Molecular mechanisms of cancer predisposition in HNPCC/Lynch syndrome

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ACADEMIC DISSERTATION

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Yliopistopaino
Helsinki 2008
To my husband and our daughter
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ABBREVIATIONS

ABL  Abelson murine leukemia viral oncogene homolog 1
ACVR2 activin type 2 receptor
APC  adenomatous polyposis coli
BCR  breakpoint cluster region
BLM  Bloom syndrome gene
BRAF  v-raf murine sarcoma viral oncogene homolog B1
CD44  cell-surface glycoprotein (indian blood group)
CDKN2A  cyclin-dependent kinase inhibitor 2A
CIMP  CpG island methylator phenotype
CIN  chromosomal instability
CTNNB1  β-catenin gene
DCC  deleted in colorectal cancer
ESR1  estrogen receptor alpha
FAP  familial adenomatous polyposis
GSTP1  glutathione S-transferase pi
HNPCC  hereditary nonpolyposis colorectal cancer
InSiGHT  International Society for gastrointestinal Hereditary Tumors
IGFIIHR  insulin-like growth factor 2 receptor
KRAS  Kirsten rat sarcoma viral oncogene homolog
LOH  loss of heterozygosity
MBD4  methyl-CpG binding domain protein 4
MINT  methylated in tumors 1
<table>
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<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>MGMT</td>
<td>O-6-methylguanine-DNA methyltransferase</td>
</tr>
<tr>
<td>MLH1, 3</td>
<td>human mutL homolog 1, 3</td>
</tr>
<tr>
<td>MLPA</td>
<td>multiplex ligation-dependent probe amplification</td>
</tr>
<tr>
<td>MMR</td>
<td>mismatch repair</td>
</tr>
<tr>
<td>MRE11A</td>
<td>meiotic recombination 11 homolog A</td>
</tr>
<tr>
<td>MSH2, 3, 6</td>
<td>human mutS homolog 2, 3, 6</td>
</tr>
<tr>
<td>MSI(-H)</td>
<td>microsatellite instability (-high)</td>
</tr>
<tr>
<td>MS-MLPA</td>
<td>methylation specific MLPA</td>
</tr>
<tr>
<td>MSP</td>
<td>methylation specific sequencing</td>
</tr>
<tr>
<td>MSS</td>
<td>microsatellite stable</td>
</tr>
<tr>
<td>MYH</td>
<td>mutY homolog (E. coli)</td>
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<tr>
<td>PTEN</td>
<td>phosphate and tensin homolog</td>
</tr>
<tr>
<td>RASSF1</td>
<td>ras association domain family 1</td>
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<tr>
<td>Rb1</td>
<td>Retinoblastoma 1</td>
</tr>
<tr>
<td>RET</td>
<td>RET proto-oncogene, “rearranged during transfection”</td>
</tr>
<tr>
<td>SNuPE</td>
<td>single nucleotide primer extension</td>
</tr>
<tr>
<td>SP-PCR</td>
<td>small-pool PCR</td>
</tr>
<tr>
<td>SSCP</td>
<td>single strand conformation polymorphism</td>
</tr>
<tr>
<td>TGFβRII</td>
<td>transforming growth factor receptor II</td>
</tr>
<tr>
<td>THBS1</td>
<td>thrombospondin 1</td>
</tr>
<tr>
<td>TIMP3</td>
<td>metalloproteinase inhibitor 3</td>
</tr>
<tr>
<td>TP53</td>
<td>tumor protein 53</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel-Lindau gene</td>
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<tr>
<td>Wnt</td>
<td>wingless-type</td>
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</table>
LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following publications:


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

Hereditary non-polyposis colorectal carcinoma (HNPCC; Lynch syndrome) is among the most common hereditary cancers in man and a model of cancers arising through deficient DNA mismatch repair (MMR). It is inherited in a dominant manner with predisposing germline mutations in the MMR genes, mainly \textit{MLH1}, \textit{MSH2}, \textit{MSH6} and \textit{PMS2}. Both copies of the MMR gene need to be inactivated for cancer development. Since Lynch syndrome family members are born with one defective copy of one of the MMR genes in their germline, they only need to acquire a so called “second hit” to inactivate the MMR gene. Hence, they usually develop cancer at an early age. MMR gene inactivation leads to accumulation of mutations particularly in short repeat tracts, known as microsatellites, causing microsatellite instability (MSI). MSI is the hallmark of Lynch syndrome tumors, but is present in approximately 15% of sporadic tumors as well.

There are several possible mechanisms of somatic inactivation (i.e. the “second hit”) of MMR genes, for instance deletion of the wild-type copy, leading to 'loss of heterozygosity’ (LOH), methylation of promoter regions necessary for gene transcription, or mitotic recombination or gene conversion. In the Lynch syndrome tumors carrying germline mutations in the MMR gene, LOH was found to be the most frequent mechanism of somatic inactivation in the present study. We also studied \textit{MLH1/MSH2} deletion carriers and found that somatic mutations identical to the ones in the germline occurred frequently in colorectal cancers and were also present in extracolonic Lynch syndrome-associated tumors. Chromosome-specific marker analysis implied that loss of the wild-type allele predominantly occurs through locus-restricted recombinational events, i.e., gene conversion, rather than mitotic recombination or deletion of the respective gene locus.

Lynch syndrome patients are predisposed to certain types of cancers, the most common being colorectal and endometrial cancer. Gastric cancer is the second most common extracolonic malignancy in individuals with Lynch syndrome but is also relatively common in the general population. Therefore, our aim was to study whether or not gastric cancer is a true Lynch syndrome spectrum malignancy. The molecular and clinicopathological profiles of gastric cancers from Lynch syndrome mutation carriers
were evaluated and compared with the profiles of sporadic gastric cancers. We found that gastric cancers from Lynch syndrome mutation carriers resembled sporadic intestinal MSI gastric cancers, except that \textit{MLH1} promoter methylation was absent and the general methylation index was lower, suggesting similar, but not identical, developmental pathways. All of these lacked the mismatch repair protein corresponding to the germline mutation and displayed high MSI. The present molecular evidence, combined with the previous demonstration of an increased incidence relative to the general population, justify considering gastric cancers as true Lynch syndrome spectrum malignancies.

Currently, it is not clear why certain tissues are more susceptible to cancer in Lynch syndrome than others. We addressed this issue by determining the molecular profiles for different tumors from a nationwide cohort of Lynch syndrome families. Here we focused on some less prevalent cancers, affecting the brain and urinary tract, and compared their molecular characteristics to those of the most common cancers, colorectal, gastric and endometrial adenocarcinomas, from the same families. Despite origin from verified MMR gene mutation carriers, the frequency of high-level microsatellite instability in tumors varied between high (100-96% for ureter, stomach and colon), intermediate (63-60% for endometrium and bladder) and low (25-0% for kidney and brain). Our results implied that different Lynch syndrome tumors develop along different routes. Uroepithelial cancers of the ureter (and bladder to lesser extent) share many characteristics of MMR deficiency-driven tumorigenesis, whereas brain tumors and kidney adenocarcinomas follow separate pathways.

In about one-third of families suspected of Lynch syndrome, mutations in MMR genes are not found. Therefore, we studied large genomic rearrangements and constitutive promoter hypermethylation as explanations for families/cases lacking germline mutations in MMR genes by conventional screening but which displayed loss of MLH1, MSH2 or MSH6 expression in tumor tissue. According to our results, large genomic deletions, mainly in \textit{MSH2}, and germline epimutations in \textit{MLH1}, together explain a significant fraction of point mutation-negative families suspected of Lynch syndrome and are associated with characteristic clinical and family features. Our findings have important implications in the diagnosis and management of Lynch syndrome families.
REVIEW OF THE LITERATURE

1 Cancer

Every fourth person in Finland will be affected by cancer at some point of their life, and the risk of cancer increases with age. Annually approximately 24000 persons are diagnosed with cancer, of which half will be cured. The prognosis varies depending on the type and the stage of the cancer. In Finland, there are approximately 170000 people who are living with cancer (The Finnish Cancer Registry, updated May 2008). However, since both cancer diagnostics and treatments are continuously improving, more than half of all cancer patients can be cured today (Finnish cancer organizations, May 2008).

Cancer is a disease with increased incidence in countries with higher life expectancy, since cancer develops over a long time and affects mainly elderly people. In Finland, the most prevalent cancers in males are prostate, lung and bronchial, colon, and urinary bladder. In females, breast cancer is by far the most common cancer type, followed by cancer of colon, endometrium, and lungs (The Finnish Cancer Registry, updated May 2008).

The human body is composed of approximately one hundred trillion cells ($10^{14}$), thus maintenance of cell homeostasis is crucial, and failure of this regulation may lead to the formation of tumors. Cancer is not a single disease but rather a collective name for a group of diseases that arise from cells with uncontrolled growth, acquired immortality and invading and metastasizing capacity (Hanahan and Weinberg, 2000). Primary cancer is according to the tissue it originates from, for instance colorectal cancer affects large intestine and rectum, gastric cancer originates from the stomach, and endometrial cancer originates from the endometrium. Similarly, classification of cancer is based on the tissue from which it originates; carcinomas are derived from epithelia; sarcomas from connective and muscle tissue; and leukemia and lymphoma from blood and lymphatic tissue (Weinberg, 2007) (Table I).
Table 1: Classification of solid tumors according to tissue of origin (modified from Syöpäjärjestöt, Tietoa Syövästä, www.cancer.fi)

<table>
<thead>
<tr>
<th>Type of tissue</th>
<th>Benign</th>
<th>Malignant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epithelial tissue</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lining epithelium</td>
<td>papilloma</td>
<td>carcinoma</td>
</tr>
<tr>
<td>glands &amp; lobules</td>
<td>adenoma</td>
<td>adenocarcinoma</td>
</tr>
<tr>
<td><strong>Connective tissue</strong> &amp; muscle tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dense connective tissue</td>
<td>fibroma</td>
<td>fibrosarcoma</td>
</tr>
<tr>
<td>cartilage</td>
<td>chondroma</td>
<td>chondrosarcoma</td>
</tr>
<tr>
<td>bone</td>
<td>osteoma</td>
<td>osteosarcoma</td>
</tr>
<tr>
<td>smooth muscle</td>
<td>leiomyoma</td>
<td>leiomyosarcoma</td>
</tr>
<tr>
<td>skeletal muscle</td>
<td>rhabdomyoma</td>
<td>rhabdomyosarcoma</td>
</tr>
<tr>
<td><strong>Neuronal tissue</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glial tissue</td>
<td>glioma</td>
<td>glioblastoma</td>
</tr>
<tr>
<td>meninges</td>
<td>meningioma</td>
<td>meningial sarcoma</td>
</tr>
</tbody>
</table>

Cells in different tissues contain the same genetic material that orchestrates cell growth, proliferation, differentiation, as well as cell death. The process of tumorigenesis is proposed to include six essential alterations in cell physiology, including self-sufficiency in growth signals, insensitivity to growth inhibiting signals, evasion of apoptosis, unlimited potential to replicate, continual angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). Most of these changes occur through changes in the genome, and therefore, cancer is considered a genetic disease. In spite of being a genetic disease, a great majority of cancers are not hereditary.

Numerous environmental factors have been associated with development of cancer, for instance a clear association has been observed between carcinogens from tobacco smoking and lung cancer (Brennan et al., 2006). Most cancers arise as a result of both genetic and environmental factors (Potter, 1999).
2 Cancer Genetics

2.1 Oncogenes and tumor suppressors

Cancer critical genes can be grouped according to their normal function in a cell. Proto-oncogenes are genes that have a role in for example regulation of cellular growth, proliferation and differentiation. Proto-oncogenes may be activated and become oncogenes through point mutation, amplification or translocation. Oncogenes are dominantly inherited, as only one mutation is sufficient cause altered cell function; however, oncogenes are rarely the cause of inherited cancer susceptibilities (Aittomaki and Peltomaki, 2006).

Tumor suppressors, on the other hand, are genes that when inactivated no longer inhibit cell proliferation in the case of, for instance, DNA damage, and thus give the tumor a growth advantage. Knudson observed that tumor suppressor genes are recessive genes requiring “two hits” (both alleles to become inactivated) for cancer development. Tumor suppressors have been described in several hereditary forms of cancer, the “first hit” being inherited and the “second hit” occurring in somatic cells (Knudson, 1996) (Figure 1).

