MOLECULAR PATHOGENESIS IN HEREDITARY NON-POLYPOSIS COLORECTAL CANCER (HNPCC) SYNDROME

Reetta Kariola

Division of Genetics
Department of Biological and Environmental Sciences
Faculty of Biosciences
University of Helsinki

Academic Dissertation

To be publicly discussed, with the permission of the Faculty of Biosciences,
University of Helsinki,
in the auditorium 1041 of the Viikki Biocenter, Viikinkaari 5, Helsinki,
on the 10th of December, 2004, at 12 o'clock noon.

Supervisor Docent Minna Nyström, Ph.D.
Department of Biological and Environmental Sciences
Division of Genetics
University of Helsinki
Finland

Reviewers Docent Nina Horelli-Kuitunen, Ph.D.
Medix Laboratories Ltd.
Espoo, Finland

Docent Jukka Partanen, Ph.D.
Department of Tissue Typing and Research Laboratory
Division of Stem Cell and Transplantation Service
Finnish Red Cross Blood Service
Helsinki, Finland

Opponent Docent Anne Kallioniemi, M.D., Ph.D.
Institute of Medical Technology
Laboratory of Cancer Genetics
University of Tampere
Finland

ISSN 1239-9469
ISBN 952-10-2139-X
ISBN 952-10-2140-3 (PDF)

Helsinki University Printing House, Helsinki 2004

"There is nothing, Sir, too little for so little a creature as man. It is by studying little things that we attain the great art of having as little misery and as much happiness as possible."

-Samuel Johnson

CONTENTS

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals I-IV:

I Kariola R, Raevaara TE, Lönnqvist KE, Nyström-Lahti M (2002) Functional analysis of *MSH6* mutations linked to kindreds with putative hereditary non-polyposis colorectal cancer syndrome. Hum Mol Genet 11:1303-1310

II Kariola R, Otway R, Lönnqvist KE, Raevaara TE, Macrae F, Vos YJ, Kohonen-Corish M, Hofstra RMW, Nyström-Lahti M (2003) Two mismatch repair gene mutations found in a colon cancer patient – which one is pathogenic? Hum Genet 112:105-109

III Kariola R, Hampel H, Frankel WL, Raevaara TE, de la Chapelle A, Nyström-Lahti M (2004) *MSH6* missense mutations are often associated with no or low cancer susceptibility. Br J Cancer 91:1287-1292

IV Kariola R*, Abdel-Rahman WM*, Ollikainen M, Bützow R, Peltomäki P, Nyström M (2004) APC and β-catenin protein expression patterns in HNPCC-related endometrial and colorectal cancers. Submitted

*These authors have contributed equally to the work

ABBREVIATIONS

APC	*adenomatous polyposis coli*
CAN1	the gene encoding canavanine resistance, Can^r
CAH	complex atypical hyperplasia
CTNNB1	*β-catenin*
CRC	colorectal cancer
DCC	*deleted in colorectal cancer*
EC	endometrial cancer
E. coli	*Escherichia coli*
Exo	exonuclease
FAP	familial adenomatous polyposis
GSK-3β	*glycogen synthase kinase -3β*
GST	glutathione S-transferase
HNPCC	hereditary non-polyposis colorectal cancer
HNPCC-CRC	HNPCC-related colorectal cancer
HNPCC-EC	HNPCC-related endometrial cancer
ICG-HNPCC	the International Collaborative Group on HNPCC
IDL	insertion-deletion loop
IGFIIR	*insulin-like growth factor II receptor*
InSiGHT	International Society for Gastrointestinal Hereditary Tumors
Lef	lymphoid enhancer factor
LOH	loss of heterozygosity
MMR	mismatch repair
MSI	microsatellite instability
MSS	microsatellite stable
NE	nuclear extract
NF-1	neurofibromatosis type 1
S. cerevisiae	*Saccharomyces cerevisiae*
Sf9	*Spodoptera frugiperda 9*
TCF4	*T-cell transcription factor 4*
TGFβRII	*transforming growth factor β receptor II*
Wnts	Wnt signal molecules
WT	wildtype

ABSTRACT

Hereditary non-polyposis colorectal cancer (HNPCC) is a dominantly inherited cancer syndrome. Germline mutations in a four different mismatch repair (MMR) genes, *MSH2*, *MSH6*, *MLH1*, and *PMS2*, are convincingly associated with HNPCC. Germline mutations in *MSH2* and *MLH1* genes are the most common and they are mainly identified in classical HNPCC families, which display the typical clinical and molecular features of the syndrome, such as early age of onset and high microsatellite instability (MSI) in tumor cells. High MSI is a consequence of a defect in DNA mismatch repair and the pathogenicity of germline mutations in HNPCC is thus linked to the malfunction of MMR. *MSH6* is the third most common suscebtibility gene in the HNPCC syndrome, but the pathogenicity of *MSH6* mutations often remains questionable, because these mutations are mainly linked to putative HNPCC families with a late age of onset, few affected family members, an atypical tumor spectrum with an excess of endometrial cancers and a lack of or low MSI phenotype. A significant proportion of all mutations identified in *MSH6* are missense alterations and their pathogenicity is often difficult to determine without functional studies.

Our main aim was to elucidate whether and how *MSH6* missense mutations cause cancer susceptibility. We clarified the functional consequences of eleven potentially pathogenic *MSH6* mutations which were found in putative HNPCC families. We also tried to find out which one of the two MMR gene mutations, one in *MSH2* and one in *MSH6*, found in a colon cancer patient could primarily underlie the cancer susceptibility in the patient's family. We studied the expression level of the protein variants, heterodimerization of MSH6 variants with MSH2 and the functionality of these MutSα complexes in a human cell-based *in vitro* MMR assay.

Ten out of eleven MSH6 protein variants were able to interact with wildtype MSH2 protein and they were also able to correct mismatches in the *in vitro* mismatch repair assay. One of those, MSH6-G566R, carrying a mutation in the MSH2-MSH6 interaction region, had a similar repair activity to wildtype protein but was poorly expressed suggesting problems in its stability. Only one mutation, MSH6-E1193K, showed marked impairment of heterodimerization with MSH2 and mismatch repair capability. This mutation occurred in two women with endometrial cancer (ages of onset 59 and 60 years) without any family history of cancer suggestive of HNPCC. Neither of the two MMR gene mutations found in a single colon cancer patient showed MMR deficiency. However, our results do not exclude the possible compound pathogenicity of the two mutations.

Our results are clinically relevant since they demonstrate that in stable *in vitro* circumstances, when the amounts of the proteins were sufficient for repair, 10 out of 11 tested *MSH6* mutations were MMR-proficient. This finding, together with clinical data of the patients and their families, leads to the conclusion that many or most missense changes in *MSH6* are clinically innocent. Some missense alterations, such as MSH6-E1193K, which lead to impaired mismatch repair, are likely to be clinically significant, but have low penetrance. However, it is also possible that while the typical HNPCC syndrome is linked to malfunction of the MMR reaction, the pathogenicity of mutations in putative HNPCC families is linked to other biochemical events preceding the repair reaction, e.g. shortage of functional protein. Especially in situations where cosegregation of mutation and disease phenotype cannot be studied, a functional test is needed to confirm that cancer susceptibility in a family is linked to a found mutation.

In HNPCC-related tumors, the inactivation of MMR genes is an early event in tumorigenesis. DNA MMR deficiency then leads to the inactivation of many growth-regulatory genes that contain coding nucleotide repeats. This promotes activation of pathways which lead to the expression of genes that favor cell growth. The molecular basis of organ selection in HNPCC tumorigenesis is poorly understood, and it is not known why activation of the mutator pathway occurs more commonly in the colon and endometrium than in other organs. Overactivation of the Wnt signaling pathway has been shown to play an important role in tumorigenesis of certain carcinomas. Abnormalities in APC or β-catenin, the main components of this pathway, are common in colorectal carcinomas (CRCs), including HNPCC-related CRCs, as well as in sporadic endometrial carcinomas (ECs), but HNPCC-related ECs are poorly characterized in this regard. We compared the expression of APC and β-catenin in 31 CRCs and 19 ECs from HNPCC patients carrying the same predisposing mutations in either *MLH1* or *MSH2*. Nuclear accumulation of β-catenin was judged to represent overactivation of the Wnt pathway and this overactivation was seen in 84% (26/31) of CRCs as compared to 53% (10/19) (P=0.02) of ECs. Accumulation of β-catenin was shown to be independent of its negative regulator, APC, in a large fraction of CRCs and even more so in ECs. In conclusion, we provide the first evidence that APC/β-catenin abnormalities are involved in HNPCC-related endometrial tumorigenesis. APC/β-catenin abnormalities in HNPCC-related ECs, although significant, are less frequent than in HNPCC-related CRCs even when the same mismatch repair gene mutation predisposed to both tumor types.

INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC) syndrome is the most common form of hereditary colon cancers (Lynch and de la Chapelle 2003). In addition to colorectal cancer (CRC), HNPCC families often display an excess of extracolonic cancers, especially endometrial cancer (EC) (Aarnio et al. 1999). Early age of onset, numerous affected family members and multiple primary tumors are characteristic features of HNPCC, as in many other hereditary cancer syndromes.

The genetic basis for HNPCC became evident in the early 1990s with the demonstration that a germline mutation in a DNA mismatch repair (MMR) gene results this syndrome (Aaltonen et al. 1993; Peltomäki et al. 1993). DNA MMR is one of several repair mechanisms that are required to maintain genomic stability in prokaryotic and eukaryotic cells. Malfunctions of the MMR system create genetic instability. This instability is mainly observed in microsatellite sequences and is called microsatellite instability (MSI) (Ionov et al. 1993; Thibodeau et al. 1993). Most microsatellite repeats in the genome are located within noncoding sequences and are thus considered to be silent. However, in MSI carcinomas, many growth-regulatory genes show instability in microsatellites within their coding regions. The tumor cells of HNPCC patients usually display a high MSI phenotype. Moreover, sporadic counterparts of carcinomas included in the HNPCC tumor spectrum may also show a high degree of MSI (Helland et al. 1997; Boland et al. 1998; Gurin et al. 1999). In these tumors, the MSI phenotype mostly results from the epigenetic silencing of MMR genes and more rarely from somatic MMR gene mutations (Liu et al. 1995; Kane et al. 1997; Veigl et al. 1998; Esteller et al. 1999; Kuismanen et al. 2000).

The MMR genes *MLH1* and *MSH2* account for the majority of HNPCC cases. Germline mutations in these genes are mainly associated with classical HNPCC families, which display the typical clinical features of the syndrome and a high MSI phenotype in tumor cells (Liu et al. 1996, Nyström-Lahti et al. 1996). *MSH6* is the third most common suscebtibility gene in HNPCC syndrome accounting for 7% of reported mutations found in the HNPCC kindreds (http://www.nfdht.nl). MSH6 protein forms a heterodimeric complex with MSH2 protein and this complex recognizes base-base mismatches and one basepair insertion-deletion loops (Acharya et al. 1996; Genschel et al. 1998). A significant proportion of all mutations identified in the *MSH6* gene are missense mutations of unknown significance. Since *MSH6* missense mutations are often associated with small and

putative HNPCC families (Kolodner et al. 1999; Wijnen et al. 1999; Wu et al. 1999; Parc et al. 2000; Wagner et al. 2001), which lack the typical molecular and clinical features of the syndrome, their pathogenicity is impossible to evaluate without a functional test. To date, only a yeast-based functional assay has been used to study the pathogenicity of *MSH6* missense mutations derived from HNPCC patients (Kolodner et al. 1999). Here, we used a human cell-based *in vitro* mismatch repair assay to clarify their pathogenicity (Lahue et al. 1989; Holmes et al. 1990). Previously this assay has been successfully used to study the pathogenicity of *MLH1* missense mutations (Nyström-Lahti et al. 2002; Raevaara et al. 2002). By complementing cells which were MSH6-deficient ($MSH6^{-/-}$) with overexpressed mutated MSH6 protein, and by using heteroduplex-DNA with a base-base mismatch as a substrate, we could study the MMR capability of MSH6 variants. With the same assay we also tried to clarify which one of the two mismatch repair gene mutations found in a single colon cancer patient could primarily underlie the cancer susceptibility in the family. Our findings are clinically relevant since they provide essential data concerning the pathogenicity of *MSH6* missense mutations.

The predisposition to HNPCC is inherited in an autosomal dominant fashion. Consistent with that, genetically predisposed individuals carry one mutant and one wildtype copy of a given MMR gene in their normal tissue, and only if the wildtype copy is also inactivated does the cell become MMR-deficient. This causes the activation of the mutator pathway, which leads to genomic instability and cancer formation. The reasons why bi-allelic gene inactivation occurs more frequently in the colon and endometrium than in other organs are not yet understood. We had a great opportunity to clarify the tumorigenesis in these two organs by using unique tumor material from HNPCC patients who shared the same germline mutation in a MMR gene. In recent years, the Wnt signaling pathway has received considerable attention because it has been reported to play an important role in the formation of certain tumors, especially colorectal cancer. The Wnt signaling pathway has been shown to be overactivated in 65% of HNPCC-related CRCs (Miyaki et al. 1999). However, until now the role of this signaling pathway had not been determined in HNPCC-related ECs. Here, the question of organ specificity and the role of the Wnt signaling pathway in ECs was addressed by studying APC and β-catenin expression patterns in colorectal and endometrial tumor samples from HNPCC patients carrying the same predisposing mutations.

REVIEW OF THE LITERATURE

HEREDITARY NON-POLYPOSIS COLORECTAL CANCER SYNDROME

Hereditary cancer

In Finland, one in four individuals will get some form of cancer during their lifetime (http://www.cancerregistry.fi). Cancer is a genetic disorder, which means that mutations in specific genes offer a selective growth advantage for specific cells. These transformed cells then form a tumor in a specific tissue. Such mutations may occur sporadically or be inherited. Susceptibility to hereditary cancers is often inherited in an autosomal dominant manner. This means that a genetically predisposed individual has one mutated and one wildtype copy of a specific cancer-susceptibility gene in every cell. However, tumor formation generally begins only if the wildtype allele of this gene is also inactivated. Thus, tumors develop as a recessive trait and require two mutations, one germinal and one somatic (Knudson 1971). Typical features of inherited cancer syndromes are early age of onset, numerous affected family members and multiple primary tumors. One exceedingly common cancer in western countries is colorectal cancer (CRC). In Finland, it is estimated that approximately 1500 new cases of CRC will occur in 2004 and that approximately 5-10% of those cases are inherited (http://www.cancer.fi). The two most common and best defined familial forms of CRC are hereditary non-polyposis colorectal cancer and familial adenomatous polyposis (FAP) syndromes.

Characteristics of hereditary non-polyposis colorectal cancer syndrome

Hereditary non-polyposis colorectal cancer, also named Lynch syndrome, is the most common form of hereditary colon cancer accounting for 1-6% of all CRCs. The syndrome is characterized by the predominance of right-sided colorectal cancers in multiple affected generations (Lynch and de la Chapelle 2003). The mean age of onset is 45 years compared to 65 years in sporadic CRCs (Lynch and de la Chapelle 1999). In addition to CRC, HNPCC families display an excess of extracolonic cancers; notably carcinoma of the endometrium, ovary, stomach, small bovel, pancreas, hepatobiliary tract, brain and upper uroepithelial tract (Watson and Lynch 1994; Aarnio et al. 1999). However, the colon and endometrium are the two most commonly affected tissues in HNPCC and it is

reported that the incidence of endometrial cancer (EC) exceeds even that of CRC in females (Aarnio et al. 1999).

The genetic basis for HNPCC became evident in the early 1990s with the demonstration that a germline mutation in a DNA mismatch repair gene results in this syndrome (Peltomäki et al. 1993). The direct consequence of defective DNA mismatch repair is the so-called mutator phenotype which creates genetic instability throughout the genome (Aaltonen et al. 1993). Susceptibility to HNPCC is inherited in an autosomal dominant fashion. An inherited alteration in a mismatch repair gene does not as such result in widespread developmental anomalies, because a wildtype allele provides sufficient DNA mismatch repair activity. Thus, before a tumor forms, the second copy of the affected DNA mismatch repair gene has to be somatically inactivated (Hemminki et al. 1994). This means that the tumor develops as a recessive trait, which requires bi-allelic gene inactivation (Knudson 1971). The reasons why this bi-allelic gene inactivation occurs more frequently in the colon and endometrium than in other organs are not yet understood.

The first event in HNPCC-related tumorigenesis is the inactivation of a MMR gene (Aaltonen et al. 1994, Shibata et al. 1994; Konishi et al. 1996; Tannergård et al. 1997, de Leeuw et al. 2000). DNA MMR deficiency then leads to the inactivation of many growth-regulatory genes that contain coding nucleotide repeats. Many of these affected genes are general tumor suppressor genes (Markowitz et al. 1995; Parsons et al. 1995; Souza et al. 1996; Chadwick et al. 2000; Guanti et al. 2000; Piao et al. 2000), as well as genes that function in DNA mismatch repair (Malkhosyan et al. 1996), Wnt signaling (Duval et al. 1999a; Liu et al. 2000) and apoptotic pathways (Rampino et al. 1997). Thus, DNA MMR deficiency promotes the activation of many pathways which lead to the expression of genes that favor cell growth.

