 3/29 (10%) tumors.

Comparisons between HNPCC associated ECs and familial site-specific ECs are shown in Table 7. These two study groups can not be easily discriminated by any other alterations than MMR defects and *PIK3CA* amplifications.

Table 7. Comparison of different EC groups studied

	HNPCC associated EC (n=60)	Familial site-specific EC (n=30)
Mean age at onset	50	63
MMR gene germline mutation	present	absent
Altered MMR expression	60/60 (100%)	12/29 (41%)
Tumors with MSI	38/60 (63%)	4/30 (13%)
Alterations in *PIK3CA*	10*/60 (17%)	9**/30 (30%)
Alterations in PTEN	37/53 (70%)	14/28 (50%)
Alterations in *KRAS*	6/60 (10%)	3/29 (10%)

* 7 mutations, 3 amplifications; ** 2 mutations, 7 amplifications

3. Second hits in HNPCC associated CRC and EC (III)

To address the question of why different organs are differently susceptible to cancer, although equally predisposed, we studied the mechanism of wild type allele inactivation in 25 HNPCC associated CRCs and 32 HNPCC associated ECs from 37 families. The study cohort represented a larger series of carriers of the Finnish founder mutations in *MLH1* (Mut1, Mut2

and Mut3; Table 8). Haplotype conservation associated with each ancestral founder mutation, as well as the nucleotide substitutions defining Mut2 and Mut3 *per se*, allowed us to determine if LOH affected the wild type or the mutant allele in each case. The clinical features of individual *MLH1* founder mutations were different (Table 8).

Table 8. Clinical features of a larger cohort of affected individuals with Mut1, Mut2, or Mut3 (331 individuals) from which the study cohort (47 individuals) was assembled

	MLH1 Mut1	*MLH1* Mut2	*MLH1* Mut3	Total (Mut1-3)
Total no of individuals	247	63	21	331
with CRC	226	51	19	296
with EC	57	20	7	84
Average age at diagnosis of				
first cancer	44.7	47.1	47.2	45.3
any CRC	45.2	49.0	50.7	46.3
any EC	51.3	46.3	48.9	49.9
CRC to EC ratio	4.4	3.0	3.4	4.0
Proximal to distal ratio (CRC)	1.4	1.6	1.8	1.5

Mut1, 3.5kb genomic deletion affecting codons 578-632 of exon 16 and flanking intron sequences; Mut2, g>a at 454-1 splice acceptor of exon 6; Mut3, T>G at 320 in exon 4, I107R.

Loss of heterozygosity was present in 31/57 (54%) of all studied HNPCC tumors, and the wild type allele was deleted more often than the mutant allele (23/57 for wild type allele vs. 8/57 for mutant allele, p=0.003). *MLH1* promoter methylation occurred in 4/55 (7%) tumors, and the tumors with *MLH1* methylation also showed significantly more methylation in other tumor suppressor genes compared to those without *MLH1* methylation (average of 10/23 methylated loci vs. average of 2/23 methylated loci, p<0.0001).

Among CRCs with a predisposing mutation in *MLH1*, LOH affected 16/25 (64%) tumors. When different predisposing mutations were analyzed separately, LOH was present in all but one of the tumors among Mut1 carriers (10/11, 91%), but it was absent in 5/10 (50%) and 3/4 (75%) of the CRCs with Mut2 and Mut3. *MLH1* promoter methylation occurred in 4/23 (17%) CRCs. *MLH1* methylation was

absent from tumors with wild type allele LOH and only affected tumors without LOH or with mutant allele LOH (0/14 vs. 4/10, p=0.02). One CRC with both LOH and methylation had lost the mutant allele suggesting that methylation affected the wild type allele.

The presence of LOH in ECs was opposite to CRCs. Most of the observed LOH affected Mut3 and Mut2 carriers (4/5, 80% and 10/14, 71%), whereas LOH among Mut1 ECs was a rare event (1/13, 8%; 14/19 for Mut2 and Mut3 together vs. 1/13 for Mut1, p=0.0003). Furthermore, Mut2 and Mut3 ECs had lost their mutant allele relatively more frequently compared to Mut1 ECs. The total frequency of LOH observed in ECs was 15/32 (47%) and none of the ECs showed *MLH1* promoter methylation.