Tumor suppressor genes can further be divided into gatekeepers, caretakers, and landscapers (Kinzler and Vogelstein, 1997; Kinzler and Vogelstein, 1998). Gatekeepers directly inhibit growth or promote cell death, an example being the APC gene that when mutated leads to familial adenomatous polyposis coli (FAP). Caretakers, in contrast, do not directly promote tumor development, but their inactivation leads to genetic instability that in turn causes increased mutation rate in both oncogenes and tumor suppressor genes, which contributes to tumorigenesis. An example of caretaker genes are the mismatch repair (MMR) genes that predispose to Lynch syndrome. Landscaper genes, as the name implies, indirectly causes cancer by changing the landscape, that is, the microenvironment, making it more advantageous for tumor formation. This phenomenon has been observed in colon cancer where the abnormal stromal environment affects the development and growth of epithelial cells, leading to neoplastic formation (Ishiguro et al., 2006).

2.2 Genetic instability

During normal cell division, spontaneous mutations occur at a rate of approximately $3 \times 10^{-13}$ per base pair per cell division. However, normal mutation rates are not sufficient to generate a cancer cell from a pre-existing normal cell (Lengauer et al., 1998; Hanahan and Weinberg, 2000). Based on the requirement of several mutations for tumorigenesis to occur and the
fact that most cancers harbor a large number of genetic and/or epigenetic changes, it is not surprising that different experiments show that cancer may contain more than 10,000 genetic and epigenetic changes (Stoler et al., 1999). A study on breast and colorectal cancers revealed that individual tumors accumulate an average of about 90 mutant genes but that only a subset of these contribute to the neoplastic process (Sjoblom et al., 2006). This has led to the hypothesis that genomic instability is required for development of sporadic cancers. The instability is assumed to be due to an increase in gene mutation rates or to an increase in the rate of production of chromosomal abnormalities (Loeb, 1991; Loeb et al., 2003). In most cancers the instability is observed at the chromosomal level (chromosomal instability) but a small share of cancers also display instability in short repetitive DNA sequences, known as microsatellites (microsatellite instability) (Lengauer et al., 1998).

**Chromosomal instability**

Chromosomal instability can be described as increased gains or losses of chromosomal material manifesting as aneuploidy, numeral changes of chromosomes, or translocations, structural abnormalities (Sieber et al., 2003). Chromosomal regions lost may contain tumor suppressor genes and gains of regions may contain oncogenes giving the tumor cell a growth advantage (Weinberg, 2007). Chromosomal aberrations caused by translocations are seen in many malignancies, particularly in leukemias and lymphomas. A typical example of this is the Philadelphia chromosome in myelogenous leukemia. It is a nonrandom aberrant translocation between the ABL proto-oncogene on chromosomes 9 and the BCR gene on chromosome 22. The BCR-ABL gene encodes a protein with uncontrolled tyrosine kinase activity causing accelerated cell division (Nowell and Hungerford, 1960; Rowley, 1975; Kurzrock et al., 2003).

**Loss of heterozygosity**

Loss of heterozygosity (LOH), or loss of the normal, functional allele at a heterozygous locus is regarded as chromosomal instability. LOH, leading to increasing homozygosity, can be a consequence of a deletion, mitotic recombination or non-disjunctual chromosome loss with or without reduplication, or it can be locus-restricted, caused by gene conversion or point mutation, or epigenetic inactivation (Tischfield, 1997; Thiagalingam et al., 2001) (Figure []). LOH has been demonstrated to be the key mechanism in inactivation of the tumor suppressor RB1 in retinoblastoma, the most common intraocular tumor in childhood (Knudson, 1971). The frequency of
LOH is observed to accumulate during tumor progression (Tamura, 2006).

LOH at the APC gene (5q21) is a frequent mechanism of gene inactivation and is commonly seen in colorectal cancer. The APC gene is an important component of the Wnt signal transduction pathway. Inactivation of the APC gene leads to an inactive APC protein, which induces stabilization of β-catenin, which is then shuttled to the nucleus, where it activates transcription factors leading to expression of genes that induces cell proliferation, and eventually carcinogenesis (Fodde, 2002; Nathke, 2004). Germline mutations in APC are found in a majority of FAP patients (Grodén et al., 1991; Joslyn et al., 1991) and somatic mutations in more than 80% of sporadic colorectal cancer patients (Nagase and Nakamura, 1993; Kinzler and Vogelstein, 1996). APC mutations are believed to occur in the earliest stage of colorectal carcinogenesis (Powell et al., 1992; Vogelstein et al., 1988). Mutations in APC alone are not sufficient for progression to carcinoma; several more alterations in other tumor pathways are required (Vogelstein and Kinzler, 2004) (see Figure 2).

Microsatellite instability

Microsatellite instability (MSI) arises when short nucleotide repeats are expanded or shortened. The repeated sequences are usually 1 to 4 basepairs in length and can be repeated up to 100 times. These repeats are scattered widely throughout the genome and may occur in both coding and non-coding regions and are hence also found in several human genes. MSI is found in 15-25% of sporadic tumors and in over 90% of Lynch syndrome tumors (Peltomaki, 2003; Aaltonen et al., 1993). Microsatellites are targets of slippage of the DNA polymerase during replication, leading to variability in length of the repeated sequence (Peltomaki, 2001). MSI can readily be detected if many cells are affected by the same change, as an indicator of clonal expansion (de la Chapelle, 2003). Recently, it has been possible to amplify small amounts of DNA that equivalents to the amount of DNA from a single cell to detect MSI in tumors. This means that tumors may have MSI but due to small number of clones with unstable alleles it remains undetected by conventional PCR (Coolbaugh-Murphy et al., 2004)

DNA mismatch repair

MSI has been studied extensively and it is caused by defects in the postreplication mismatch repair mechanism responsible for the fidelity of DNA replication (Jiricny, 1998). In humans, at least six different MMR genes (MLH1, MSH2, MSH6, MLH3, PMS2 and MSH3) have been identified. The MMR proteins interact with each other to form protein complexes that mediate
Figure 1: A. According to Knudson’s 'two hit' theory, both copies in a tumor suppressor need to be inactivated for a normal cell to transform into a tumor cell. In hereditary cancer, a person has a germline mutation in one allele (the “first hit”), and only a “second hit” is needed for tumorigenesis: whereas in sporadic cancer the likelihood of both copies of the same gene becoming inactivated is rare. B. Different mechanisms of second hit. 1. Mitotic non-disjunction where the paternal chromosome is lost, 2. mitotic recombination or gene conversion, the mutated locus replaces the normal locus, 3. deletion of the normal allele, 4. sporadic mutation in paternal allele, 5. gene silencing by promoter methylation of the wild-type allele (Modified from Aittomaki and Peltomaki, 2006).

distinct functions in repairing insertion/deletion and single-base substitution mismatches (Peltomaki, 2001). MLH1 protein together with PMS2 directs the mismatch recognition complex to repair insertion-deletion loops, whereas MSH2 together with MSH6 repair single-base mismatches (Peltomaki, 2001; Chao and Lipkin, 2006). MMR proteins also participate in additional mechanisms that could contribute to carcinogenesis, most notably initiation of apoptosis in response to DNA damage (Fishel, 2001; Li, 1999).

MSI at microsatellites is estimated to be more than 100-1000 times higher in MMR deficient cells compared to proficient cells (Parsons et al., 1993). MSI is used to directly test whether MMR deficiency (a mutator phenotype) is involved in specific tumor types. Almost all described *MLH1*/*MSH2* mutations cause high degree of MSI (MSI-H) in tumors (Liu et al., 1999; Lipkin et al., 2004). Currently it is thought that MMR gene
mutations are recessive on a cellular level, and therefore, both alleles need to be inactivated for the development of malignant phenotype.

2.3 Cancer epigenetics

Epigenetic changes are modifications in the DNA that are passed on to the daughter cell during cell division that do not involve a change in DNA sequence. DNA methylation involves the addition of a methyl group to DNA at CpG sites. During the last decades it has become evident that DNA methylation has an important role in regulation of oncogenes and tumor suppressor genes by means of hypo- and hypermethylation, and loss of imprinting (Feinberg, 2004). CpG islands are GC-rich regions that display unmethylated CpG-nucleotides, and these CpG islands are predominantly located in promoters, close to the transcription site (Esteller, 2008; Jones and Laird, 1999; Antequera and Bird, 1993).

Epigenetic silencing (hypermethylation) of tumor suppressors and reactivation (hypomethylation) of oncogenes contribute to cancer development. Global genomic hypomethylation at CpG dinucleotides was the first epigenetic abnormality identified in cancer cells (Christman et al., 1977; Feinberg, 2004). Hypomethylation is observed to occur at an early stage in colorectal neoplasia, leading to reactivation of genes critical for tumorigenesis, which in normal cells are silenced by methylation (Feinberg et al., 1988; Hernandez-Blazquez et al., 2000). Hypermethylation of promoter region CpG islands in turn has been observed to play an important role in inactivation of tumor suppressor genes (Esteller et al., 2001; Costello et al., 2000; Esteller, 2002; Herman and Baylin, 2003; Myohanen et al., 1998). CIMP (CpG island methylator phenotype) was originally defined as methylation at three or more of seven “cancer-specific” loci (MINT1, MINT31, p14 gene, p16 gene, THBS1, MLH1, and TIMP3) (Toyota et al., 1999). A clear association has been described with family history and colorectal cancer patients with CIMP (Rashid and Issa, 2004; Wynter et al., 2006; Weisenberger et al., 2006; Frazier et al., 2003). As described in Figure 1, epigenetic silencing is another mechanism for the “second hit” to inactivate a tumor suppressor (Jones and Laird, 1999).

Hypermethylation of a gene in the germline, reflected in the presence in somatic cells throughout the body, is known as germline epimutation. Epimutations in MLH1 and MSH2 have been described as a possible mechanism for cancer susceptibility (Chan et al., 2006; Gazzoli et al., 2002; Miyakura et al., 2004; Hitchins et al., 2007; Valle et al., 2007; Morak et al., 2008).
Colorectal cancer is one of the most common cancers world wide, and the third most common cause of cancer-related death in the Western world (Globcan 2002 database, International Agency for Research on Cancer, www-dep.iarc.fr). It is mainly considered a disease of the elderly, as the risk of colorectal cancer increases with age. In Finland roughly 2000 new cases are diagnosed every year (Syöpäjärjestöt, www.cancer.fi). A majority of the cases are sporadic and believed to be due to environmental factors (Weitz et al., 2005), and around 20–25% of colorectal cancers occur in individuals with positive family histories and therefore, are considered to have an inherited susceptibility (de la Chapelle, 2004; Lynch and de la Chapelle, 2003). About 5% of colorectal cancer with inherited predisposition is caused by highly penetrant mutations in single genes, giving rise to hereditary syndromes that are mostly transmitted as autosomal dominant traits. The two most common being, Lynch syndrome/HNPCC (hereditary nonpolyposis colon cancer) which is caused by predisposing mutations in the MMR genes, and FAP that is caused by mutations in the APC gene. Other hereditary colorectal cancers are, MYH-associated polyposis, Juvenile polyposis, Peutz-Jeghers, Bannayan-Ruvalcaba-Riley, and Cowden syndrome (Abdel-Rahman and Peltomaki, 2004; de la Chapelle, 2004). Low penetrance susceptibility genes, together with modifier genes and environmental factors, account for a large share of inherited or sporadic colorectal cancer (de la Chapelle, 2004).

Tumorigenesis is a multi-step process, where consecutive genetic alterations drive the progressive transformation of normal cells into highly malignant cancer cells (Hanahan and Weinberg, 2000). Colorectal cancer is probably the most studied cancer type. The studies on carcinogenesis of colorectal cancer through sequential accumulation of genetic alterations have improved our understanding in cancer genetics and have provided useful models also for other cancers. One reason for this is that colorectal cancers occur, at least to some extent, through a well-known adenoma to carcinoma sequence (Fearon and Vogelstein, 1990). Furthermore, biopsies from different stages of colorectal cancer can be obtained relatively easily and can thus be subjected to genetic analyses (de la Chapelle, 2004).