Clinically, HNPCC syndrome is not as clearly defined as for example FAP, the other common hereditary colon cancer syndrome, with hundreds to thousands of colorectal polyps (Groden et al. 1991; Nishisho et al. 1991). In HNPCC, there is no tendency for extensive polyposis, and hence it is not easily distinguished from sporadic CRC. For the uniform diagnosis of HNPCC, the first international criteria were defined in 1991 (Vasen et al. 1991). These so-called Amsterdam criteria I were later considered to be too restrictive and the new, less stringent Amsterdam criteria II were established in 1999. These criteria also accept, in

addition to CRC, extracolonic carcinomas, notably ECs (Table 1) (Vasen et al. 1999). Microsatellite instability is the most characteristic feature of HNPCC-related tumors, even if it also occurs in about 20% of sporadic counterparts of tumors belonging to the HNPCC tumor spectrum (Helland et al. 1997; Boland et al. 1998; Gurin et al. 1999; Peltomäki 2001). Thus, MSI testing is an important tool in identifying HNPCC patients who do not meet the Amsterdam criteria, but whose tumors still show the MSI phenotype. The Bethesda guidelines were formulated in 1997 and revised in 2004 to identify CRC patients who should be tested for MSI (Table 1) (Rodriguez-Bigas et al. 1997; Umar et al. 2004). MSI testing is also frequently used to select cancer patients for mutation analysis of HNPCC genes (Aaltonen et al. 1998; Wijnen et al. 1998).

Table 1 Clinical criteria for the diagnosis of the HNPCC syndrome

Amsterdam criteria I (Vasen et al. 1991)
At least three relatives must have histologically verified colorectal cancer:
One must be a first degree relative of the other two
At least two successive generations must be affected
At least one of the relatives with colorectal cancer must have received the diagnosis before the age of 50 years
Familial adenomatous polyposis should have been excluded
Amsterdam criteria II (Vasen et al. 1999)
At least three relatives must have a cancer associated with HNPCC (colorectal, endometrial, stomach, ovary, ureter or renal pelvis, brain, small-bowel, hepatobiliary tract or skin):
One must be a first degree relative of the other two
At least two successive generations must be affected
At least one of the relatives with cancer associated with HNPCC should have received the diagnosis before the age of 50 years
Familial adenomatous polyposis should have been excluded in any relative with colorectal cancer
Presence of tumor should be verified whenever possible
Bethesda criteria (Umar et al. 2004)
Tumors from individuals should be tested for MSI in the following situations:
CRC diagnosed in a patient who is less than 50 years of age
Presence of synchronous, metachronous CRC, or other HNPCC-related tumors, regardless of age
CRC with high MSI histology diagnosed in a patient who is less than 60 years of age
CRC diagnosed in one or more first-degree relatives with an HNPCC-related tumor, with one of the cancers being diagnosed under age 50 years
CRC diagnosed in two or more first- or second-degree relatives with HNPCC-related tumors, regardless of age

The table is adapted and modified from Lynch and de la Chapelle 2003

DNA MISMATCH REPAIR

Overview of DNA mismatch repair

DNA mismatch repair is one of the repair processes that are required to maintain genomic stability in prokaryotes and eukaryotes. The main function of MMR is to correct single base mismatches and insertion-deletion loops (IDLs), which arise during replication and escape from the proofreading activity of DNA polymerases. Mismatches and IDLs can also result from recombination or DNA damage.

Cells deficient in MMR exhibit a mutator phenotype in which the spontaneous mutation rate is greatly elevated. This can be recognized from frameshift mutations in many regions throughout the genome where there are short tandemly arranged, identical segments of DNA. These repeating units are called short tandem repeats or microsatellites and they are from one to four base pairs in length. In addition, MMR-deficient cells can be hyper-recombinogenic because MMR normally prevents recombination between divergent sequences, thus promoting genetic stability (Sugawara et al. 1997). The subset of proteins involved in the MMR reaction are also implicated in other functions, such as double-strand break repair (Marsischky et al. 1999), meiotic and mitotic recombination (Wang et al. 1999a), the transcription-coupled nucleotide-excision repair pathway (Mellon et al. 1996; Mellon and Champe 1996), cell-cycle regulation (Davis et al. 1998) and the TP53-dependent apoptotic response to a variety of forms of damage to DNA. (Duckett et al. 1999). Thus, the MMR proteins play a critical role in many fundamental cellular processes.

The MMR mechanism was originally identified in the bacterium *Escherichia coli* (*E. coli*) (Glickman et al. 1980). The importance of this repair system in humans became evident with the demonstration that its inactivation is the primary cause of hereditary non-polyposis colorectal cancer syndrome (Aaltonen et al. 1993; Peltomäki et al. 1993). The key components of the MMR mechanism are conserved in evolution, which has been a great help in attempting to define the corresponding system in eukaryotes. Multiple homologs of the *E. coli* MMR genes have been found in yeast and mammals. Yeast MMR has been characterized most extensively in *Saccharomyces cerevisiae* (*S. cerevisiae*) (Schar et al. 1997) and knowledge of the human system is mainly based on studies in this organism. The

key MMR components have now been identified in yeast and human. However, many specific proteins and their major activities need still to be specified.

DNA mismatch repair reaction

The repair process consists of four principal steps: (1) mismatch recognition, (2) repairosome assembly, (3) strand discrimination linked to degradation of the newly synthesized strand and (4) resynthesis of excised strand. Some major activities and components of the DNA mismatch repair reaction in bacteria, yeast and humans are listed in Table 2. Figure 1 shows a putative model of the human MMR mechanism.

Table 2 The proteins required for DNA mismatch repair in *E. coli*, *S. cerevisiae* and *H. sapiens*

Activity or function	***E. coli***	***S. cerevisiae***	***H. sapiens***
Mismatch recognition	MutS (ATPase) β-clamp	MutSα (Msh2+Msh6) MutSβ (Msh2+Msh3) PCNA?	MutSα (MSH2+MSH6) MutSβ (MSH2+MSH3) PCNA?
Coupling of mismatch recognition to the subsequent steps of repair	MutL (ATPase)	MutLα (Mlh1+Pms1) MutLβ (Mlh1+Mlh3) MutL (Mlh1+Mlh2)	MutLα (MLH1+PMS2) MutLβ (MLH1+PMS1) MutL (MLH1+MLH3)
Strand discrimination	MutH (GATC endonuclease)	Strand discontinuity? PCNA?	Strand discontinuity? PCNA?
Unwinding of DNA	DNA helicase II (uvrD/MutU)	DNA helicase?	DNA helicase?
Excision of mismatch containing portion	ExoVII or RecJ (5′-3′) ExoI, ExoVII or ExoX (3′-5′)	ExoI (5′-3′) RAD27? (5′-3′) DNA Polymerase δ (3′-5′) DNA Polymerase ε (3′-5′)	HEXI (EXOI) (5′-3′) FEN1 (RAD27)? (5′-3′) DNA Polymerase δ (3′-5′) DNA Polymerase ε (3′-5′)
Protection of single-stranded template	SSB	RPA	RPA
Repair synthesis	DNA polymerase III	DNA polymerase δ DNA polymerase ε?	DNA polymerase δ DNA polymerase ε?
Ligation	DNA ligase	DNA ligase I?	DNA ligase I?

?, remains to be identified
Exo, exonuclease
PCNA, proliferating cell nuclear antigen
RPA, replication protein A
SSB, single-strand DNA-binding protein