The major differences in second hits between HNPCC associated CRCs and ECs were as follows. The wild type allele LOH predominated in CRC irrespective of the predisposing *MLH1* mutation whereas wild type and mutant allele LOH were roughly equally common in EC. Among CRCs, the LOH status of *MLH1* did not correlate with the age at onset, however, ECs with wild type allele LOH were diagnosed at a significantly earlier age (mean 43.2 years) compared to ECs with no LOH (mean 53.6 years; p=0.003). For Mut1, LOH was significantly more frequent in CRC than EC (10/11 for CRC vs. 1/13 for EC, p>0.0001) whereas for Mut2 and Mut3 LOH was slightly more common in EC. Although *MLH1* promoter methylation rarely occurred, all four tumors displaying *MLH1* methylation were CRCs. The average number of methylated loci among Mut2 and Mut3 carriers was higher in CRC than EC (5.8 vs. 2.6, p=0.017) that is opposite to the observed LOH frequencies among the same tumors.

DISCUSSION

1. Molecular classification of familial CRC

The most common hereditary form of CRC is HNPCC, but up to half of the families meeting the diagnostic criteria for HNPCC fail to show any germline mutations in DNA MMR genes (Abdel-Rahman *et al.* 2006). HNPCC tumors with MMR defects represent the MSI pathway in tumorigenesis and are virtually all lacking gross chromosomal gains and losses (Aaltonen *et al.* 1993). On the contrary, sporadic MMR proficient (MSS) tumors show chromosomal instability as a major feature (Abdel-Rahman *et al.* 2001; Lengauer *et al.* 1997). It has been considered that these two instability pathways, MSI and CIN, are independent (Abdel-Rahman *et al.* 2001; Lengauer *et al.* 1997). Which pathways then lead to the HNPCC phenotype without a MMR defect?

We studied the role of five pathways (MMR, Wnt, p53, CIN, and PI3K/AKT) in MMR mutation positive and negative HNPCC tumors. While the Wnt pathway is reported to be active in the majority of CRCs, regardless of the MSI status (Huang *et al.* 1996; Morin *et al.* 1997; Rowan *et al.* 2000; Sparks *et al.* 1998), the MMR mutation negative tumors studied here showed inactivity of the Wnt signal pathway in a majority of tumors. The same tumors were also distinguished by the absence of chromosomal instability (CIN-) and *TP53* mutations. Moreover, *TP53* mutations were absent even in tumors with p53 stabilization, shown by immunohistochemistry, in this “stable” subset. These findings were significantly different from those observed in CRCs from (1) the MMR gene mutation positive families where the Wnt signaling pathway was activated in 81% of the tumors and p53 stabilization was due to *TP53* mutation in all cases and from (2) sporadic CRCs where the majority of tumors show an active Wnt pathway (Hao *et al.* 2002; Iwamoto *et al.* 2000) mutated *TP53* (Konishi *et al.* 1996; Leslie *et al.* 2003; Salahshor *et al.* 1999), and chromosomal instability (CIN+)(Leslie *et al.* 2003; Schlegel *et al.* 1995). Such a high occurrence (9/17, 56%) of stable carcinomas with no apparent changes in the four major pathways (MMR, Wnt, p53, and CIN) is unique. The only comparable report we could find showed only an occurrence of 3/50 (6%) (Leslie *et al.* 2003), the difference being highly significant (p=0.00008).

Parsons *et al.* (2005) found that nearly 40% of sporadic colorectal tumors had alterations in one of eight PI3K pathway genes. The role of the PI3K/AKT pathway in our MMR gene mutation positive and negative tumors was significant. Although we focused on only three of these genes, *PIK3CA, PTEN*, and *KRAS*, more than half of the tumors showed alterations. Our observation of a mutually exclusive relationship between *PIK3CA* mutations and amplifications in familial CRCs is consistent with studies on sporadic CRCs. Of *PIK3CA* alterations, amplifications and mutations were equally common in both of our CRC study groups. This finding is in contrast to published reports for sporadic cancers (Samuels *et al.* 2004; Velho *et al.* 2005) in which amplifications are rare or completely absent, providing a possible distinguishing feature between the familial tumors and their sporadic counterparts.