APC mutations, together with β-catenin activation, are believed to occur in the earliest stage, initiating the neoplastic process of colorectal carcinogenesis, and tumor progression results from mutations in other genes, such as, KRAS and TP53 (Powell et al., 1992; Vogelstein et al., 1988; Vogelstein and Kinzler, 2004) (Figure 2). KRAS indirectly regulates cell
cycle progression by recruiting and activating proteins necessary for the propagation of growth factor receptor signals. *TP53* controls cell cycle progression by activating DNA repair processes in case of DNA damage and initiating apoptosis. *KRAS* mutations are observed to arise during the adenoma stage, and mutations in *TP53* and deletions on chromosome 18q coincide with transition to malignancy. Mutations in *KRAS* and *TP53* are identified in approximately 50% of colorectal cancer (reviewed in Chung, 2000). Conversely, loss of chromosome 18q occurs later in the sequence of development from adenoma to carcinoma. Loss of the 18q region is thought to contribute to inactivation of the *DCC* tumor-suppressor gene, which is reported to be mutated in approximately 70% of colorectal cancers (Fearon and Vogelstein, 1990; Takayama *et al.*, 2006).

Defective MMR leads to tumor development as a result of the accumulation of widespread mutations within short repetitive sequences, in for instance *TGFβRII*, and as well as other genes involved in signal transduction, DNA repair, transcriptional regulation, protein stability and immune surveillance (Chao and Lipkin, 2006).

![Sequential genetic and epigenetic changes leading to colorectal tumorigenesis](modified from Kinzler and Vogelstein, 1996; Chung, 2000; Takayama, 2006; Kondo and Issa, 2004.)

**Figure 2:** Sequential genetic and epigenetic changes leading to colorectal tumorigenesis (modified from Kinzler and Vogelstein, 1996; Chung, 2000; Takayama, 2006; Kondo and Issa, 2004.)

In the colon, aberrant DNA methylation arises early and contributes to later stages of colon cancer formation and progression through CIMP. Most colorectal cancers have epigenetic abnormalities, together with the genetic changes such as *TP53*, *KRAS* and β-catenin mutations (Issa, 2000; Kondo and Issa, 2004).
3.1 Lynch syndrome

Genetic characteristics of Lynch syndrome

Lynch syndrome, or HNPCC, is among the most common hereditary cancers in man and is caused by germline mutations in MMR genes \textit{MLH1}, \textit{MSH2}, \textit{MSH6} and \textit{PMS2} (Abdel-Rahman \textit{et al.}, 2006). A database has been established for the known mutations in \textit{MLH1}, \textit{MSH2} and \textit{MSH6} that are associated with Lynch syndrome (The International Society for Gastrointestinal Hereditary Tumors (InSiGHT), Database for MMR mutations: \url{www.insight-group.org}). Mutations in \textit{MLH1} and \textit{MSH2} account for approximately 90\% of all MMR gene mutations (de la Chapelle, 2004). Some frequent mutations have been described in Lynch syndrome including founder mutations inherited from a common ancestor. These can be tested for as the first step in high-risk individuals from isolated populations (Peltomaki and Vasen, 2004). However, among families clinically diagnosed with Lynch syndrome, the proportion of MMR mutation varies considerably between different studies, ranging from approximately 30\% to 90\% (e.g., Lynch and de la Chapelle, 2003; Renkonen \textit{et al.}, 2003; Nystrom-Lahti \textit{et al.}, 1996). In MMR mutation-negative families, the etiology remains to date unknown, but possible explanations are certain complex structural alterations (e.g., inversions) (Wagner \textit{et al.}, 2002) as well as regulatory changes in non-coding regions (Shin \textit{et al.}, 2002). MSI is used to directly test whether MMR deficiency (a mutator phenotype) is involved in specific tumor types.

Although all cells in Lynch syndrome patients carry a MMR gene mutation (“first hit”), tumor development additionally requires somatic inactivation of the remaining wild-type allele (“second hit”) in a target tissue. Germline mutation in MMR gene leads to accumulation of replication errors, mainly at short repetitive DNA sequences in the tumor cells, and gives rise to the molecular hallmark of Lynch syndrome, MSI. MSI contributes to cancer via an increased mutation rate in both microsatellites and in genes important in cancer suppression. The revised Bethesda guidelines (Table \ref{table2}) are used for identification of individuals with Lynch syndrome who should be tested for microsatellite instability (Umar \textit{et al.}, 2004a). MSI is present in 15-25\% of corresponding sporadic tumors as well (Peltomaki, 2003).

Clinical characteristics and tumor spectrum of Lynch syndrome

When Lynch syndrome was first described, it was considered a familial syndrome characterized by an increased incidence of colorectal and endometrial cancer. At present there is disagreement on defining which other tumors
belong to Lynch syndrome. However, tumors that have sufficiently high relative risk compared to the general population are to be considered Lynch syndrome tumors (Watson and Riley, 2005). Therefore, the term hereditary nonpolyposis colorectal cancer may be too restrictive, and Lynch syndrome has been proposed as a more appropriate name to cover this syndrome (Umar et al., 2004a). Epidemiological studies have found that tumors of colon and rectum, endometrium, stomach, ovary, ureter/renal pelvis, brain, small bowel, hepatobiliary tract, and skin (sebaceous tumors) are associated with Lynch syndrome (Vasen et al., 1999; Aarnio et al., 1999). Of these, cancers of the colon and rectum, endometrium, ureter, renal pelvis, and small bowel have the highest relative risk compared to the average population, and are, therefore, the most specific for Lynch syndrome.

Uniform clinical criteria were needed to provide consistency in collaborative studies. For this purpose the Amsterdam criteria were developed in 1991, before the genetic basis of the disorder was known (Vasen et al., 1991).
Amsterdam criteria I were found to be too stringent as the importance of extracolonic cancers became clear and thus the 'Amsterdam criteria II' were developed in 1998 (Table 2) (Vasen et al., 1999). Whereas the definition of Lynch syndrome tumor spectrum remains controversial, the consensus is that at least cancers of the colon and rectum, endometrium, small intestine, ureter and renal pelvis (Figure 3) are associated with sufficiently high relative risk compared with the average population to justify their inclusion in the clinical consensus criteria for the syndrome (Amsterdam criteria II; Vasen et al., 1999). The diagnosis of Lynch syndrome is made by considering the family history using Amsterdam criteria and when the mutation in the family is known, genetic testing can be used for diagnosis. The Bethesda guidelines were introduced in 1996 to help aid in the decision process for whether individuals with cancer in families that do not fulfill Amsterdam criteria should undergo genetic testing (Rodriguez-Bigas et al., 1997). The revised Bethesda guidelines were developed for several reasons: to identify patients who were at risk for hereditary cancer, to include a complete spectrum of colonic and extracolonic cancers, and to identify MSH2 and MLH1 germline-mutation carriers in patients with cancers who might or might not fulfill the Amsterdam II criteria (Umar et al., 2004a).

The International Workshop developed the Bethesda panel of five mono- and dinucleotide markers to determine MSI status in tumors. Tumors with two or more unstable markers are considered to have high frequency of MSI (MSI-H), whereas those with no unstable markers were microsatellite stable (MSS) (Boland et al., 1998; Umar et al., 2004a).

The average age of onset of colorectal cancer from Lynch syndrome patients is about 45 years, whereas in sporadic cancers it is 20 years later (Lynch and de la Chapelle, 1999; Peltomaki et al., 2001). The tumors are mainly located in the proximal colon, and multiple synchronous and metachronous tumors are common (Jarvinen et al., 2000). Lynch syndrome is also characterized by frequent occurrence of extracolonic tumors, mainly in the endometrium (Aarnio et al., 1999). The risk of colorectal cancer in MMR gene mutation carriers is estimated to be around 80% by the age of 70, the risk being slightly higher in males than females. The lifetime risk of endometrial cancer in predisposed females is 42% in MLH1 mutation carriers and 61% for MSH2 mutation carriers (Aarnio et al., 1999; Vasen et al., 1996). The risk of other extracolonic, extraendometrial cancers is estimated to be approximately 2 to 10% (Aarnio et al., 1999; Watson and Lynch, 2001) (Table 3).

The risk of extracolonic cancers in Lynch syndrome has been found to be higher in MSH2 mutation carriers than MLH1 mutation carriers (Vasen et al., 2001; Watson et al., 2008), but the correlation between germline
Table 2: The clinical consensus criteria for diagnosis of Lynch syndrome (Vasen et al., 1991; Vasen et al., 1999)

<table>
<thead>
<tr>
<th>Amsterdam Criteria I and II:</th>
<th>Revised Bethesda guidelines</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. At least three relatives with Lynch syndrome associated cancer (in colorectum, endometrium, small bowel, ureter and renal pelvis)</td>
<td>1. Colorectal cancer diagnosed in a patient before 50 years of age</td>
</tr>
<tr>
<td>2. One should be a first degree relative of the other two</td>
<td>2. Presence of synchronous, metachronous colorectal, or other Lynch syndrome-associated tumors, regardless of age</td>
</tr>
<tr>
<td>3. At least two successive affected generations</td>
<td>3. Colorectal cancer with the MSI-H histology diagnosed in a patient before 60 years of age</td>
</tr>
<tr>
<td>4. At least one Lynch syndrome-associated cancer diagnosed under 50 years</td>
<td>4. Colorectal cancer diagnosed in one or more first-degree relatives with an Lynch syndrome-associated tumor, with one of the cancers diagnosed before age 50</td>
</tr>
<tr>
<td>5. FAP should be excluded</td>
<td>5. Colorectal cancer diagnosed in two or more first- or second-degree relatives with Lynch syndrome associated tumors, regardless of age</td>
</tr>
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<td>6. Tumors should be verified by pathological examination</td>
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</table>

Table 3: The cumulative incidence of Lynch syndrome associated tumors by age 70 (Aarnio et al., 1999)

<table>
<thead>
<tr>
<th>Site of Tumor</th>
<th>Finnish population (%)</th>
<th>Lynch syndrome families (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon and rectum</td>
<td>1.6</td>
<td>82</td>
</tr>
<tr>
<td>Endometrium</td>
<td>1.3*</td>
<td>60*</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.8</td>
<td>13</td>
</tr>
<tr>
<td>Ovary</td>
<td>1.3*</td>
<td>12*</td>
</tr>
<tr>
<td>Bladder, ureter and urethra</td>
<td>0.7</td>
<td>4.0</td>
</tr>
<tr>
<td>Brain</td>
<td>0.9</td>
<td>3.7</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.8</td>
<td>3.3</td>
</tr>
<tr>
<td>Biliary tract, gallbladder</td>
<td>0.2</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*Women only
mutation and clinical phenotype is generally poor in the case of Lynch syndrome. It has also been shown that $MSH6$ mutations are associated with families with a higher incidence of endometrial carcinoma (Wijnen et al., 1999).

The cancer spectrum also varies between Lynch syndrome families geographically, which is partly due to different environmental factors. For example, it is well-known that Asian families generally have a higher incidence of stomach cancer compared to Western countries, where colorectal cancer predominates (Cai et al., 2003; Lynch et al., 1988a; Lynch et al., 1988b; Lynch et al., 1998). For this reason, these countries have developed modified diagnostic criteria in addition to the Bethesda guidelines (reviewed in Umar et al., 2004b; Lee et al., 2005; Liu et al., 2004; Zhang et al., 2005).

An approximately four-fold increased incidence of brain tumors, mainly gliomas, has been reported in Lynch syndrome families (Vasen et al., 1996; Vasen et al., 1996; Aarnio et al., 1999). Colon cancer and concurrent malignant glioma/glioblastoma have been reported to occur in the Lynch syndrome variant of Turcot syndrome, in association with mutations in $MLH1$, $MSH2$, $PMS2$, or $MSH6$ (Hamilton et al., 1995; Chan et al., 1999; De Rosa et al., 2000; Hegde et al., 2005). Colon polyposis and medulloblastoma are associated with heterozygous $APC$ gene mutations in the FAP variant of Turcot syndrome (Mori et al., 1994).

Muir-Torre syndrome is a rare subtype of Lynch syndrome, and in addition to the Lynch syndrome tumor spectrum, patients also display skin tumors, mainly sebaceous gland tumors or keratoacanthomas (Cohen et al., 1995; Schwartz et al., 1989). Muir-Torre tumors display MMR defects with accompanying MSI (Kruse and Ruzicka, 2004).