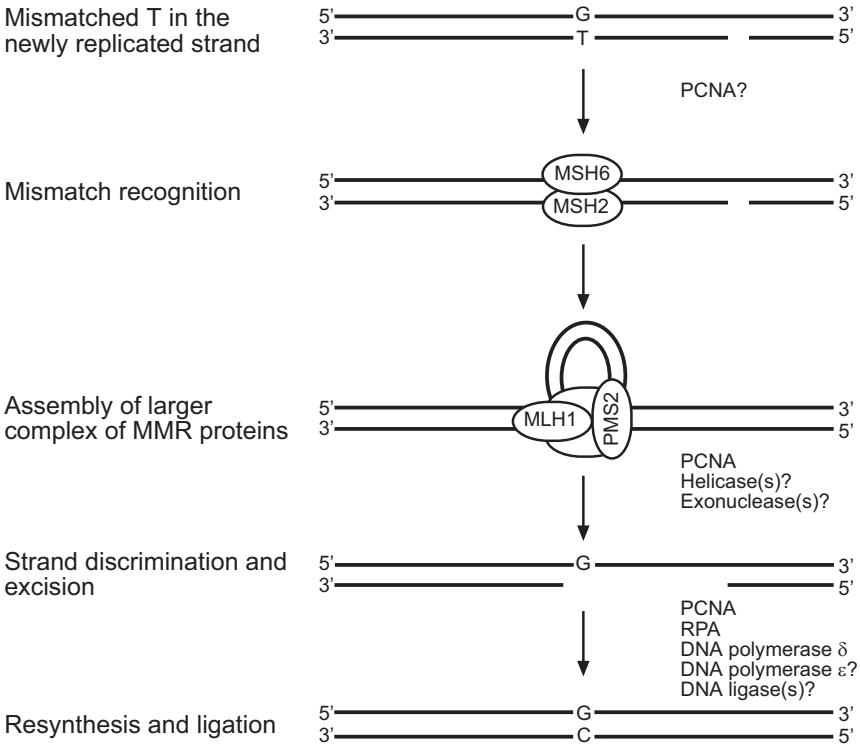

Figure 1 Putative model of the human MMR reaction. MSH2-MSH6 heterodimer (MutSα) recognizes the mismatched T-G pair, where the T is in the newly replicated strand. Prior to mismatch recognition, MSH2-MSH6 has formed a ternary complex with proliferating cell nuclear antigen (PCNA) and fully base-paired DNA. Once this ternary complex encounters the mispair, the MSH2-MSH6 is transferred from PCNA to the mispair, PCNA exits the complex and the MMR pathway is activated (Lau and Kolodner 2003). The heterodimer undergoes a conformational change driven by ATP and slides along DNA forming a loop (Iaccarino et al. 1998). The assembly of MLH1-PMS2 complex (MutLα) onto MSH2-MSH6 at the mispair couples mismatch recognition to further steps along the pathway(Fang and Modrich 1993; Li and Modrich 1995). Excision of a large, newly replicated DNA fragment containing the mismatched T is initiated by DNA exonuclease(s) and DNA helicase(s). DNA exonuclease cleaves nucleotides one at a time from the end of the polynucleotide chain and DNA helicase separates the strands of DNA. The method of discrimination between the template and the newly synthesized strand is unknown. It is possible that strand discontinuities mark the nascent strand during replication *in vivo* (Holmes et al. 1990; Thomas et al. 1991). Other model suggests that PCNA acts as a link between the MMR complex and DNA polymerase at the replication fork, possibly facilitating recognition and repair of the nascent strand (Umar et al. 1996). Resynthesis of the excised strand appears to be executed by DNA polymerase δ and possibly DNA polymerase ε (Thomas et al. 1991; Modrich 1997), PCNA and replication protein A (RPA) (Lin et al. 1998). The DNA ligase(s) that completes the MMR reaction remains to be identified. As a result of the MMR reaction, the mismatched T has been corrected to C, which is the normal pair of G. Other candidate proteins engaged in human DNA mismatch repair are listed in Table 2.

Main protein complexes in human DNA mismatch repair

In human cells, the mismatch recognition occurs by two MutS homologs whose specificities are very similar to their yeast counterparts. MutSα, a heterodimeric complex of the MSH2 and MSH6 proteins, recognizes base-base mismatches and one basepair IDLs, whereas MutSβ, a heterodimeric complex of the MSH2 and MSH3 proteins, preferentially binds to 2-4 basepair IDLs (Acharya et al. 1996; Genschel et al. 1998). The MSH6 and MSH3 proteins are functionally redundant so that MSH6 can partially compensate for the function of MSH3 but not *vice versa* (Marsischky et al. 1996; Edelmann et al. 1997; Das Gupta and Kolodner 2000; Edelmann et al. 2000). The cellular level of the MutSα complex is much higher than MutSβ indicating that it has a major role in mismatch recognition (Drummond et al. 1997; Marra et al. 1998). After recognition, a second heterodimeric complex, formed by MLH1 and PMS2 (MutLα), couples mismatch recognition with the subsequent steps of the repair process (Fang and Modrich 1993; Li and Modrich 1995). The specific proteins that function in strand discrimination, unwinding of DNA, excision of the mismatch containing portion, repair synthesis and ligation are not yet known (Table 2, Figure 1).

The three above-mentioned heterodimers, MutSα, MutSβ and MutLα, are of a particular interest in the human MMR reaction. It has been hypothesized that because the MSH3 and MSH6 functions are partially redundant, inactivation of either gene leads to only a weak mutator phenotype. Accordingly, the cells deficient in both MSH3 and MSH6 proteins have a high mutation rate, similar to that observed in the MSH2-deficient cells (de Wind et al. 1999). MSH6-deficiency typically causes single base substitutions, MSH3-deficiency causes gains or losses of short repeat units, whereas MSH2-deficiency causes both single substitutions and gains and losses. MLH1 can form a heterodimeric complex with PMS1, PMS2 or MLH3. Of those, MLH1-PMS2 is a major component of MMR (Kondo et al. 2001). MLH1-MLH3 has been shown to participate in the repair of IDLs with MutSβ in yeast but its role in human MMR, as well as the role of the MLH1-PMS1 complex, still remains putative (Lipkin et al. 2000). MLH1 is, however, a common subunit to all MutL complexes and its deficiency leads to a severe mutator phenotype similar to MSH2 deficiency. Interestingly, only the MSH2 and MLH1 proteins are stable as monomers, while the other proteins, MSH6, MSH3 and PMS2, are unstable in the absence of their partners. Thus, the MSH2-deficient cells also show reduced levels of MSH3 and MSH6 (Marra et al. 1998; de Wind et al.

1999; Chang et al. 2000) and, in the absence of MLH1, PMS2 is vulnerable to degradation (Räschle et al. 1999).

MMR genes and predisposition to HNPCC syndrome

Germline mutations in MMR genes

Germline mutations in four different MMR genes, *MSH2*, *MLH1*, *MSH6* and *PMS2*, are convincingly associated with HNPCC. Three further genes, *MLH3*, *PMS1* and *MSH3*, have also been implicated in predisposition to HNPCC, but their roles are less clear (Peltomäki and Vasen 2004). The previous International Collaborative Group (ICG) on HNPCC, the current International Society for Gastrointestinal Hereditary Tumors (InSiGHT), maintains a database of syndrome-associated pathogenic mutations and polymorphisms (http://www.nfdht.nl). The database was established in 1994, and nowadays it contains information on more than 450 different MMR gene alterations that occur in 750 families from different parts of the world (Peltomäki et al. 1997; Peltomäki and Vasen 2004). Two genes, *MLH1* and *MSH2*, account for 90% of all mutations. The third most common susceptibility gene, *MSH6*, accounts for 7% of all mutations and the remaining MMR genes for less than 5% (Table 3).

Table 3 Number of germline mutations in different MMR genes (http://www.nfdht.nl)

MMR gene	Chromosomal location	Number of germline mutations (% of total) in 1996	in 2004
MLH1	3p21-23	75(60%)	241 (53%)
MSH2	2p21	48 (38%)	169 (37%)
MSH6	2p21	0(0%)	32 (7%)
MLH3	14q24.3	0(0%)	10 (2%)
PMS2	7p22	2(2%)	5 (1%)
PMS1	2q31-33	1(1%)	1 (0,2%)
Total		126 (101%)	458 (100,2%)

Mutations in MMR genes are scattered throughout the coding sequence. Frameshift and nonsense mutations that lead to truncated proteins are a common type of germline mutation in the *MLH1*, *MSH2* and *MSH6* genes. Also a great proportion of the alterations, especially in the *MSH6* and *MLH3* genes, consist of missense change that cause a single amino acid substitution (Figure 2) (Peltomäki and Vasen 2004; http://www.nfdht.nl). Truncating mutations that are present in large HNPCC families are usually considered pathogenic changes. However, an increasing proportion of mutations listed in the database are of the missense type that occurs in small and atypical HNPCC families (Peltomäki and Vasen 2004). Those mutations and their pathogenicity pose important questions to both clinicians and researches.