Our studies suggest that MMR gene mutation negative HNPCC families are not a single separate entity, but they can be divided into at least two distinct groups according to chromosomal stability, β-catenin localization, and *TP53* mutation frequency. In addition, the group forming a majority of the MMR mutation negative tumors (MSS/CIN-), have a tendency to affect the right colon and are diagnosed at earlier age.

Collectively, at least the MMR, Wnt, p53, PI3K/AKT, and CIN pathways are not the main ones giving rise to the MMR proficient HNPCC phenotype. Linkage studies (Huang *et al.* 2001; Lewis *et al.* 1996) suggest that there might be novel loci that could account for the unexplained fraction of HNPCC families with no detectable MMR gene mutations. Part of such families may be due to shared environment that has been shown to account for approximately 8% of familial cases (Lichtenstein *et al.* 2000). Interactions between genes and environment are also shown to be very important in cancer predisposition (Potter 1999). There are also studies on rare variants or low-penetrance alleles that are seen in increased frequencies in families with clustered CRC compared to control population. One example is *TGFBR1**6A variant. Although it is associated with a modest risk of CRC in Caucasians (de la Chapelle 2004) it is more prevalent in patients meeting Amsterdam criteria but lacking MMR gene mutation than in MMR gene mutation positive HNPCC patients (Bian *et al.* 2005). Some of the rare variants may not predispose to CRC when occurring alone, giving an explanation why they also are seen in healthy controls to some extent. When these rare variants aggregate they may have an important role in cancer susceptibility. Although our findings

provide new insights into the mechanisms of colorectal carcinogenesis, the nature of the predisposing gene(s) still remains unknown and further studies in larger series of CRC families are needed.

After our data was published (I), Johnson *et al.* (2005) published a study in which they did not find similar molecular associations as we reported. Nuclear β-catenin expression did not correlate with MMR gene mutations or with p53 expression. Their group of familial CRCs without molecular diagnosis of HNPCC was more heterogeneous and larger than our MMR gene germline mutation negative CRC group. Thus, the molecular definition and size of the study groups were different between Johnson *et al.* (2005) and us, which may account for the differences between these studies.

2. Molecular classification of familial EC

The genetic background of EC has not been studied as extensively as that of CRC. A few studies reporting two different genetic models that may play a role in the development of familial EC exist (Boltenberg *et al.* 1990; Gruber and Thompson 1996; Sandles *et al.* 1992). According to these models familial EC can be divided into two classes: one representing the HNPCC syndrome, which is associated with germline mutations in MMR genes and the other representing familial site-specific endometrial carcinoma, which genetic background is completely uncharacterized. We studied the role of MMR genes as the first step in the molecular dissection of familial site-specific EC.

In the study of 22 families with EC clustering only, around half (12/22) showed a hint of a MMR defect based on the lack of MMR expression by immunohistochemistry. In ten families, however, no germline mutation could be found by sequencing, direct mutation testing (for mutations common in the Finnish population not detectable by direct sequencing), or by MLPA (for large genomic deletions). In addition, 8/12 (67%) of the ECs from these ten families displayed stable microsatellites (MSS or MSI-low) despite showing altered MMR gene expression by immunohistochemistry. When analyzing the families more closely, we found that in four families the immunohistochemical changes were discrepant arguing against inherited MMR defect. The analyses of the remaining six families with expression changes were based on one individual only. Thus no

firm conclusions could be made, although we could speculate that these families are very unlikely to be associated with HNPCC since no mutations were found and only one of the tumors showed MSI. All five tumors with lost MSH2 and/or MSH6 and 2/7 tumors with loss of MLH1 expression showed LOH at the nearest markers. Altogether five of the tumors with lost MLH1 expression were later shown to be hypermethylated at the *MLH1* promoter (unpublished data), probably suppressing the gene expression, shown in immunohistochemistry. Altogether 2/12 families with MMR expression changes were found to carry a predisposing germline mutation in the respective genes, *MSH2* in Family 13 and *MSH6* in Family 15. These two families represent 9% of all 22 families studied, selected by the presence of EC in at least one first-degree relative of the index patient (in the absence of other cancers). Thus, the majority of families with site-specific EC may be due to hereditary defects in other genes, nongenetic factors, or chance.