### 4 Tissue specificity in hereditary cancer syndromes

A feature that all hereditary forms of cancer have in common is that they predispose to only certain types of tumors. However, the genes responsible for the syndrome are usually expressed in all tissues and regulate fairly universal processes like DNA repair and cell cycle control (Chao and Lipkin, 2006). Apart from Lynch syndrome, there are other hereditary cancer syndromes that predispose to specific types of cancer, such as Li-Fraumeni, Von Hippel-Lindau, and multiple endocrine neoplasia 2 that are well-known hereditary cancer syndromes. Li-Fraumeni syndrome is a rare autosomal dominant syndrome characterized by early-onset tumors including bone and soft tissue sarcomas, breast cancer, brain cancer, leukemia and childhood adrenocortical tumors (Li and Fraumeni, 1969). Germline mutations in
the tumor suppressor gene p53 account for the majority of Li-Fraumeni syndrome families (Varley, 2003). Von Hippel-Lindau syndrome is caused by germline mutations of the VHL tumor suppressor gene (Latif et al., 1993; Stolle et al., 1998). It is an autosomal dominant trait distinguished by renal cell carcinoma, hemangioblastoma, and pheochromocytoma (Maher et al., 1991). Germline mutations in the RET proto-oncogene are associated with multiple endocrine neoplasia type 2, which is characterized by medullary thyroid carcinoma, pheochromocytoma (both of neural crest origin), and parathyroid hyperplasia (Eng et al., 1996).

Eng and Ponder (1993) discussed several explanations for the tissue specificity of cancer occurrence although the mutation in the cancer susceptibility genes occurs in the germline, and is thus, being present in all tissues of the body. In some cases it may be explained by common origin of cells but often the underlying mechanism is more complex. The variable expression and penetrance of phenotypes may be modulated by different germline mutations within the susceptibility gene, mutations causing target protein conformational change, or modulation of expression by modifier genes (Ribeiro et al., 2001; Eng and Ponder, 1993).
4.1 Tissue specificity & MMR defect

There are several separate mechanisms that contribute to tissue specificity in MMR deficient cells but they contribute in different ways in different tissues (reviewed in Chao and Lipkin, 2006). Because mutation rates are significantly higher in MMR defective versus proficient cells, it is thought that the cell types with the highest overall proliferation rate (and therefore the highest total number of genomic replication cycles) would be most susceptible to cancer due to MMR deficiency (Parsons et al., 1993). This is believed to be the case in the gastrointestinal epithelium, having the highest known proliferation rate of all cell types (Lipkin, 1973). In the gastrointestinal and genitourinary epithelium, exposure to mutagens in the diet and concentration of environmental mutagens in the urine has been associated with carcinogenesis (Watson et al., 2004; Lynch and Smyrk, 1998). Helicobacter pylori infection has also been shown to be related to gastric carcinoma (Lynch et al., 2005).

Since MMR defects particularly affect genes which contain short repetitive sequences, the dependence of these genes in different organs has been suggested to be critical for cancer development due to MSI (Schwartz et al., 1999; Yamamoto et al., 2000; Duval et al., 2002). For instance, TGFβRII and ACVR2, sharing common regulatory mechanisms (Deacu et al., 2004), are frequently mutated in colorectal tumors, causing frameshift mutations (Markowitz et al., 1995; Mori et al., 2002; Mori et al., 2001). Several other genes with presumed tumor suppressor functions and containing coding microsatellites have been shown to be mutated at various frequencies in both sporadic MSI-H and Lynch syndrome associated colorectal, gastric and endometrial cancers. These include additional genes such as IGFIIR, PTEN (Kuismanen et al., 2002; Souza et al., 1996) involved in signal transduction, MSH3, MSH6, MBD4, BLM, MRE11 important for DNA repair (Abdel-Rahman et al., 2008; Baranovskaya et al., 2001; Malkhosyan et al., 1996), as well as genes that play roles in transcriptional regulation, protein stability and immune surveillance.
AIMS OF STUDY

This study was conducted to elucidate the genetic background and tissue specificity of MMR deficient tumors from Lynch syndrome families. The specific aims were:

1. To investigate the frequency and nature of inactivation of the wild-type allele in tumors from MMR gene germline mutation carriers (I-IV)

2. To examine the mechanisms of cancer predisposition in Lynch syndrome patients by determining the molecular profiles for different tumors from a nationwide cohort of Lynch syndrome families, including tumors of brain, urinary tract, stomach, colorectum and endometrium (II, III)

3. To study the role of large genomic rearrangements and germline epimutations in MMR genes in mutation-negative patients with suspected Lynch syndrome (IV)
MATERIALS & METHODS

For detailed information on materials and methods used in the present study, please refer to the Materials and Methods section of each paper.

1 Patients & samples

The material for studies I-IV were selected from the Hereditary Colorectal Cancer Registry of Finland that includes 194 verified Lynch syndrome families classified according to the Amsterdam criteria (Vasen et al., 1999; Vasen et al., 1991) or revised Bethesda guidelines (Umar et al., 2004a), in which a germline change in the MMR genes has been detected by exon-specific screening methods in 148 families. A sample cohort consisting of 7 Finnish Lynch syndrome patients and 16 Swiss putative Lynch syndrome patients were studied to determine proportion of genomic rearrangements and to study LOH as a second somatic hit in Lynch syndrome (publication I).

MMR gene mutation carriers (n=15) with gastric cancer were selected in order to study whether gastric cancer is a true Lynch syndrome associated malignancy (publication II). The Lynch syndrome associated gastric cancers were compared to sporadic gastric carcinomas (n=46). Sporadic gastric cancers were initially divided into three different subgroups based on histology and MSI status: intestinal MSI (n=10), intestinal MSS (n=20) and diffuse MSS (n=16).

All available brain tumor samples (n=7) and urological tumor samples (n=14) (publication III) from patients belonging to verified Finnish Lynch syndrome families were selected. The urinary tract tumors included 4 renal cell adenocarcinomas and 10 uroepithelial cancers (5 ureter and 5 urinary bladder). All tumor samples had corresponding normal tissue when available. Colorectal carcinoma samples (n=48) and endometrial carcinoma samples (n=60) from well-characterized Lynch syndrome families, all fulfilling the Amsterdam and Bethesda criteria, were selected in order to compare findings in brain and urological tumors from Lynch syndrome families.

Lynch syndrome MMR mutation carriers with colorectal cancer and endometrial cancer (n=51) were also chosen to make clinical comparisons
with putative Lynch syndrome families lacking germline mutations in MMR genes by conventional screening (Holmberg et al., 1998; Renkonen et al., 2003) but displaying loss of MLH1, MSH2 or MSH6 expression in tumor tissue (n=45, publication IV). Among the original 81 kindreds ascertained through family history suggestive of Lynch syndrome, 11 families showed no detectable germline changes, despite aberrant MMR protein expression and MSI in tumor tissue. A diagnostic cohort of 34 patients with suspected Lynch syndrome was included, displaying loss or MMR protein expression, in whom no MMR gene mutations have been found by conventional methods (publication IV).

Formalin-fixed paraffin-embedded and fresh frozen specimens of tumor and matching normal tissues were collected from the pathology departments of different hospitals in Finland for all the studies. Representative areas of the paraffin-embedded tissue from both the cancerous and normal areas were selected for immunohistochemistry and DNA extraction. DNA was prepared from archival tissue samples according to the method of Isola et al. (1994). DNA was extracted from lymphocytes and lymphoblasts as described in (Lahiri and Nurnberger, 1991).

Informed consent was obtained from patients participating in this study, and the appropriate institutional review boards approved this study.

2 Protein expression analyses & histology

2.1 Immunohistochemistry (I-IV)

Immunohistochemistry was used to confirm the presence or absence of MMR proteins, β-catenin and p53 in tumor tissue. Immunohistochemical results for the MMR proteins were interpreted using nuclear staining of normal tissue and stromal cells included in each tumor section as a reference for the evaluation of the staining results. β-catenin expression was considered aberrant if there was nuclear staining in more than 10% (not observed in the matching normal tissue). In reporting p53 protein stabilization, a cut-off level of more than 10% positive tumor cells was used.

2.2 Helicobacter pylori status & tumor grading (II, III)

Gastric carcinomas were classified according to Laurén (1965) into intestinal and diffuse types of tumors, and the differentiation grade was determined according to the WHO classification (Fenoglio-Preiser et al., 2000). Chronic gastritis, atrophy, intestinal metaplasia and H. pylori infection were evaluated and graded according to the Sydney classification (Dixon et al., 1996). H. pylori status was verified using hematoxylin and eosin and
<table>
<thead>
<tr>
<th>Study Method</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
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<tbody>
<tr>
<td>1. DNA extraction</td>
<td>x</td>
<td>x</td>
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<tr>
<td>2. Protein expression/histology</td>
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<tr>
<td>2.1 Immunohistochemistry</td>
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<tr>
<td>MLH1, MSH2, MSH6</td>
<td>x</td>
<td>x</td>
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<tr>
<td>p53, β-catenin</td>
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<td>2.2 Histology and <em>H. pylori</em> status</td>
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<tr>
<td>3. Mutation analysis</td>
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<tr>
<td>3.1 Exon-specific sequencing</td>
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<tr>
<td>MLH1, MSH2, MSH6</td>
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<td>x</td>
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<tr>
<td>KRAS, BRAF, CTNNB1</td>
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<td>3.2 SSCP</td>
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<tr>
<td>KRAS, BRAF, CTNNB1</td>
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<td>4. MSI analysis</td>
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<tr>
<td>4.1 (BAT25, BAT26, D2S123, D5S346, D17S250)</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
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<td>4.2 Small-pool PCR</td>
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<td>4.3 Frameshift-prone target genes</td>
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<tr>
<td>ACVR2, MRE11A, PTEN</td>
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<tr>
<td>MBD4, BLM, TGFβRII</td>
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<td>5. LOH analysis</td>
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<td>5.1 MLPA (deletion MLH1 ex16, MSH2 ex3-5)</td>
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<td>x</td>
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<tr>
<td>5.2 SNUPE (MLH1, APC)</td>
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<td>5.3 APC microsatellite markers (D5S346, D5S1965)</td>
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<tr>
<td>5.3 MSH2 microsatellite markers (D2S2378, CA7, D2S123)</td>
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<td>6. Methylation analysis</td>
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<tr>
<td>6.1 MS-MLPA</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
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<tr>
<td>6.2 Bisulfite modification &amp; sequencing</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
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<tr>
<td>6.3 MSP</td>
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<td>x</td>
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<tr>
<td>7. Deletion detection</td>
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</tr>
<tr>
<td>7.1 MLPA</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>7.2 Long-Range genomic PCR</td>
<td></td>
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<td>x</td>
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</table>
Alcian-blue/periodic acid Schiff stains added with modified Giemsa. Cancers of the bladder and ureter were of transitiocellular type, and graded according to the WHO. Kidney tumors were adenocarcinomas and graded using Fuhrman (1982). Brain tumors were graded according to the most commonly used classification system for brain tumors developed by the WHO.

3 Mutation detection analysis

3.1 Exon-specific sequencing (I-IV)

Immunohistochemistry was first performed on tumor tissue to identify the affected MMR gene. After the deficient MMR gene was identified, the respective MMR genes were screened for point mutations using DNA amplification, genomic sequencing, and specific testing for the founder mutation, as described previously (Chadwick et al., 2001; Vahteristo et al., 2001; Nystrom-Lahti et al., 1995).

3.2 Single strand conformation polymorphism (II, III)

KRAS, BRAF, and CTNNB1 were initially screened for hot spot mutations (Deng et al., 2004; Abdel-Rahman et al., 2005; publication II) by single strand conformation polymorphism (SSCP) analysis of genomic DNA. Samples screened with SSCP were first PCR amplified and then separated on a polyacrylamide gel with 1 x MDE Gel Solution (Cambrex Bio Science Rockland Inc., Rockland, ME) to improve sensitivity to conformational changes. For detection of DNA, gels were stained using silver nitrate. Changes observed on the SSCP gels were then sequenced to determine the exact nucleotide changes.

4 Microsatellite instability analyses

4.1 Bethesda panel for studying MSI (I-IV)

The Bethesda panel (Boland et al., 1998; Umar et al., 2004a) of five mono- and dinucleotide markers was used to determine MSI status in tumors since it is the hallmark of MMR deficiency and recommended by the International Workshop (Boland et al., 1998). The microsatellite markers were run on an ABI 3730 automatic DNA sequencer and analyzed with GeneMapper 4.0 software (both Applied Biosystems, Foster City, CA). Tumors with two or more unstable markers were considered to have MSI-H, whereas those with no unstable markers were MSS.
4.2 Small-pool PCR (III)

Small-pool PCR (SP-PCR) is a sensitive method for detection and quantification of MSI in somatic cells (Coolbaugh-Murphy et al., 2004). SP-PCR was conducted on all brain tumor samples and their matching blood samples. The fluorescently labeled dinucleotide markers D5S346 and D2S123 (Boland et al., 1998) were studied using SP-PCR to genotype individual alleles. The small-pool PCR reactions were essentially performed as described earlier (Coolbaugh-Murphy et al., 2004), in a 25µl reaction volume and 39 parallel aliquots per sample, using Expand High Fidelity PCR System Enzyme Mix (Roche, Mannheim, Germany) and 50 pg of DNA template. The annealing temperature was 62°C. From each aliquot (clone), 0–4 alleles amplified, of which those that deviated more than 1 bp from the constitutional allele size (determined by standard PCR) were considered unstable.