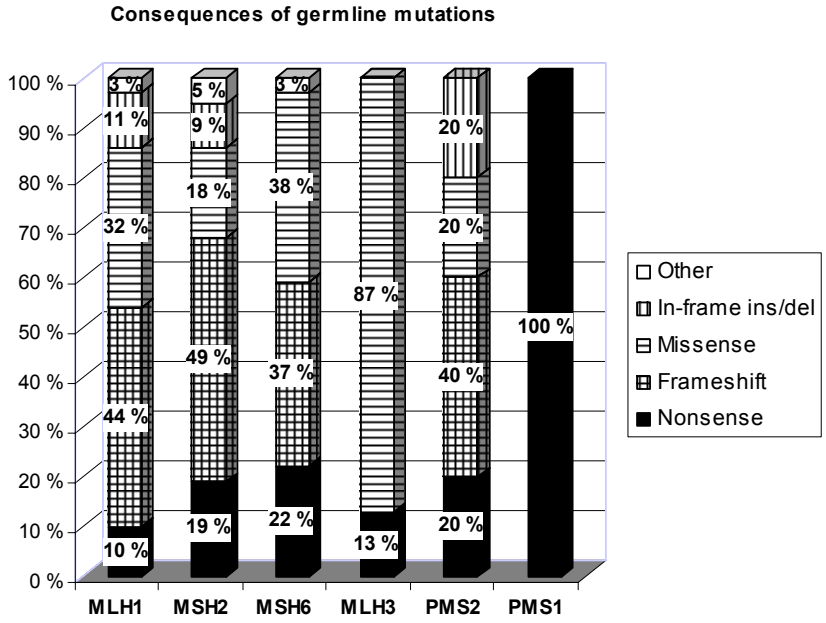

Figure 2 Types of germline mutations in HNPCC-associated MMR genes (Peltomäki and Vasen 2004)

Two mismatch repair gene mutations in the same patient

Rarely, a patient can carry two mutations either in a single MMR gene or in two separate MMR genes. The patients who carry homozygous mutations in both alleles of *MLH1* or *MSH2* have been shown to express an atypical tumor spectrum with hematological malignancy and neurofibromatosis type 1 (NF-1), suggesting that development of hematological malignancies and NF-1 are also a consequence of the mutator phenotype caused by MMR deficiency (Ricciardone et al. 1999; Wang et al. 1999b; Vilkki et al. 2001; Whiteside et al. 2002; Raevaara et al. 2004). The few reports from patients who carry a compound heterozygous mutation in *MLH1* or *PMS2* demonstrate that in these cases, tumors (breast, brain, colon) display the MSI phenotype, but the risk for colorectal cancer seems to be no greater than in heterozygous mutation carriers (Hackman et al. 1997; De Rosa et al. 2000). Neither do patients who have inherited two mutations in different MMR genes display a clinically more severe phenotype than heterozygous mutation carriers in general (Wu et al. 1999, 2001; Samowitz et al. 2001).

MLH1 and *MSH2* mutations are associated with classical HNPCC families

Germline mutations in *MLH1* and *MSH2* are mainly associated with classical HNPCC families that fulfill the Amsterdam criteria I (Table 1) and display a high MSI phenotype in tumor cells (Liu et al. 1996, Nyström-Lahti et al. 1996). A high degree of MSI is defined as instability at $\geq$ 2/5 or $\geq$ 30-40% of studied microsatellite loci (Boland et al. 1998). Both *MLH1* and *MSH2* encode protein products that are essential in the mismatch repair reaction and indispensable subunits of the heterodimeric complexes (Figure 1, Table 2), which explains the severe consequences of mutations in these genes.

MSH6 mutations are associated with putative HNPCC families

The role of the *MSH6* gene in HNPCC predisposition is not so obvious. *MSH6* mutations are rarely found in HNPCC kindreds that fulfill the original Amsterdam criteria I (Akiyama et al. 1997; Miyaki et al. 1997; Goodfellow et al. 2003) or even the revised, less stringent criteria (Table 1) (Wijnen et al. 1999; Wu et al. 1999; Plaschke et al. 2004), but are identified mostly in families with putative or atypical HNPCC (Kolodner et al. 1999; Verma et al. 1999).

Germline *MSH6* mutations appear to be associated with a later age of cancer onset than *MLH1* or *MSH2* mutations. The mean age at diagnosis is 55 years, which is approximately ten years later than in *MLH1* or *MSH2* mutation carriers, but still significantly less than in sporadic cases (Miyaki et al. 1997; Wijnen et al. 1999; de Leeuw et al. 2000; Wagner et al. 2001; Goodfellow et al. 2003; Cederquist et al. 2004). Patients with *MSH6* mutations remarkably often display endometrial cancer or atypical endometrial hyperplasia, the precursor of endometrial carcinoma (Miyaki et al. 1997; Wijnen et al. 1999; Wagner et al. 2001; Berends et al. 2002; Cederquist et al. 2004). The significance of *MSH6* in the development of EC is emphasized by the observation that lack of MSH6 protein characterizes ECs but not CRCs in HNPCC patients carrying the *MLH1* mutation (Schweizer et al. 2001).

A substantial proportion of *MSH6*-deficient colorectal and endometrial cancers show a lack of or only a low degree of MSI (Wu et al. 1999; Plaschke et al. 2000; Wagner et al. 2001). Because of the redundant function of MSH6 and MSH3 proteins, MSH6 deficiency mainly causes base substitutions (Marsischky et al. 1996; Sia et al. 1997) and instability in mononucleotide repeats (Akiyama et al. 1997; Miyaki et al. 1997; Verma et al. 1999; de Leeuw et al. 2000; Plaschke et al. 2000, 2004; Cederquist et al. 2004). However, it has also been reported that, in

MSH6-deficient cells, instability in dinucleotide repetitive regions is as frequent as that of mononucleotide repeats (Berends et al. 2002).

THE PATHOGENICITY OF INHERITED MMR GENE MUTATIONS

The question of the pathogenicity of a sequence alteration

Inherited mutations in mismatch repair genes are distributed throughout the coding sequence and most mutations are unique (Peltomäki et al. 1997; Peltomäki and Vasen 2004; http://www.nfdht.nl). This means that usually the entire genes need to be screened for the detection of a predisposing mutation in a new family. A sequence alteration that results in a truncated protein is considered pathogenic, because it typically causes the loss of important functional domains. Furthermore, it generally leads to the absence of any detectable gene product in tumor tissue, which supports the assumption that the mutation is pathogenic. Although all alterations in the genome are potentially deleterious changes, the pathogenic significance of missense mutations is often difficult to determine. They do not necessarily affect the expression or stability of the protein, which can result in positive immunohistochemical staining in the tumor even though the function of the protein may be defective.

A significant proportion of all mutations identified in *MLH1*, *MSH2* and *MSH6* are of the missense type (Figure 2). Particularly, interpreting the pathogenicity of missense alterations, which are linked to small and putative HNPCC families lacking the typical clinical and molecular features of the syndrome, is very difficult (Kolodner et al. 1999; Wijnen et al. 1999; Wu et al. 1999; Parc et al. 2000; Wagner et al. 2001). It can also be impossible to interpret the significance of missense alteration found in patients who carry two different MMR gene mutations. It is often far from clear which one of the identified mutations is pathogenic, or if both of them are needed to cause MMR deficiency and cancer susceptibility (Wu et al. 1999, 2001; Samowitz et al. 2001).

It is most important for clinicians and genetic counsellors that pathogenic alterations can be distinguished from nonpathogenic variants, since counseling must be based on a reliable assessment of the pathogenicity of the mutation. The optimal approach to evaluate the pathogenicity of a nontruncating mutation is to clarify whether the detected mutation and the disease cosegregate in a family.

Unfortunately, segregation studies are often hindered by an insufficient family size or the unavailability of clinical specimens.

Many reports classify missense mutations as most likely to be pathogenic if they fulfill the following criteria: (1) the mutated codon is located in an evolutionarily conserved residue; (2) the amino acid substitution is significant, for example it causes a change in polarity; (3) the mutation is not present in healthy control individuals; (4) other MMR germline mutations are excluded in the kindred and (5) the mutation segregates with the disease phenotype (Kolodner et al. 1999; Wu et al. 1999; Trojan et al. 2002). In addition to these criteria, the location of the mutated codon in a critical functional domain may be relevant for protein activity. In many cases, these criteria are not fulfilled but the mutation is still listed as pathogenic rather than as a polymorphism in the HNPCC mutation database (http://www.nfdht.nl). Determining a missense mutation found in an HNPCC family as pathogenic is often difficult without additional confirmatory studies. Thus biochemical characterization and functional analyses of the mutated MMR proteins are necessary.

Functional tests to determine the pathogenicity of MMR gene mutations

In vitro and *in vivo* MMR protein expression assays

A decreased in the amount of mutated MMR protein compared to wildtype protein might be an indication of the mutation's pathogenicity (Brieger et al. 2002; Nyström-Lahti et al. 2002; Raevaara et al. 2002; Trojan et al. 2002). Several groups have used different expression systems to produce mutated MSH2 and MLH1 proteins (Palombo et al. 1996; Iaccarino et al. 1998; Marra et al. 1998; Brieger et al. 2002; Nyström-Lahti et al. 2002; Räschle et al. 2002; Trojan et al. 2002; Raevaara et al. 2003). For protein production, the target cells are cotransfected with MSH2 and MSH6 or MLH1 and PMS2 expression vectors, to produce heterodimeric complexes of MutSα and MutLα, respectively, since both MSH6 and PMS2 are unstable in the absence of their partner proteins (Marra et al. 1998; de Wind et al. 1999; Räschle et al. 1999; Chang et al. 2000).