Our study may give an underestimation of MMR mutations in the above mentioned families, since all tumors were not tested for mutations, but immunohistochemistry was used to guide mutation analyses. Based on our own studies and the experience of others (Berends *et al.* 2001; Berends *et al.* 2003; de Leeuw *et al.* 2000; Schweizer *et al.* 2001), however, germline mutations constantly lead to abnormal expression of the respective protein which can be observed with immunohistochemistry. In contrast to what is observed in CRC, the correlation between MMR gene expression and MSI was incomplete, since only one third (4/12, 33%) of the studied ECs with observed MMR protein loss showed MSI. This could be explained possibly by the earlier detection of EC compared to CRC, when the MSI pattern may not be well developed and by the observation that MMR deficient ECs have a lower mutational rate than MMR deficient CRCs (Duval *et al.* 2002). The panel of microsatellite markers used here and recommended by the National Cancer Institute may not be optimal for ECs, since the panel was originally developed for CRCs. Therefore MSI detected by the Bethesda panel markers might not be a definite indicator of a MMR defect in ECs. Other reasons for the incomplete correlation by MMR protein expression and MSI may include normal tissue contamination and clonal heterogeneity.

We studied HNPCC associated EC and familial site-specific EC for alterations in the PI3K/AKT pathway members *PIK3CA* and PTEN, and in *KRAS* that may modulate the pathway. The pathway was affected in comparable proportions in

both EC groups studied (43/60 [72%] for HNPCC associated ECs vs. 20/30 [67%] for familial site-specific ECs) and the mutation frequencies were analogous to that reported for sporadic ECs (Hayes *et al.* 2006; Muller *et al.* 2007; Oda *et al.* 2005; Velasco *et al.* 2006). In contrast, amplifications were characteristic of familial site-specific ECs without a MMR gene germline mutation, being more frequent in such ECs compared to HNPCC associated ECs (7/24 vs. 3/57, p=0.015). Moreover, when a recent publication on sporadic ECs showed amplifications in 1/9 (11%) tumors with poor differentiation (Oda *et al.* 2005), the amplifications that we observed occurred in well differentiated and local ECs with endometrioid histology – features that generally correlate with a better prognosis. Notably, all but one of the tumors with high-level (more than 4-fold) amplification were MSS, supporting the idea of amplifications being a manifestation of CIN rather than a manifestation of MSI (Lengauer *et al.* 1998).

Apart from the differences in the MMR defect and *PIK3CA* amplifications HNPCC associated ECs and familial site-specific ECs seem broadly comparable regarding their molecular characteristics. This is not surprising since the tumors from these two groups show also minor clinical diversity. In addition, very slight differences are found even when HNPCC associated ECs are compared to sporadic ECs (Rijcken *et al.* 2006).

The existence of familial site-specific EC as a separate entity still remains equivocal, until the predisposing gene (or genes) is found. It is possible that familial site-specific EC is not due to one highly penetrant susceptibility gene, however, but it might result from inherited polymorphic alleles that may act alone or in different combinations. Further studies are clearly needed for the full molecular characterization of this proposed EC entity.

3. Second hits and selective organ susceptibility in HNPCC

Selective organ susceptibility in HNPCC is poorly understood. MMR genes are ubiquitously expressed and their function in DNA repair is important in all tissues. Still mutations in MMR genes lead to a selective development of tumors in particular tissues. In HNPCC a wide spectrum of cancers is found. The diagnostic criteria for HNPCC only list certain tumors (colorectal, endometrial, small bowel, ureter, and renal pelvis) to be a part of the HNPCC tumor spectrum.