The average total number of alleles analyzed per tumor specimen was 15 and 34 for D2S123 and D5S346, respectively, and 40 and 64 per blood specimen. Mutation frequency was calculated by dividing the number of alleles with MSI by the total number of alleles in each sample.

\[
\text{Mutation frequency} = \frac{\text{No. alleles with MSI}}{\text{Total no. of alleles}}
\]

4.3 Mononucleotide repeats (II, III)

Eight mononucleotide repeats in six frameshift-prone target genes were PCR amplified and the products were run on an automated capillary electrophoresis sequencer and analyzed with the GeneMapper 4.0 program (Applied Biosystems, Foster City, CA).

5 Loss of heterozygosity analysis

5.1 Multiplex ligation-dependent probe amplification (I-IV)

Multiplex ligation-dependent probe amplification (MLPA) (MRC Holland, Amsterdam, the Netherlands) was used to detect locus restricted LOH in cases with verified MMR germline mutation. Two kits, SALSA MLPA P003 and P008, were used. SALSA MLPA kit P003 has probes for all exons of \(MLH1\) and \(MSH2\) as well as 7 control probes mapping to different chromosomes. SALSA MLPA kit P008 has probes for all exons of \(MSH6\) as well as for 13 of the 15 \(PMS2\) exons; in addition there are probes for \(MUTYH\ (MYH), MLH3, MSH3\) and a probe for \(APC\). All reactions were done...
following the manufacturer’s instructions (http://www.mrc-holland.com; Schouten et al., 2002). The principle of MLPA is as follows: each MLPA probe pair consists of two target sequence specific oligonucleotides that can be ligated to each other when hybridized to a target sequence. All ligated probes have identical sequences at their 5’ and 3’ ends (a universal PCR primer sequence), enabling simultaneous amplification using only one primer pair in a PCR reaction. Furthermore, for every probe pair, one of the probes also contains a stuffer sequence, giving the fragments produced unique sizes ranging from 130 to 480 bp (see Figure 4A for outline).

In brief, the DNA is first denatured and fragmented after which the MLPA probes are hybridized to their target sequences overnight. If no deletion is present, the probes can hybridize to their target sequences and will, after addition of ligase, be ligated. Only ligated probes are amplified in the PCR reaction. One PCR primer is fluorescently labeled, producing a PCR product that was run on an automated sequencer and analyzed with GeneMapper software (version 4.0, Applied Biosystems, Foster City, CA). Each probe produces a product of a unique size that can be detected using GeneMapper software. The whole reaction of more than 40 probes is visualized as a peak diagram (Figure 4B).

Relative peak values were calculated for each sample by dividing the peak area of a given probe pair \( (P_x) \) by the sum of the peak areas of all probes in that sample \( (P_{all}) \). This value was then divided by the mean relative peak value of normal DNAs from healthy controls, to obtain a dosage ratio \( (D) \). To calculate LOH, the dosage ratio of a patient’s tumor sample was divided with the patient’s normal sample. Forty percent reduction in fluorescence intensity was indication of loss of heterozygosity.

\[
D = \frac{\text{sample } P_x}{\text{control } P_x} \times \frac{P_{all}}{P_{all}}
\]

5.2 Single nucleotide primer extension (II, III, IV)

The extension reaction of single nucleotide primer extension (SNuPE) is based on the incorporation of a single dideoxy nucleotide that is selected to produce differential extension of a primer annealed close to the polymorphic site. In MLH1 associated cases, LOH was determined using the I219V A/G polymorphism (Renkonen et al., 2003; Ollikainen et al., 2007). The APC gene was examined by utilizing a coding polymorphism C/T at nucleotide 1458 (Renkonen et al., 2005). LOH ratios \( (L) \) of allelic peak areas \( (A_1 \text{ for allele 1, } A_2 \text{ for allele 2}) \) in normal relative to tumor were calculated, and values at or below 0.6 or above 1.67 (indicating that the transcript of one
Each probe pair hybridizes to their target sequences. The two parts of hybridized probe are ligated by a thermostable ligase. All ligation products are amplified by PCR using only one primer pair. The amplification product of each probe has a unique length (130-480 bp).

**Figure 4:** Outline of the MLPA reaction (modified from Schouten et al., 2002). The principle of the MLPA method (A.) and an example of a peak diagram (B.).
allele had decreased 40% or more) were considered strict LOH (Ollikainen et al., 2005) and ratios between 0.61-0.75 and 1.66-1.33 were considered putative LOH (Cleton-Jansen et al., 2001). SNuPE was also applied when performing allele-specific expression analysis on MLH1 in study IV.

\[
L = \frac{\text{tumor A1/A2}}{\text{normal A1/A2}}
\]

5.3 Microsatellite markers in detecting LOH (II, III)

In MSH2 or MSH6 mutation carriers, LOH was analyzed using flanking microsatellite markers, D2S2378, CA7 and D2S123 (Ollikainen et al., 2005). In addition to SNuPE, microsatellite markers next to the APC gene, D5S1965 (200 kb upstream of APC) and D5S346 (<100 kb downstream of APC), were also used to study LOH of APC (Renkonen et al., 2005). LOH was calculated as described above (section 5.2) comparing peak areas of normal relative to tumor, using the same ratios for putative and strict LOH.

6 Methylation detection

6.1 Methylation-specific MLPA (II-IV)

The methylation-specific MLPA (MS-MLPA) (MRC Holland, Amsterdam, the Netherlands) was used to detect methylation in MMR gene promoter regions using probes that contain one or two digestion sites for the methylation-sensitive HhaI enzyme. The kit ME011 (32 probes) includes five probe pairs for MLH1, three probe pairs for MSH2, and three probe pairs for MSH6, as well as ten probes for MLH3, PMS2, MSH3 and MGMT. In addition, the kit contains 11 different control probes that do not have HhaI enzyme digestion site. The ME001B kit (41 probes), on the other hand, contains probes for 24 different tumor suppressor genes, including two probe pairs for MLH1. The present tumor suppressor genes were selected because their inactivation has been found to be important in a wide variety of human cancers (Verma and Srivastava, 2002; Laird, 2003). Furthermore, the genes included were found relevant in relation to the tumors analyzed in the present study either through their role in DNA repair, apoptosis or cell cycle regulation. Table 5 includes detailed information on all 24 tumor suppressor genes included in the ME001B kit. The kit includes another 15 probes that function as control probes because they lack an HhaI digestion site. Normal DNA specimens derived from lymphocytes of healthy controls were included as controls in every assay.
### Table 5: All tumor suppressor genes included in the MS-MLPA ME001B kit (modified from Joensuu et al., 2008)

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Protein Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Participates in Wnt signaling, free beta-catenin level regulator, involved in cell adhesion and active cell migration</td>
</tr>
<tr>
<td>ATM</td>
<td>Protein kinase, DNA damage sensor and damage response regulator, checkpoint signaling activator at double strand breaks, apoptosis and genotoxic stresses, involved in signal transduction and cell cycle control</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Central role in DNA repair by facilitating cellular response to DNA repair, mediator of E2-dependent ubiquitination, lipid synthesis inhibitor</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Involved in double-strand break repair and/or homologous recombination</td>
</tr>
<tr>
<td>CASP8</td>
<td>Protease in the activation cascade of caspases responsible for apoptosis</td>
</tr>
<tr>
<td>CD44</td>
<td>Receptor for hyaluronic acid, mediator of cell-cell and cell-matrix interactions, role in cell migration, tumor growth and progression</td>
</tr>
<tr>
<td>CDH13</td>
<td>Calcium dependent cell adhesion protein</td>
</tr>
<tr>
<td>CDKN1B</td>
<td>Involved in cell cycle arrest, cellular response to DNA damage, regulator of cell cycle, effector of signal transduction pathways that control cell differentiation</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>(encoding p14ARF) Induces cell cycle arrest in G1 and G2 phases, negative regulator of the proliferation of normal cells</td>
</tr>
<tr>
<td>CDKN2B</td>
<td>Regulator of cell cycle, passage through the G1 checkpoint, tightly linked and highly homolog to CDKN2A</td>
</tr>
<tr>
<td>CHFR</td>
<td>E3 ubiquitin-protein ligase, cell arrest in early prophase</td>
</tr>
<tr>
<td>DAPK1</td>
<td>Calcium/calmodulin-dependent serine/threonine kinase, positive regulator of apoptosis</td>
</tr>
<tr>
<td>ESR1</td>
<td>Nuclear estrogen receptor α, involved in gene expression regulation, cellular proliferation and differentiation</td>
</tr>
<tr>
<td>FHIT</td>
<td>Diadenosine 5’,5”’-P1,P3-triphosphate hydrolase, involved in purine metabolism</td>
</tr>
<tr>
<td>GSTP1</td>
<td>Involved in conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles</td>
</tr>
<tr>
<td>HIC1</td>
<td>Transcriptional repressor</td>
</tr>
<tr>
<td>IGSF4 (=CADM1)</td>
<td>Cell adhesion molecule</td>
</tr>
<tr>
<td>MLH1</td>
<td>DNA mismatch repair protein</td>
</tr>
<tr>
<td>PTEN</td>
<td>Protein and lipid phosphatase, cell cycle progression and cell survival modulator</td>
</tr>
<tr>
<td>RARB</td>
<td>Receptor for retinoic acid, gene expression regulator</td>
</tr>
</tbody>
</table>
Table 5: cont. All tumor suppressor genes included in the MS-MLPA ME001B kit (modified from Joensuu et al., 2008)

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Protein function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RASSF1(A)</strong></td>
<td>Required for death receptor- dependent apoptosis Isoform A inhibits proliferation by negatively regulating cell cycle progression</td>
</tr>
<tr>
<td><strong>TIMP3</strong></td>
<td>Metalloproteinase inhibitor</td>
</tr>
<tr>
<td><strong>TP73</strong></td>
<td>Involved in apoptotic response to DNA damage</td>
</tr>
<tr>
<td><strong>VHL</strong></td>
<td>Transcriptional repressor, ubiquitin conjugating protein, cell cycle regulator</td>
</tr>
</tbody>
</table>

As in ordinary MLPA reactions, the MS-MLPA protocol starts with DNA denaturation and overnight hybridization of probes complementary to their specific DNA targets. The reaction is then divided into two parts. One set is processed as a standard MLPA reaction, described in section 5.1 providing information on copy number changes. The other set of the MS-MLPA hybridization reaction is incubated with the methylation sensitive HhaI endonuclease and simultaneously the hybridized probes are ligated. A sample that is not methylated is digested by the HhaI enzyme, while methylated samples are not digested and therefore, are ligated. Digested probes will not be amplified by PCR and hence will not generate a signal when analyzed by capillary electrophoresis. In contrast, if the sample DNA is methylated, the methylation of DNA prevents the DNA from being digested, and will generate a signal. The MS-MLPA reaction outline is shown below (Figure 5).

\[
DM = \frac{\text{digested } P_x}{\text{undigested } P_x} = \frac{P_x}{P_{ctrl}}
\]

The methylation dosage ratio (DM) was calculated by dividing the peak area of a given probe (P_x) by the sum of all peak areas of probes lacking a digestion site (P_{ctrl}). The ratio of P_x to P_{ctrl} of HhaI digested sample is then divided by the ratio of an undigested sample (above).
Figure 5: Outline of the MS-MLPA method (modified from Nygren, et al., 2005)
Based on our titration experiments with genes known to have methylation or complete lack of methylation, a dosage ratio of 0.15 (corresponding to 15% of methylated DNA) was regarded to indicate promoter methylation. This threshold value also provided the best discrimination of tumor DNA relative to paired normal DNA where no methylation was generally expected. The MS-MLPA technique was also independently validated by another method, methylation-specific PCR.