The Baculovirus expression system is one of the most widely used expression systems to produce human MMR proteins in cultured insect cells (Palombo et al. 1996; Iaccarino et al. 1998; Marra et al. 1998; Nyström-Lahti et al. 2002; Räschle et

al. 2002). This expression system is based on recombinant baculovirus constructs, in which the heterologous gene is placed under the transcriptional control of the strong polyhedrin promoter and abundantly expressed during transient transfection. The produced proteins are processed, modified and targeted to their appropriate cellular locations, where they are functionally similar to their authentic counterparts (Bac-to-Bac® baculovirus expression system, Instruction Manual, 2003).

The human homologous expression system has been used to study the biochemical properties of MSH2 and MLH1 protein variants (Brieger et al. 2002; Trojan et al. 2002, Raevaara et al. 2003). In these studies, mismatch repair-deficient cell lines, LoVo ($MSH2^{-/-}$), HCT 116 ($MLH1^{-/-}$) or the human embryonic kidney fibroblast cell line 293T ($MLH1^{-/-}$), are transiently transfected with different expression vector constructs to study the expression and stability of mutated MSH2 or MLH1, respectively. Here, the amount of some MSH2 and MLH1 variants has been shown to decrease compared to wildtype protein levels, suggesting that mutant proteins or mutant mRNAs are less stable than the corresponding wildtype molecules (Brieger et al. 2002; Trojan et al. 2002; Raevaara et al. 2003).

The Baculovirus expression system is a very efficient method for producing high amounts of protein, and intracellular proteins in particular, when compared to other eukaryotic expression systems (Luckow et al. 1993). Production of the mutated MMR proteins *in vivo* in the human expression system is not as efficient as in the baculovirus system (Trojan et al. 2002; Raevaara et al. 2003). Since functional assays generally require more recombinant protein than can be produced *in vivo*, the Baculovirus expression system is widely used. Moreover, the human expression system always requires a suitable cell line, which is deficient for the MMR gene of interest, in order to produce that mismatch repair protein, and hence it is not appropriate for all MMR genes.

***In vitro* interaction assays**

Guerrette et al. (1998, 1999) have used a glutathione *S*-transferase (GST) fusion protein interaction assay to evaluate the effects of *MLH1* and *MSH2* missense alterations on protein interactions. In the assay, the recombinant GST fusion protein is incubated with ^{35}S-labeled cell lysate in the presence of glutathione-agarose beads. The proteins are allowed to associate, and are then fractionated by

polyacrylamide gel electrophoresis and subjected to autoradiography. It was shown that both MSH2-MSH6 and MSH2-MSH3 heterodimers have two distinct interaction regions (Guerrette et al. 1998), while the interaction between the two components of the MLH1-PMS2 heterodimer is located in one region, in the carboxyl terminus of both proteins (Guerrette et al. 1999). Several *MSH2* missense alterations found in HNPCC patients and located in the consensus interaction regions showed no defect in the MSH6-MSH2 or MSH3-MSH2 interactions, whereas deficiencies in the interaction of MLH1 with PMS2 was shown to be associated with HNPCC (Guerrette et al. 1998,1999).

Coimmunoprecipitation assays provide a different method to study the effect of a MMR missense mutation on protein-protein interactions (Brieger et al. 2002; Nyström-Lahti et al. 2002). A protein of interest is specifically immunoprecipitated from the cell extracts using a specific antibody and the immunoprecipitates are then resolved on an polyacrylamide gel and detected by western blotting with antibodies directed against the protein of interest and its associated proteins.

In the glutathione *S*-transferase fusion protein interaction assay, the amino-terminal GST fusion of the MMR protein construct may change the native folding of the protein and thus affect its interactions. Furthermore, this *in vitro* assay is not quantitative and cannot determine the effects of an altered interaction on the MMR process *in vivo*. The major advantage of the coimmunoprecipitation assay compared to GST fusion assay is that it allows studying the proteins in their native conformation. However, it may not be sensitive enough to detect weak interactions between proteins (Guerrette et al. 1998). It also requires an appropriate antibody for coimmunoprecipitation. So far, none of the commercial anti-MSH2-antibodies are effective enough to coimmunoprecipitate MSH2 together with MSH6 protein (Acharya et al. 1996; Brieger et al. 2002), whereas this interaction can be shown with monoclonal anti-MSH6-antibody (Matton et al. 2000).

In vivo bacterial- and yeast-based functional assays

Quaresima et al. (2003) have described a simple method to test the effect of overexpressed wildtype or mutated human MLH1 protein upon the mismatch repair mechanism in *E. coli*. This assay is based on the presumption that human MLH1 protein is able to interact with bacterial MutL and MutS proteins *in vivo*. In

the assay, the *E. coli* strain, which carries a *lac* +1 frameshift mutation (CCC to CCCC), is transfected with a plasmid expressing wildtype or mutated human MLH1 protein. The Lac+ reversion rate is used as an indication of functionality of the tested protein (Longerich et al. 1995). The wildtype human MLH1 as well as functional MLH1 variants display the mutator phenotype due to their ability to interact with MutL and MutS, whereas pathogenic variants show weaker interaction with bacterial MMR proteins and have no effect on the Lac+ reversion rate (Quaresima et al. 2003).

Shimodaira et al. (1998) have described a yeast-based functional assay to test human *MLH1* mutations. This assay is based on the assumption that the function of human MLH1 can be monitored in *S. cerevisiae*, because the MMR function is conserved between these species. In the assay, human wildtype or mutated *MLH1* cDNA is overexpressed in a MMR-proficient yeast strain. A defective MMR protein causes an increase in the mutation rate of the canavanine resistance (*CAN1*) gene, and in the reversion of the *hom3-10* allele in yeast. Highly expressed wildtype human MLH1 increases the rate of accumulation of *CAN1* mutations and Hom+ reversions and thus causes the dominant mutator phenotype. The mutations in human MLH1 which prevent the proper protein function totally or partially inactivate this dominant mutator effect. The assay is thus based on interference of functional human MLH1 protein, which competes with native yeast MLH1 protein and results in only partial MMR activity.

Shcherbakova and Kunkel (1999) have used a different kind of yeast-based assay to examine the functional consequences of human *MLH1* missense mutations. They showed that overexpression of yeast *MLH1* in a wildtype yeast strain increases the mutation rate, possibly by sequestering and preventing other proteins from participating in the MMR reaction. Here, human *MLH1* mutations are introduced into the homologous sequence in yeast *MLH1* and stay pathogenic if the mutation results in a strong mutator phenotype.

Ellison et al. (2001) have evaluated the effect of missense alterations in MLH1 function by using a reporter gene which measures the stability of an in-frame $(GT)_{16}$ tract in *S. cerevisiae*. Besides introducing the missense changes at the homologous yeast codon, a hybrid human-yeast MLH1 protein was constructed. The hybrid proteins were shown to be functional in MMR *in vivo*.

Kolodner et al. (1999) have also described a yeast-based assay but in distinction from the other assays, which are used to test *MLH1* mutations, this was developed for the testing of *MSH6* missense mutations. Here, the mutations are introduced into the *S. cerevisiae MSH6* gene; the mutated plasmids are transformed into a MMR-deficient *msh3 msh6* double mutant strain and the rate of reversion of the *hom3-10* frameshift mutation is determined by fluctuation analysis (Marsischky et al. 1996). The amino acid substitution is classified as a loss of function mutation if the mutated plasmid fails to complement the MMR-deficient phenotype. The wildtype MSH6 plasmid is able to fully complement this defect.

All of the bacterial- and yeast-based functional tests rely on the expression of a particular phenotype in a heterologous system. Quaresima's and Shimodaira's assays assume that the function of a human MMR protein can be monitored in different organisms (Shimodaira et al. 1998; Quaresima et al. 2003). Shcherbakova's and Kolodner's assays require that affected amino acid residues are evolutionarily conserved and assume that homologous mutations in *S. cerevisiae* give the same phenotype in yeast as the original mutations in human cells (Kolodner et al. 1999; Shcherbakova and Kunkel 1999). Ellison's test has overcome the limitation of studying only evolutionarily conserved codons by constructing human-yeast hybrid proteins (Ellison et al. 2001). All of these assays are quantitative, since the result is based on the rate of reversion of frameshift mutations. They are also facile and less time-consuming than *in vitro* functional assays.