Gastric, ovarian, hepatobiliary tract, and brain tumors also occur in excess in HNPCC (Aarnio *et al.* 1999; Sijmons *et al.* 1998; Vasen *et al.* 1996; Watson and Lynch 1993). In addition, cancer of the breast and prostate may belong to the HNPCC tumor spectrum (Scott *et al.* 2001; Soravia *et al.* 2003; Vasen *et al.* 2001).

Although in general a poor correlation between the type of germline mutation and clinical phenotype in HNPCC exists (Liu *et al.* 1996; Peltomaki *et al.* 2001), in some cases the predisposing mutation may play an important role in tissue selection. *MSH2* mutation carriers seem to have a higher risk of developing cancer of the urinary tract, ovaries, stomach, and brain than *MLH1* mutation carriers (Vasen *et al.* 2001). It has also been shown that *MSH6* mutations are associated with families with a higher incidence of EC (Wijnen *et al.* 1999). Moreover, families with different *MLH1* mutations show different CRC to EC ratios according to the predisposing mutation (Table 8).

In addition to the effect of inherited susceptibility, accumulation of mutations within tumorigenesis may have an effect on organ selection. A MMR defect leads to accumulation of small insertion/deletion mutations in repeat tracts, which occur, in addition to non-coding microsatellites, in coding or promoter regulatory sequences of certain genes. These target genes very likely contribute to the tissue specificity according to their expression pattern and functional importance in different cell types. The spectrum of microsatellite mutator pathway target genes in CRC vs. EC is to some extent overlapping, but for example *TGFβRII* and *TCF-4* genes are strongly associated with CRC (Abdel-Rahman *et al.* 1999; Duval *et al.* 1999; Markowitz *et al.* 1995), and *PTEN* with EC (Kuismanen *et al.* 2002; Zhou *et al.* 2002).

The proliferation rate of a tissue is also a very important factor in selective organ susceptibility in HNPCC. Moreover, the rate of change of cell proliferation may have an important effect (Frank 2004). In the endometrium the monthly cycle of rapid proliferation followed by rapid apoptosis and regression offers a good environment for mutation accumulation in MMR deficient cells. Epithelial cells of the colorectum have also been characterized with variable changes in the proliferation rate (Lipkin 1973). In the tissues with rapid changes in cell proliferation rates genes critical for terminating the cell cycle and initiating apoptosis would be more frequently mutated than in tissues with a steady proliferation rate. Thus, the endometrium and colorectum have a selection

advantage for tumor formation, but what determines the differences between EC and CRC susceptibility?

MMR genes mainly act as tumor suppressor genes, requiring two hits (one in each allele) before tumor initiation. In HNPCC the first hit is inherited and the second hit is somatic (Hemminki *et al.* 1994). We focused on the differential susceptibility to HNPCC associated CRCs and ECs by studying the role of LOH and *MLH1* methylation as second hits required for tumor initiation. Our study agreed nicely with Knudson's two hit theory. LOH more frequently affected the wild type allele than the mutant allele (p=0.003), and it was inversely associated with *MLH1* promoter methylation. Moreover, the only tumor showing both LOH at *MLH1* locus and *MLH1* methylation had lost the mutant allele, suggesting that methylation affected the wild type allele.

The classical two hit model may, however, not always be fully applicable. For example, elevated MSI levels have been detected in subjects heterozygous for a germline mutation, suggesting haploinsufficiency of MMR in cancer initiation (Alazzouzi *et al.* 2005). In the case of haploinsufficiency, loss of one allele of a tumor suppressor is sufficient to accelerate tumorigenesis. Haploinsufficiency can be partial or complete and it may vary according to tissue type. This might be the case in our HNPCC series, in which LOH and methylation together served as a second hit more frequently in CRCs compared to ECs (p=0.001). Haploinsufficiency in EC could also explain why the wild type allele LOH predominated in CRC irrespective of the predisposing mutation, while the wild type and mutant allele LOH were roughly equally common in ECs from Mut2 and Mut3 carriers. It has also been shown for other genes that site and type of predisposing germline mutation may affect phenotypic features (Albuquerque *et al.* 2002). According to our LOH analyses, a predisposing germline mutation might have some effect on the dosage sensitivity. In tumors with Mut1, LOH affected mainly CRC, with only one EC showing LOH (p<0.0001), whereas among tumors with Mut2 and Mut3 LOH was more common in ECs. We did not address somatic mutations as second hits, but their role would probably be very modest. Yuen *et al.* (2002) and Potocnik *et al.* (2001) observed very few *MLH1* somatic mutations in tumors with a *MLH1* germline mutation.