6.2 Bisulphite modification and sequencing (IV)

DNA was modified using the CpGenome DNA Modification Kit (Chemicon, Temecula, CA). For methylation analysis of the MLH1 promoter region, a fragment from -370 to -49 relative to the initiating ATG was amplified from bisulphite-modified DNA using methylation-unbiased primers MLH1 DEG 5' and MLH1 DEG 3' from Suter et al. (2004). Patient F36 displayed a deletion of exons 1–2 and flanking areas, and hence the methylation unbiased primers only amplified the wild-type allele. For analysis of the deletion-containing allele, the MLH1 DEG 5’ primer was combined with a methylation-unbiased reverse primer (study IV) from intron 2 beyond the deletion. The PCR products obtained with methylation-unbiased primers were sequenced to determine the methylation status.

To examine allele-specificity of methylation, bisulphite-converted DNA amplified with primers MLH1 DEG 5' and MLH1 DEG 3' was first cloned into a pCR2.1-TOPO vector using the TOPO TA Cloning System (Invitrogen, Carlsbad, CA), followed by sequencing of the cloned DNA.

For methylation analysis of the MSH2 promoter, a region from -651 to -42 relative to ATG site was evaluated in two overlapping PCR fragments, using methylation-unbiased primers NP1-F and NP1-R, from Chan et al. (2006) for the area distal to initiating ATG, as well as a forward and a reverse primer for the area proximal to initiating ATG (see study IV).

6.3 Methylation-specific PCR (IV)

Methylation-specific PCR (MSP) was performed using HotStarTaq DNA polymerase (Qiagen, Valencia, CA) and bisulphite modified DNA. The manufacturer’s standard protocol was applied to designing PCR conditions for HotStarTaq polymerase. Primers from Kim et al. (2006) were used for methylation analysis of the MLH1 promoter. Primers for the methylated reaction (M) covered a region from -269 to -206 and those for the unmethylated reaction (U) a region from -288 to -192 relative to the initiating ATG. PCR products were visualized on an agarose gel with UV transillumination. The RKO cell line with verified MLH1 promoter methylation
and lymphocyte-derived DNA from a healthy control served as positive and negative controls, respectively.

7 Deletion detection

7.1 MLPA (I-IV)
MLPA, described in section 5.1, was also used to screen DNA samples for large genomic rearrangements in all exons of the MMR genes in cases in which no mutations in the MMR genes had been found, despite lack of MLH1, MSH2 and MSH6 protein expression and MSI. The dosage ratio was calculated as described above and a reduction of 40% or more indicated that the transcript of one allele had decreased.

7.2 Long-range PCR (IV)
To determine the breakpoints for MLH1 deletion observed by MLPA, long-range PCR was carried out using the Expand Long Template PCR System (Roche Diagnostics, Mannheim, Germany). The fragments specific for the mutant allele (1.1 kb) and wild-type allele (7.5 kb) were determined in an agarose gel. The product corresponding to the mutant allele was cut out, purified, and sequenced.

8 Statistical analysis (I-IV)
Statistical significance for differences between groups (p-value) was determined using Fisher’s or t test using VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html), as appropriate. All reported p-values were two-tailed, and values less than 0.05 were considered significant.
RESULTS

1  Mechanisms of somatic MMR gene inactivation (I-IV)

1.1  LOH and acquired promoter methylation as second hits

In the Lynch syndrome associated tumors of stomach, urinary tract and brain LOH was found to be the primary mechanism for the inactivation of the second allele of MLH1 and MSH2. In Lynch syndrome associated gastric cancers the frequency of LOH affecting the MLH1 gene was 50% (4/8). On the contrary, in sporadic MSI gastric cancers the MLH1 inactivation was probably due to MLH1 promoter methylation since it was present in 70% of the cases (7/10). Furthermore, the dosage ratio of MS-MLPA suggested that both alleles were methylated. No significant MLH1 promoter methylation was seen in the Lynch syndrome gastric cancers.

LOH of MLH1 also seemed to be an important mechanism for somatic inactivation of the wild-type allele in Lynch syndrome associated brain tumors. In urinary tract tumors, however, LOH accounted for somatic inactivation mainly in urological tumors. In informative urinary tract tumors LOH at MLH1 and MSH2 loci was observed in 83% (5/6) of MLH1 carriers and in 100% (2/2) of MSH2 carriers, respectively. Only three cases were available and informative among the brain tumors. LOH at the MLH1 locus was found in 67% of the cases (2/3) of which both were MLH1 mutation carriers.

1.2  Mitotic recombination vs. gene conversion as a mechanism of somatic inactivation

Based on the results obtained in study I, gene conversion was found to be the mechanism of wild-type inactivation in a set of Lynch syndrome tumors. Seven Finnish Lynch syndrome patients including four tumors of colon, two endometrial tumors and one gastric cancer carrying germline deletions of one or several exons of MSH2 or MLH1 were studied for somatic deletions. All patients displayed MSI-H and six carried MLH1 deletion and
one MSH2 deletion. The frequency of loss of heterozygosity as a second, somatic event, in tumors was studied using MLPA. The somatic deletions were mostly identical to the ones found in the germline. Biallelic deletion of MLH1 was found in two of the four colon tumors (2/7, 29%), indicated by the low dosage ratios in tumor tissue. The remaining tumors lacked somatic deletions. In the 16 Swiss Lynch syndrome samples, biallelic deletions were found in seven tumors, giving a total overall frequency of somatic deletions of 50% (9/18) in this study, of which most were found in colorectal tumors (6/11, 55%).

Eight highly polymorphic short tandem repeat markers flanking the gene loci on chromosome 3 for MLH1 and chromosome 2 for MSH2 were investigated to distinguish between the possible mechanisms leading to loss of heterozygosity in the tumor. None of the tumors showed allelic loss at the flanking markers at MLH1 and MSH2, indicating that a locus-restricted event had occurred. Instead of mitotic recombination, gene conversion had taken place in all cancers that were homozygous for the germline deletion.

2 Tumor spectrum in Lynch syndrome (II, III)

At present, cancer of stomach, bladder, brain and kidney are not included in the tumor spectrum of Lynch syndrome according to Amsterdam criteria. For a better understanding of carcinogenesis associated with Lynch syndrome and deficient MMR in general, gastric, brain and urological carcinomas were molecularly characterized and compared with common tumors, such as colorectal and endometrial tumors, from the same families. The tumors (Figure 6) originated from a well-characterized series of Lynch syndrome families from a nationwide registry with the predominant involvement of MLH1 and a high rate of shared mutations. Exon 16 deletion of MLH1, known as Mutation 1, was present in half of the families (Holmberg et al., 1998).

2.1 MMR status in gastric cancer

The gastric cancers were from patients belonging to families with verified Lynch syndrome and all families were known to have germline mutations in the MMR genes. After initial screening, MMR gene germline mutations were found in 87% (13/15) of Lynch syndrome associated gastric cancers. All tumors with mutation were MSI-H and lacked MMR protein expression corresponding to germline mutation. One MSH2 mutation carrier further lacked expression of MSH6 protein (Schweizer et al., 2001). MLH1 was the predisposing mutation in all but two cases, the remaining two being
MSH2 mutation carriers. These tumors were mainly of intestinal histology (12/13, 92% and 1 diffuse). Two out of 15 cancer patients did not have the predisposing MMR gene germline mutation of their families, giving a phenocopy frequency of 13%. The phenotypes of these two patients were identical to the other Lynch syndrome patients, however, these tumors were MSS and showed no evidence of abnormal MMR protein expression.

Out of 46 sporadic gastric cancers, 10 tumors showed MSI-H and were of intestinal histology. The sporadic intestinal MSI gastric cancers were analyzed in comparison to Lynch syndrome gastric cancers, and all showed loss of the MLH1 protein by immunohistochemistry. As described above, the mechanism of MLH1 inactivation was found to be promoter methylation in 70% (7/10) of sporadic MSI gastric cancers in contrast to Lynch syndrome gastric cancers.

2.2 MMR status urinary tract & brain tumors

All urinary tract and brain tumors from Lynch syndrome patients carried MMR gene germline mutation, MLH1 being most commonly affected. However, the relative share of MSH2 was higher among uroepithelial cancers of the bladder and ureter (3/10, 30%), and in brain tumors (3/7, 43%) compared to for instance colorectal cancers (3/35, 9%) and endometrial cancer (3/42, 7%). Urological cancer was rarely the first or only cancer in the patients studied. All tumors of the urinary tract and brain lacked the MMR protein corresponding to the germline mutation. Additionally, the expected loss of MSH6 in the absence of the MSH2 protein was found in 33% (4/12) tumors. The frequency of MSI varied greatly between the different urinary tract and brain tumors. The frequency was highest in ureter (5/5, 100%), intermediate in bladder (3/5, 60%) and low in kidney and brain (1/4, 25% and 0/7, 0%, respectively). Brain tumors were studied with small-pool PCR, and appeared to have a different pattern of MSI, observed by the presence of low share of unstable clones that was masked by the normal alleles present.

2.3 Comparison of molecular profiles in Lynch syndrome associated tumors

Ureter, stomach and colon tumors showed similar levels of MSI, whereas MSI values of bladder tumors resembled those of endometrial tumors. In brain tumors, no MSI was observed by standard PCR. Kidney carcinomas were almost exclusively MSS. The difference is most likely due to tissue-specific patterns of tumor growth (Kuismanen et al., 2002).
As expected, frameshift mutations in mononucleotide repeats followed the general MSI frequency in the tumors, as was seen in sporadic gastric cancers. For example, ACVR2 frameshift mutations were significantly more common in gastric and colorectal cancers compared to ureter cancer (32/33, 97% vs. 1/5, 20%; p = 0.00033). PTEN inactivation is one of the major genetic changes responsible for endometrial tumorigenesis. PTEN involvement was characteristic of, and practically limited to, endometrial carcinoma (Kuismanen et al., 2002, and Figure 6). The Wnt signaling pathway is commonly deregulated in human carcinogenesis either due to inactivating mutations in APC or activating mutations in CTNNB1 genes, leading to the stabilization and accumulation of β-catenin in the nucleus. Previous studies have implicated the role of the Wnt signaling pathway in several types of tumors, both sporadic and hereditary (Giles et al., 2003). Brain and urological tumors seldom displayed nuclear β-catenin in contrast to gastrointestinal (stomach and colon) and endometrial carcinomas; the difference between urinary tract (0/13, 0%) and gastrointestinal (stomach and colon; 25/41, 61%) cancers and between urinary tract (0%) and endometrial carcinomas (10/19, 53%) was statistically significant (p-values 0.000066 and 0.0016, respectively).

Strict or putative LOH at APC occurred significantly more often in the urological samples (7/11, 64%), compared with colon cancers (2/14, 14%; p = 0.016) or endometrial cancers (1/15, 7%; p = 0.0032). As APC-LOH, however, showed a generally poor correlation with nuclear β-catenin in the present series, APC-LOH may reflect general chromosomal instability instead of providing a targeted mechanism to inactivate the wild-type copy of APC. In the gastric cancer study, however, APC-LOH was found to be frequent in intestinal gastric tumors independent of whether the tumor was hereditary or sporadic and whether it was MSI or MSS (20/39, 51%), and was significantly less common in diffuse gastric tumors (2/17, 12%, p=0.001).

In contrast to the gastrointestinal and endometrial carcinomas, KRAS mutations were absent in the brain and urinary tract tumors. The difference between urinary tract cancers (0/14, 0%) and colon cancer (15/48, 31%) was statistically significant (p = 0.028). Furthermore, KRAS mutations were only found in MSI gastric carcinomas. Compared to gastrointestinal cancers (6/41, 15%), p53 protein stabilization occurred at a similar rate in brain tumors (1/6, 17%) but significantly more often in the urinary tract cancers (33–75%, p = 0.0047).

Among 24 tumor suppressor genes examined for promoter methylation (Figure 7), the highest average methylation frequencies were observed in the colorectal (4.0) and gastric carcinomas (4.1), and the lowest in the urinary
Figure 6: Summary of molecular data on MMR gene germline mutation-positive cancers. Proportion of tumors with alterations is shown in percentage. ND, not determined.
tract (1.0–2.3; p = 0.010 relative to gastrointestinal tumors) and brain tumors (1.4; p = 0.039 relative to gastrointestinal tumors). Promoter methylation was significantly associated with MSI gastric cancers, when compared to MSS gastric cancers. *RASSF1* was one of the most commonly methylated genes in the analyzed tumors, with the highest methylation frequencies in kidney (75%) and endometrial carcinoma (71%). Except for kidney, *APC* promoter 1A methylation was found in all carcinomas (especially in stomach, colorectal and endometrial carcinoma, 48-62%), whereas no *APC* methylation was detected in brain tumors. Some genes were methylated in a tissue-specific manner (Table 6), for instance significant *CD44* promoter methylation was detected in kidney cancer only (75%). *ESR1* methylation was limited to adenocarcinomas (especially those of the gastrointestinal tract) with no methylation in urothelial and brain tumors. The highest frequency of *GSTP1* promoter methylation was found in brain tumors (57%).