***In vitro* MMR assays**

Nyström-Lahti et al. (2002) have adapted and used the human cell-based *in vitro* mismatch repair assay (Lahue et al. 1989; Holmes et al. 1990) to study the effect of *MLH1* mutations in the MMR reaction. In this assay, the total protein extract of baculovirus-infected insect cells, which contains overexpressed recombinant human MLH1, is incubated together with nuclear extract from the mismatch repair-deficient cell line HCT116, which lacks functional MLH1. The repair reaction is carried out *in vitro* using a nicked circular heteroduplex molecule, which contains a G•T mismatch, as the substrate (Lu et al. 1983; Lahue et al. 1989; Holmes et al. 1990). If HCT116 nuclear extract complemented with MLH1 variant can correct the mismatch, a specific restriction enzyme is able to cut the heteroduplex, and the repair efficiency can be measured by the cleavage

efficiency. MMR-proficient nuclear extract and *MLH1*-deficient nuclear extract complemented with wildtype MLH1 are used as positive controls in the assay. *MLH1*-deficient nuclear extract is used as a negative control.

Trojan et al. (2002) have described a modified version of the *in vitro* MMR assay. The repair reaction uses a bacteriophage heteroduplex containing a G•T mismatch in the coding sequence of the *lacZ* α-complementation gene fragment as the substrate, and cytoplasmic extract from *MLH1*-deficient cell line is complemented with recombinant human MLH1 expressed in insect cells. The purified DNA is introduced into the *E. coli* strain by electroporation and plated along with the α-complementation strain. If no repair occurs, a high percentage of mixed plaques, containing both blue and colorless progeny, are observed. A reduction in the percentage of mixed plaques and the concomitant increase in single-color plaques are indicative of repair (Thomas et al. 1991; Marra et al. 1998).

The *in vitro* MMR assays allow studying the functionality of the mutated proteins in a homologous human system. Their advantages compared to bacterial-based, yeast-based and interaction assays are that here it is possible to study the protein-protein interaction of any two known components of the human MMR system. Furthermore, all in-frame mutations from missense mutations to small exonical insertions/deletions, irrespective of their evolutionary conservation status or location in a gene, can be investigated (Nyström-Lahti et al. 2002; Raevaara et al. 2002; Trojan et al. 2002).

TUMORIGENESIS IN HNPCC

DNA MMR deficiency as the first step in tumorigenesis

The predisposition to HNPCC is inherited in an autosomal dominant fashion. According to Alfred Knudson's two hit hypothesis, tumor formation requires two hits (one germinal and one somatic) in a MMR gene (Knudson 1971; Hemminki et al. 1994). The direct consequence of defective DNA mismatch repair is the mutator phenotype characterized by high microsatellite instability in tumor tissue (Ionov et al. 1993; Thibodeau et al. 1993). Mutation rates in MMR-deficient cells are 100 - 1000-fold higher as compared with normal cells (Parsons et al. 1993; Bhattacharyya et al. 1994). Sporadic counterparts of carcinomas included in the HNPCC tumor spectrum can also show a high degree of MSI, and approximately 15% of sporadic colon and 25% of sporadic endometrium carcinomas display the

mutator phenotype (Helland et al. 1997; Boland et al. 1998; Gurin et al. 1999). The majority of sporadic MMR-deficient tumors are due to bi-allelic silencing of the *MLH1* gene, which mostly results from promoter hypermethylation and more rarely from somatic mutations or loss of heterozygosity (LOH) (Liu et al. 1995; Kane et al. 1997; Veigl et al. 1998; Esteller et al. 1999; Kuismanen et al. 2000).

Most microsatellite repeats in the genome are located within noncoding or intronic sequences and are thus considered to be silent. However, many genes also contain microsatellites within their coding regions and these microsatellite repeats are observed to be mutated in MSI carcinomas. Several of these mutated genes are well-known tumor suppressor genes, which require two genetic events (one in each allele) for their inactivation. In HNPCC-related tumors, the inactivation of MMR genes is an early event in tumorigenesis (Aaltonen et al. 1994, Shibata et al. 1994; Konishi et al. 1996; Tannergård et al. 1997, de Leeuw et al. 2000). This causes the activation of the mutator pathway leading to a cascade of accumulating frameshift mutations in many growth-regulatory genes with coding microsatellites, promoting genome-wide genetic instability.

Mutational and immunohistochemical analyses of target genes have been performed mainly in the colon and endometrium, the two most commonly affected tissues in HNPCC (Aarnio et al. 1999). The majority of the investigated tumors derived from HNPCC patients with a predisposing *MSH2* or *MLH1* mutation (de Leeuw et al. 2000; Schweizer et al. 2001; Kuismanen et al. 2002). Because the genetic basis of organ selection in HNPCC is poorly understood, the comparative studies of affected target genes in HNPCC-related endometrial cancers (HNPCC-ECs) and colorectal cancers (HNPCC-CRCs) with an identical genetic predisposition have supplied unique data about tumor progression in these two organs (Schweizer et al. 2001; Kuismanen et al. 2002).

Kuismanen et al. (2002) have shown that the target gene pattern is more heterogenous and involves a lower proportion of unstable markers per tumor in HNPCC-ECs than HNPCC-CRCs despite the same predisposing MMR mutation in the patients. Some target genes, such as *insulin-like growth factor II receptor* (*IGFIIR*) and the MMR genes *MSH3* and *MSH6*, which contain coding mononucleotide repeats, seem to be fairly evenly involved in these tumors (de Leeuw et al. 2000; Kuismanen et al. 2002), whereas some others show considerable tissue specificity. For example, *transforming growth factor β receptor II* (*TGFβRII*) is the most frequently targeted tumor suppressor gene in colorectal cancer, but this

is not as common in ECs (Konishi et al. 1996; Tannergård et al. 1997; Kuismanen et al. 2002; Paoloni-Giacobino et al. 2002). Its alterations affect growth inhibition, cell death and differentiation in intestinal epithelial cells (Wu et al. 1992; Markowitz and Roberts 1996). The most common target gene for endometrial cancer seems to be *PTEN* (Kuismanen et al. 2002; Zhou et al. 2002), a phosphatase which signals through the phospoinositol-3-kinase/Akt pathway and mediates cell cycle arrest and cell death (Li et al 1998; Stambolic et al. 1998; Weng et al. 1999). The considerable differences between affected target genes and their inactivation frequencies support the suggestion that tumor progression occurs via distinct mechanisms in these two organs (de Leeuw et al. 2000; Schweizer et al. 2001; Kuismanen et al. 2002).

General adenoma-carcinoma sequence in colon cancer formation

The general model of colon cancer development from a pre-existing benign tumor to a malignant form is one of the best characterized models of tumor progression. Colon cancer formation is a multistep process resulting from a number of specific mutational events in many different genes which are members of pathways that control cell growth and death. Colon cancer is most commonly initiated by alterations in the signaling elements of the Wnt signaling pathway (Iwamoto et al. 2000; Hao et al. 2002). It then progresses as a result of the accumulation of sequential events that either inactivate tumor suppressor genes or activate oncogenes, which require only one genetic event for their activation (Sparks et al. 1998). The *adenomatous polyposis coli* (*APC*) gene is one of the key components in the Wnt signaling pathway and alterations in *APC* result in overactivation of this pathway (Morin et al. 1997).

Mutational inactivation of the *APC* gene appears to initiate about 70% of sporadic colon adenocarcinomas (Miyaki et al. 1994). Germline mutations in *APC* predispose to familial adenomatous polyposis syndrome displaying numerous colorectal and small intestinal tumors (Groden et al. 1991; Nishisho et al. 1991). Mutations in *APC* have been detected in the smallest adenomas studied, indicating its very early role in colon tumorigenesis (Powell et al. 1992; Ichii et al. 1993; Levy et al. 1994). In sporadic cases, over 90% of *APC* mutations result in a premature stop codon and truncated gene product (Miyoshi et al. 1992; Powell et al. 1993).

The other alterations that have been convincingly shown to promote colon carcinogenesis affect *K-ras*, genes on chromosome 18q, and *TP53*. *K-ras* is an oncogene and has been shown to be activated in about 40% of sporadic colon tumors. In activated form it promotes cell proliferation and mediates adenoma growth (Bos et al. 1987; Arber et al. 2000). LOH on 18q occurs in approximately 70% of all colon cancers. This deleted region contains a number of tumor suppressor genes, for example *deleted in colorectal cancer* (*DCC*), *SMAD2* and *SMAD4*, which have been shown to play a significant role in tumor progression from intermediate adenoma to late-stage adenoma (Vogelstein et al. 1988; Fearon et al. 1990; Takagi et al. 1996). *TP53* is the best-studied tumor suppressor gene, whose function is affected in about 50% of all human tumors (Soussi et al. 2000). *TP53* encodes a nuclear transcription factor that is involved in maintaining genomic stability by controlling cell cycle arrest and apoptosis, in response to various intra- and extracellular stress signals (Kastan et al. 1991; Yonish-Rouach et al. 1991; Kuerbitz et al. 1992). Mutations in *TP53* occur as a late event in colon tumorigenesis and can be found in 50-60% of sporadic colorectal carcinomas (Soussi et al. 2000). Furthermore, a variety of genetic alterations have been described in advanced carcinoma and an unknown number of mutational steps are further needed for the metastatic stage.