In summary, our findings of distinctive frequencies of second hits (LOH and *MLH1* methylation) in CRC vs. EC suggest different dosage requirements of *MLH1* in these two tissues. Moreover, the predisposing germline mutation may modify the dosage sensitivity. Taken together, the patterns of LOH and methylation of tumor suppressor genes are dependent on tissue type and germline mutation, and may in part explain the differential tumor susceptibility of different organs in HNPCC.

CONCLUSIONS AND FUTURE PROSPECTS

This PhD project aimed to molecularly characterize familial colorectal and endometrial cancers, in both MMR deficient and proficient tumors. The main findings may be summarized and concluded as follows:

MMR proficient familial CRC consists of two molecularly distinct groups that differ from MMR deficient tumors. Group A shows paucity of common molecular and chromosomal alterations characteristic of colorectal carcinogenesis. Group B shows molecular features similar to classical microsatellite stable tumors with gross chromosomal alterations. Our finding of a unique tumor profile in group A suggests the involvement of novel predisposing genes and pathways in colorectal cancer cohorts not linked to MMR gene defects. Further studies in larger series of families are needed to define the nature of predisposing genes that remained unknown.

Endometrial cancer may be the only manifestation of HNPCC. Among 22 families with clustering of EC, two were due to MMR gene germline mutations. The remaining familial site-specific ECs are largely comparable with HNPCC associated ECs, the main difference between these groups being MMR proficiency vs. deficiency. The existence of site-specific endometrial carcinoma as a separate entity remains equivocal until predisposing genes are identified. It is possible that no single highly penetrant gene for this proposed syndrome exists. It may, however, be due to a combination of multiple low penetrance genes. Candidates for familial site-specific EC susceptibility genes may be searched for by genetic linkage or association based approaches or, alternatively, by tumor-based studies such as we used here for familial MMR proficient CRCs.

A more refined molecular classification of cancers can be obtained by using microarrays to perform genome-wide expression profiling, as shown for various cancers (Boussioutas *et al.* 2003; Schaner *et al.* 2003; Sorlie *et al.* 2001; van 't Veer *et al.* 2002). In regard to CRC, tumors with different MSI phenotype have been shown to express different gene subsets by microarray analysis (Mori *et al.* 2004). Microarray data has also been used to identify clinically relevant tumor subgroups (Bertucci *et al.* 2004). These analyses may provide a useful tool for defining new genes and pathways in the subset of familial CRCs with stable microsatellites and chromosomes as well as in the familial site-specific EC. In

addition, while providing improved classification of CRC and EC, a more comprehensive molecular understanding of carcinogenesis may one day facilitate individualized treatment of CRC and EC.

Important determinants of the HNPCC tumor spectrum are, in addition to different predisposing germline mutations, organ specific target genes and different instability profiles, loss of heterozygosity at *MLH1* locus, and *MLH1* promoter methylation. It will be interesting to see if, besides CRC and EC, the mechanisms of somatic inactivation also differ between the various other tumors that are overrepresented in HNPCC.

In conclusion, this study provided more precise molecular classification of families with clustered CRC and EC that is highly relevant for the proper genetic counseling of the affected patients and their families. Part of the familial accumulation of EC is due to a germline mutation in a MMR gene and these families need to be counseled similar to other HNPCC families. In the case of ECs the testing for MSI is not as informative predictor of a MMR gene mutation as it is in CRCs where practically all tumors with MMR deficiency show unstable microsatellites. Among familial ECs, tumors with stable microsatellites may as well harbor a MMR gene germline mutation. The families with accumulated MMR proficient CRCs also need surveillance since individuals in such families are at increased risk of developing CRC, although the genetic predisposition still remained unknown. Our observations on familial CRC and EC are likely to have broader significance that extends to sporadic CRC and EC as well.

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