**Table 6:** Genes with promoter methylation in a minimum of 30% of tumors in at least one tumor type. Numbers shown are percentages, bold numbers refer to over 30% methylation.

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Bladder</th>
<th>Ureter</th>
<th>Kidney</th>
<th>Brain</th>
<th>Stomach</th>
<th>Colon</th>
<th>Endometrium</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>RASSF1</em></td>
<td>20</td>
<td>60</td>
<td>75</td>
<td>43</td>
<td>23</td>
<td>30</td>
<td>71</td>
</tr>
<tr>
<td><em>APC</em></td>
<td>20</td>
<td>20</td>
<td>25</td>
<td>62</td>
<td>56</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td><em>ESR1</em></td>
<td>20</td>
<td>25</td>
<td>46</td>
<td>38</td>
<td>22</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><em>CDH13</em></td>
<td>20</td>
<td>25</td>
<td>57</td>
<td>31</td>
<td>26</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td><em>GSTP1</em></td>
<td>20</td>
<td>25</td>
<td>31</td>
<td>15</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>CD44</em></td>
<td>75</td>
<td>15</td>
<td>54</td>
<td>22</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>CHFR</em></td>
<td></td>
<td></td>
<td></td>
<td>54</td>
<td>22</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><em>PTEN</em></td>
<td>14</td>
<td>31</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 7:** Average number of methylated genes, out of 24 tumor suppressor genes studied, per tumor.
3 Nature of germline defects in point mutation-negative families suspected of Lynch syndrome (IV)

It has previously been observed that in one-third of Lynch syndrome families fulfilling the Amsterdam criteria and in a majority of those not fulfilling these criteria, no mutations in the MMR genes are found, even after comprehensive mutation screening by advanced methods (Renkonen et al., 2003; Holmberg et al., 1998). We searched for germline changes, i.e., the “first hit”, in the MMR genes using deletion and methylation analyses in tumors lacking MMR mutation.

3.1 Germline deletions and epimutations in Lynch syndrome patients

All 45 index patients were suspected of having Lynch syndrome and were mutation-negative by exon-specific genomic sequencing and specific testing for the prevalent founder mutation (Mutation 1). They also lacked any of the three MMR proteins in tumor tissue. Twelve out of 45 patients (27%) were found to have large genomic rearrangements, of which four patients (4/11, 36%) were from the research cohort and eight patients (8/34, 24%) from the clinic-based cohort. Deletions were present in 3/25 (12%), 9/16 (56%), and 0/4 (0%) among index patients lacking MLH1, MSH2, or MSH6 expression, respectively. The difference in deletion frequency between MLH1 and MSH2 was statistically significant (p = 0.0043).

Two patients with MLH1 protein loss in tumor tissue (one from each cohort) showed a germline epimutation in MLH1. Of the five tested CpG-containing regions, three were methylated in patient F36 and all in patient N2. Among the index patients with MSH2 protein loss in tumor tissue, only one displayed promoter methylation in a region that has not been associated with lost expression of MSH2 in the literature. Subsequent bisulphite sequencing of the MSH2 promoter region around HhaI sites monitored by MS-MLPA showed no evidence of methylation at the surrounding CpG sites. There were no cases of MSH6 promoter methylation. The overall frequency of epimutations was therefore 2/45 (4%) in our series.

In patient F36, methylation was found in blood, normal and tumor tissue for three sites out of five studied MLH1 promoter regions, and the higher methylation dosage ratio in tumor tissue implied somatic loss of the unmethylated allele. Primer extension analysis revealed loss of the wild-type allele in tumor DNA. Long-range PCR on genomic DNA from blood of the same patient subsequently showed that the regions not showing
methylation by MS-MLPA were located within a large genomic deletion occurring in the same patient. In conclusion, patient F36 had both a large genomic deletion and a germline epimutation, which overlapped in the same allele, and the observed loss of expression from the mutant allele could result from either change.

In patient N2, all five tested CpG-containing regions were methylated in blood, normal colonic mucosa and colorectal tumor, and the dosage level suggested monoallelic methylation. To prove that methylation affected one allele and left the other allele intact, blood DNA from patient N2 was subjected to bisulphite conversion, after which the promoter region was amplified with methylation-unbiased primers (Suter et al., 2004), cloned, and sequenced. Sequencing of the individual clones showed that either all CpG sites or none were methylated, proving our hypothesis. The patient was heterozygous for the c.655 polymorphism in MLH1 exon 8, which made it possible to investigate the mRNA expression pattern of the two MLH1 alleles in blood; only one allele was expressed, thus the other allele was silenced by methylation. Patient N2 displayed two different cancers of the Lynch syndrome spectrum, both diagnosed at an early age, but the family history was atypical. In addition, none of five first- or second-degree relatives of N2 had the epimutation.

The present index patients with abnormal MLH1 or MSH2 expression were divided into groups with and without large genomic deletions and compared to index individuals from our nationwide registry known to carry MLH1 or MSH2 point mutations. In both MLH1- and MSH2-associated groups, deletion patients showed clearly stronger family histories than those without deletions. An interesting feature of MSH2 point mutation carriers was the predominance of distal over proximal colorectal tumors, and the same was true for patients with large genomic deletions of MSH2 (p = 0.021 for difference between MLH1 and MSH2, with point mutation and large deletion groups combined).
DISCUSSION

1 Gastric, urinary tract and brain tumors & the Lynch syndrome tumor spectrum

In order to include a given tumor in the Lynch syndrome tumor spectrum, there should be increased evidence of the tumor type in Lynch syndrome compared to the average population. Furthermore, evidence is necessary to support the idea that a certain type of tumor from a MMR gene germline mutation carrier has MMR deficiency as the driving force for tumorigenesis (Watson and Riley, 2005).

All available gastric, urinary tract, and brain tumors were collected from families known to have germline mutations in the MMR genes. We started out by determining the mutation status of the respective patients in order to exclude possible phenocopies. The phenocopy rate was 13% (2/15) among patients with gastric cancers, and no phenocopies were found in the patients with urinary tract and brain tumors. All subsequent molecular analyses were based on verified MMR gene mutation carriers.

1.1 Gastric cancers

At present gastric cancer is not included in the Amsterdam II criteria (Vasen et al., 1999), but is included in the revised Bethesda guidelines (Umar et al., 2004a). An overall lifetime risk of gastric cancer by age 70 has been reported to vary between 4% and 19% in Lynch syndrome (Watson et al., 2008; Vasen et al., 2001; Park et al., 2000; Aarnio et al., 1999; Goecke et al., 2006). In Korea and China, gastric cancer is observed to be the most common extracolonic cancer in Lynch syndrome families (Park et al., 2000; Zhang et al., 2005), which probably reflects the high incidence of gastric cancer in the general population of these countries.

Lynch syndrome associated gastric cancers analyzed in this study shared many features of colorectal cancers from Lynch syndrome patients, such as MSI-H, loss of MMR protein expression, high frequency of instability within repeat-containing target genes (ACVR2, TGFβRII, MRE11A) and a high number of methylated promoters (Planck et al., 2000; Kuismanen et al.,
2002; Gonzalez-Aguilera et al., 2003; Schulmann et al., 2005). The Wnt-signaling pathway is activated in 90% of sporadic colorectal cancers (Giles et al., 2003) and is also found to be activated in about 65% of Lynch syndrome colorectal cancers (Miyaki et al., 1999). In the gastric cancers of the present study, the Wnt pathway was found to be involved in nearly half (27/59, 46%), through either alterations in β-catenin expression (nuclear localization) or APC-LOH. Furthermore, LOH in the APC region was associated with intestinal histology regardless of MSI (p=0.007). Altogether, these findings suggest that gastric cancers of Lynch syndrome are similar in many ways to colorectal cancers, which are verified Lynch syndrome tumors. Furthermore, the tumor profile of our Lynch syndrome gastric cancers was compatible with MMR defect as the driving force shown by MMR gene germline defect, MSI-H and loss of MMR protein expression, suggesting that gastric cancers represent true Lynch syndrome spectrum malignancies.

1.2 Urinary tract cancers

In the general population, bladder cancer is by far the most frequent cancer of the urinary tract, whereas in Lynch syndrome renal pelvis and ureter cancer, which are included in Amsterdam criteria II, are more common than bladder cancer (Watson and Lynch, 1993; Aarnio et al., 1999; Vasen et al., 1999). Sijmons et al. (1998) also noted an increased risk for transitional cell cancer of the upper urinary tract in Lynch syndrome. In a recent study, cancers of the urinary tract (kidney, renal pelvis, ureter or bladder) had an overall lifetime risk of 8%, cancer risk rates were higher in males than in females and 7-fold higher in MSH2 than in MLH1 associated families. The lifetime risk estimate for male carriers in MSH2 families was nearly 28% (Watson et al., 2008). Aarnio et al. (1999) also found a higher risk of bladder and ureter cancer (4%), compared to kidney cancer (3.3%) in Lynch syndrome families.

The present study, ureter and bladder cancers from Lynch syndrome families carried MMR gene germline mutation, lacked MMR protein expression and displayed MSI-H. In addition, our findings are compatible with the available literature reports of four urothelial tumors of the ureter or bladder from verified or putative carriers of MSH2 mutations (Hartmann et al., 2003; Planck et al., 2000; Yuen et al., 2002; Mongiat-Artus et al., 2006a) and five urothelial tumors of the ureter and renal pelvis from carriers of a single MSH6 mutation (Wagner et al., 2001) that show high degree MSI and/or MMR protein loss in these tumors as well. Kidney cancer, or renal cell carcinoma, did however differ clearly from the other urinary tract
tumors. Despite being from MMR gene germline mutation carriers, the tumors were mainly MSS and stable at repeat-containing target genes. Our findings suggest that uroepithelial cancers of the ureter (and bladder to a lesser extent) share many features of tumorigenesis driven by MMR deficiency, whereas renal cell carcinomas are unlikely to be part of the Lynch syndrome tumor spectrum.

1.3 Brain tumors

Watson and Lynch (1993) did not find any excess risk of developing brain tumor amongst Lynch syndrome families. However, Hamilton et al., (1995) suggested a possible association between glioblastoma multiforme and Lynch syndrome, i.e., Turcot syndrome. Vasen et al. (1996) found a statistically significantly increased relative risk of brain tumors and concluded that brain tumors belong to the tumor spectrum of Lynch syndrome. A recent study noted that putative mutation carriers did not have a higher incidence of disease than their first-degree relatives, hence these tumors were not considered to be associated with Lynch syndrome (Watson et al., 2008), despite significant excess of brain tumors in Lynch syndrome reported relative to the average population (Vasen et al., 1996; Aarnio et al., 1999; Vasen et al., 1996).

The Lynch syndrome related brain tumors analyzed by us were MSS despite MMR gene germline mutation, loss of wild-type copy and the associated loss of MMR protein expression. One explanation may be clonal heterogeneity, i.e., occurrence of multiple subclones within the tumor (Barnetson et al., 2000). This could be the case in brain tumors as they displayed MSI when analyzed using small-pool PCR. MSI was not visible in a standard PCR probably due to the presence of a minor share of clones with instability and the majority of clones with normal alleles, thus resulting in a MSS pattern. The brain tumors analyzed in our study lacked most molecular alterations tested for, even the methylation pattern differed in brain tumors from all other tumor types. Our data thus suggest different pathways of tumorigenesis for brain tumors compared to other tumors from Lynch syndrome families.

1.4 Overall conclusion

Colorectal and endometrial cancers have been studied extensively in Lynch syndrome families. Here we used colorectal and endometrial cancers, which are unequivocal components of the Lynch syndrome tumor spectrum, as comparison for the less common tumors of Lynch syndrome families. All colorectal cancers displayed MMR gene germline mutation and nearly all
displayed loss of MMR protein expression and MSI-H. Gastric cancer and uroepithelial tumors of the ureter and bladder shared several features of Lynch syndrome colorectal cancers. In contrast, tumors of the brain and kidney were mainly MSS despite loss of MMR protein expression. This suggests that brain tumors and kidney adenocarcinomas develop through different pathways.

2 Differences and similarities between Lynch syndrome and sporadic cancer

Microsatellite instability, resulting from an MMR defect, is found at high frequencies in tumors of Lynch syndrome patients and is also present to a lesser degree in sporadic tumors (Peltomaki, 2003). The frequency of MSI varies according to tissue, with high general fluctuation. As expected, the MSI frequencies were generally higher in our Lynch syndrome tumors compared to findings in literature on their sporadic counterparts (Table 7). Brain and kidney tumors were exceptions, displaying roughly equal MSI frequencies in sporadic vs. Lynch syndrome tumors. Another difference between sporadic and Lynch syndrome tumors is the mechanism of inactivation of the wild-type allele. In MSI sporadic cancer, \textit{MLH1} promoter methylation is a major mechanism, whereas LOH of MMR genes predominates in Lynch syndrome tumors (Ollikainen \textit{et al.}, 2007; Pinto \textit{et al.}, 2008; Leung \textit{et al.}, 1999; Fleisher \textit{et al.}, 1999; Suzuki \textit{et al.}, 1999; Carvalho \textit{et al.}, 2003).

In the literature, the frequencies of MSI in sporadic gastric cancer ranges from 5 to 50\% (Zaky \textit{et al.}, 2008; Ottini \textit{et al.}, 2006; Bacani \textit{et al.}, 2005; Oda \textit{et al.}, 2005), whereas our Lynch syndrome gastric cancers displayed MSI in all cases studied. The Lynch syndrome gastric cancers analyzed in this study closely resembled sporadic intestinal MSI gastric cancers, except that \textit{MLH1} promoter methylation was absent and general epigenetic changes less frequent, suggesting similar but not identical developmental pathways. Similar to our findings on sporadic gastric cancer, Falchetti \textit{et al.} (2008) observed in their sporadic gastric cancers that MSI-H was associated with the intestinal type, according to the Lauren classification, and that MSI-H was strongly associated with loss of MLH1 expression. In agreement with the general notion discussed above, in sporadic intestinal MSI gastric cancer, biallelic methylation of \textit{MLH1} appeared to be the mechanism of MMR inactivation, whereas in Lynch syndrome gastric cancers somatic inactivation of \textit{MLH1} was LOH.

The bladder and ureter tumors included in this study showed MSI fre-
Table 7: MSI in different tissues in tumors from Lynch syndrome vs. sporadic tumors.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Lynch syndrome (our data)</th>
<th>Sporadic (literature)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>100%</td>
<td>5 - 50%</td>
<td>(Oda et al., 2005; Zaky et al., 2008; Ottini et al., 2006; Bacani et al., 2005)</td>
</tr>
<tr>
<td>Ureter</td>
<td>100%</td>
<td>4 - 27%</td>
<td>(Rouprêt et al., 2005; Ericson et al., 2005; Hartmann et al., 2003; Hartmann et al., 2002; Catto et al., 2003; Mongiat-Artus et al., 2006)</td>
</tr>
<tr>
<td>Colorectum</td>
<td>96%</td>
<td>15 - 25%</td>
<td>(Boland et al., 1996; MacDonald et al., 2000)</td>
</tr>
<tr>
<td>Endometrium</td>
<td>63%</td>
<td>9 - 45%</td>
<td>(Gonzalez-Zulueta et al., 1993; Catto et al., 2003)</td>
</tr>
<tr>
<td>Bladder</td>
<td>60%</td>
<td>0 - 10%</td>
<td>(Uchida et al., 1994; Diakoumis et al., 1998; Leach et al., 2002; Thrash-Bingham et al., 1995)</td>
</tr>
<tr>
<td>Kidney</td>
<td>25%</td>
<td>8 - 18%</td>
<td>(Zhu et al., 1996; Lundin et al., 1998; Malmer et al., 2001; Szybka et al., 2003; Eckert et al., 2007; Martinez et al., 2007)</td>
</tr>
<tr>
<td>Brain</td>
<td>0%</td>
<td>0 - 30%</td>
<td>(Zhu et al., 1996; Lundin et al., 1998; Malmer et al., 2001; Szybka et al., 2003; Eckert et al., 2007; Martinez et al., 2007)</td>
</tr>
</tbody>
</table>

Quencies that exceed those reported for the corresponding sporadic tumors (Gonzalez-Zulueta et al., 1993; Hartmann et al., 2002; Hartmann et al., 2003; Catto et al., 2003; Ericson et al., 2005; Roupret et al., 2005; Mongiat-Artus et al., 2006b). This may indicate that somatic MMR inactivation is a minor pathway in the development of sporadic upper urothelial cancer (Ericson et al., 2005). The occurrence of MSI in sporadic kidney cancer in literature and our Lynch syndrome associated kidney cancer was rare (Uchida et al., 1994; Diakoumis et al., 1998; Thrash-Bingham et al., 1995; Leach et al., 2002). Additionally, loss of function of MLH1 and MSH2 does not usually occur in sporadic kidney cancer, either by promoter methylation or exonic mutation (Rubio-Del-Campo et al., 2008). Together with the infrequent occurrence of MSI in sporadic renal cell carcinomas, our findings suggest that renal cell carcinomas are unlikely to be part of Lynch syndrome, contrary to ureter and possibly bladder carcinoma.

In contrast to brain tumors analyzed in this study where no MSI was observed by standard PCR, MSI frequencies for sporadic gliomas have been reported to range up to 30% (Zhu et al., 1996; Lundin et al., 1998; Malmer et al., 2001; Szybka et al., 2003; Eckert et al., 2007; Martinez et al., 2007), and is reported to be absent or rare in gangliogliomas [0%, (Zhu et al., 1996)] and meningiomas [1–2%, (Zhu et al., 1996; Kirsch et al., 1997)]. However, nearly all Lynch syndrome brain tumors we studied displayed
loss of MMR protein corresponding to germline mutation, whereas in other studies loss of MMR protein expression is reported to be infrequent in brain tumors (Eckert et al., 2007; Martinez et al., 2007).

3 Germline defects in mutation-negative Lynch syndrome patients

We found large genomic deletions in nearly a third of the tumors from suspected Lynch syndrome families, not including the Finnish founder mutation (MLH1 del ex16; Nystrom-Lahti et al., 1995) which was excluded at the outset. Our frequency is in the upper range of deletion rates reported for comparable series in literature (Gille et al., 2002; Baudhuin et al., 2005; Grabowski et al., 2005; Pistorius et al., 2007). Germline epimutations in MLH1 were found in two patients (4%), the frequency being in the range of previous studies (Gazzoli et al., 2002; Miyakura et al., 2004; Hitchins et al., 2007; Valle et al., 2007). Even though methylation in our epimutation carriers was found to be present in all tested tissues (blood, normal mucosa and colorectal cancer), it did not show evidence of heritability, because epimutation was absent in studied first- and second-degree relatives. Some studies provide evidence of heritability or transgenerational epigenetic inheritance of germline epimutation (Chan et al., 2006; Hitchins et al., 2007). The remaining investigations indicate the absence of epimutation in other family members (Suter et al., 2004; Miyakura et al., 2004; Valle et al., 2007; Hitchins et al., 2005). Morak et al. (2008) described MLH1 epimutation cases that were compatible with either heritability, de novo methylation, mosaic or incomplete methylation, or lack of heritability.

No genetic or epigenetic germline defect was detectable in nearly half of our cases with lost MSH2 protein and in a majority of MLH1 or MSH6-associated cases, leaving their genetic etiology unknown. Wagner et al. (2002) described a Lynch syndrome family caused by an inversion disrupting the MSH2 gene. Hence, certain structural alterations, such as inversions, as well as regulatory changes in non-coding regions (Shin et al., 2002) remain theoretical possibilities for MMR gene inactivation. Loss of PMS2 protein in tumor tissue could point to germline mutations in PMS2, which have been found to account for 5% of Lynch syndrome cases, however, the penetrance appears to be lower than that for the other MMR genes (Cladenning et al., 2008; Truninger et al., 2005; Hendriks et al., 2006). In our study, the instability of PMS2 was probably secondary to the loss of MLH1, since all cases with absent PMS2 also lacked MLH1 (Chang et al., 2000). As we generally did not have tumor samples available from several affected
family members, we were unable to evaluate concordance for immunohistochemical staining patterns and MSI within families. It is possible that MMR gene silencing in tumor tissue occasionally resulted from completely somatic mechanisms, since most of our cases only met the revised Bethesda guidelines at best. In our research cohort we had tumor samples available and found that, at least for \textit{MLH1}, somatic hypermethylation was a probable mechanism for MMR gene silencing.
CONCLUSIONS & FUTURE PROSPECTS

Despite origin from verified MMR gene mutation carriers, the frequency of high-level microsatellite instability in tumors varied between high (100–96% for ureter, stomach and colon), intermediate (63-60% for endometrium and bladder) and low (25-0% for kidney and brain). Also, promoter methylation of tumor suppressor genes distinguished the tumors in an organ-specific manner. Whereas, all Lynch syndrome colorectal cancers displayed MMR gene germline mutation and nearly all displayed loss of MMR protein expression and MSI-H, tumors of brain and kidney were mainly MSS, despite loss of MMR protein expression. This suggests that brain tumors and kidney adenocarcinomas develop through different pathways. On the other hand, gastric cancer and uroepithelial tumors of ureter and bladder shared several features of Lynch syndrome colorectal cancers.

Gene conversion has been described as a mechanism of both germline and somatic inactivation of genes (Cavenee et al., 1983), but little is known about its role in cancer (Chen et al., 2007). Analysis of cancer specimens from two independent sets of Swiss and Finnish MLH1/MSH2 deletion carriers revealed that somatic deletions nearly identical to the ones in the germline occur frequently in colorectal cancers. Chromosome-specific marker analysis further implied that loss of the wild-type allele predominantly occurred through locus-restricted recombinational events, i.e., gene conversion, rather than mitotic recombination or deletion of the respective gene locus. This may be helpful for the identification of germline mutations of the MMR genes.

Large genomic deletions (mainly MSH2) may explain a significant proportion and germline epimutations (in MLH1) a smaller fraction of point mutation-negative families/cases with MMR protein loss in tumor tissue. In contrast to genomic deletions, which are associated with strong family histories for Lynch syndrome, epimutations occurred in patients with multiple early-onset tumors without any significant family history. Still no genetic or epigenetic germline defect was detectable in almost half of the cases suspected of Lynch syndrome with lost MMR protein expression,
leaving their genetic etiology unknown. Further characterization of these tumors is necessary to identify the underlying defects to improve the diagnosis, counseling, and management of the patients and their families.

The same approach as used for gastric, urinary tract and brain tumors in the present study could be performed to investigate the possible association of other tumors in Lynch syndrome, such as breast cancer. Breast cancer has also been observed in Lynch syndrome family members and, in some cases, the inherited mismatch repair gene mutation has been shown to have played a role in its development (Vasen et al., 2001; Boyd et al., 1999). Other studies provide no evidence that Lynch syndrome cases are at higher risk for breast cancer than members of the general population (Watson et al., 2008). Hence, Lynch syndrome-associated elevation of breast cancer risk has not been convincingly demonstrated and therefore, it would be important to further study the role of breast cancer in Lynch syndrome.

Germline epimutations in tumor suppressor genes other than MMR genes may be interesting to study as possible contributing mechanisms to non-syndromic types of familial cancer where no clear underlying mechanism has yet been found. Since germline epimutations lack a uniform pattern of inheritance (Morak et al., 2008) and present with rather diverging clinical characteristics, they could hence serve plausible explanations for non-syndromic types of hereditary cancer. Also, the availability of new methods, such as MS-MLPA, to study the role of epigenetics in tumor development, enables screening of several tumor suppressor genes simultaneously.

In conclusion, this study provides better understanding of Lynch syndrome associated tumors, particularly gastric cancer, urinary tract cancers and brain tumors. Gastric cancer was observed to be a true Lynch syndrome tumor with MMR deficiency as the driving force of tumorigenesis. The uroepithelial tumors of bladder and ureter displayed MMR gene germline mutation and subsequent loss of MMR protein and high frequency MSI, compatible with MMR tumorigenesis. Brain tumors and kidney carcinoma, on the other hand, were mostly MSS despite MMR gene mutation and loss of protein expression, implying the possibility of alternative routes of tumor development. The findings of the present study present possible implications in clinical cancer surveillance.
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detection and familial predisposition: Development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res. 58:5248-5257.


of four novel mutations in hereditary nonpolyposis colorectal cancer. Mutations in Brief No. 144. Online. Hum. Mutat. 11:482.


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