Wnt signaling pathway

The function of the Wnt pathway was originally described during embryogenesis. The *Wnt* genes (homologous to *wingless* in Drosophila) encode secreted glycoprotein factors that regulate cell growth. Wnt signaling molecules bind to transmembrane receptors called Frizzleds and activate the pathway by a so far largely unknown mechanism, leading to stabilization of the protein β-catenin, which upregulates the expression of many target genes that favor cell growth. In recent years, the Wnt signaling pathway has received considerable attention because its overactivation has been demonstrated to play an important role in the formation of certain tumors, especially colorectal cancer. Although mutation or misexpression in *Wnt* genes has not directly been associated with tumorigenesis, mutations in many components of this pathway have been shown to be critical in many forms of cancer (Miller 2001; Lustig and Behrens 2003).

The main components of the Wnt signaling pathway are β-catenin, APC, AXIN2 and glycogen synthase kinase -3β (GSK-3β), which form a macromolecular complex in the cytoplasm. In the absence of Wnt signal molecules (Wnts), this

macromolecular complex directs β-catenin toward proteosomal degradation (Rubinfeld et al. 1996; Aberle et al. 1997; Kishida et al. 1998), thus keeping the cytoplasmic concentration of free β-catenin in normal cells very low. The presence of Wnts or mutations in *APC*, *β-catenin* (*CTNNB1*) or *AXIN2* cause activation of this signaling pathway resulting in the stabilization of β-catenin (Morin et al. 1997; Liu et al. 2000), which enters into the nucleus and associates with transcription factors leading to the overexpression of Wnt target genes (Figure 3). In general, only one of these genes is mutated in a given tumor tissue, reflecting their role in a common pathway (Miyaki et al. 1999; Akiyama et al. 2000; Liu et al. 2000).

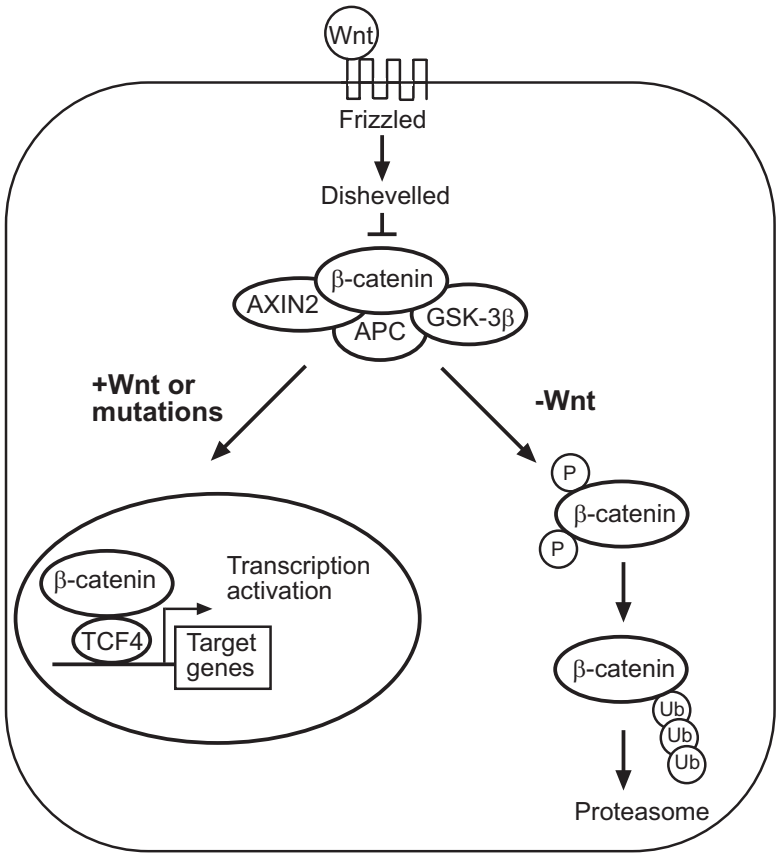

Figure 3 Overview of the Wnt signaling pathway. In the absence of Wnt signal molecules (-Wnt), APC interacts with β-catenin and they together form a macromolecular complex with AXIN2 and GSK-3β. In this complex, as a result of phosphorylation by GSK-3β, β-catenin is consequently directed toward ubiquitin(Ub)-mediated proteosomal degradation (Rubinfeld et al. 1996; Aberle et al. 1997; Kishida et al. 1998). Thus, the cytoplasmic concentration of free β-catenin is very low in normal cells. Binding of Wnts to Frizzled receptors activates Dishevelled, which blocks the function of the macromolecular complex. Therefore, in the presence of Wnts (+Wnt) or after mutations in *APC*, *β-catenin* or *AXIN2*, formation of the macromolecular complex is prevented. As a

result, the amount of β-catenin protein increases in the cytoplasm with subsequent nuclear translocation and accumulation (Morin et al. 1997; Liu et al. 2000). In the nucleus, activated β-catenin can interact with transcription factors like T cell factor/Lymphoid enhancer factor (TCF/Lef) (Behrens et al. 1996; Korinek et al. 1997), leading to overexpression of several target genes that have been shown to promote tumor formation. TCF4 is the predominant TCF family member expressed in the colonic epithelium (Korinek et al. 1997), and it upregulates growth-regulatory genes such as *C-MYC* and *CYCLIN D1* (He et al. 1998; Mann et al. 1999; Tetsu and McCormick 1999). Consequently, mutations in Wnt signaling components have an essential role in tumor development.

Tumor progression in HNPCC-related colorectal cancer

The genetic alterations from adenoma to carcinoma stage in HNPCC-related colon tumors with the microsatellite mutator phenotype are quite different from that of sporadic microsatellite stable (MSS) colon tumors (see above), even if they do share several genetic alterations (Figure 4) (Yamamoto et al. 1998). However, in both sporadic and HNPCC-related microsatellite instable colon cancers, the inactivation of MMR genes is an early event in tumorigenesis (Aaltonen et al. 1994, Shibata et al. 1994; Konishi et al. 1996; Tannergård et al. 1997).

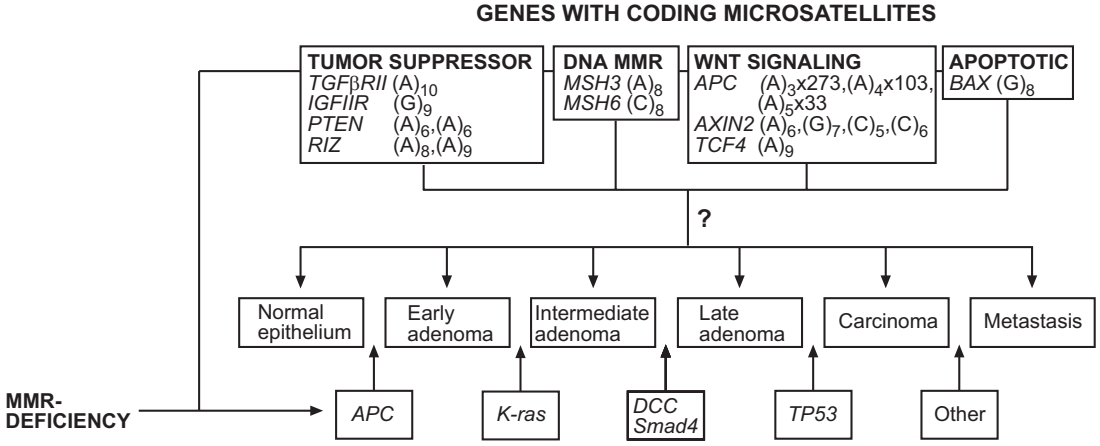

Figure 4 Putative model of genetic changes associated with HNPCC-related colorectal cancer tumorigenesis. The most obvious difference between sporadic and HNPCC-related colon tumorigenesis is that HNPCC-tumors are mismatch repair-defective and progress via the microsatellite mutator pathway. Inactivation of a MMR gene is an early event in tumorigenesis. DNA mismatch repair deficiency leads to inactivation of many growth-regulatory genes that contain coding mononucleotide repeats. Many of these affected genes are general tumor suppressor genes, namely *TGFβRII* (Markowitz et al. 1995; Parsons et al. 1995), *IGFIIR* (Souza et al. 1996), *PTEN* (Guanti et al. 2000), *retinoblastoma protein-interacting zinc finger* (*RIZ*) (Chadwick et al. 2000; Piao et al. 2000), as well as genes that function in DNA mismatch repair (Malkhosyan et al. 1996), Wnt

signaling (Duval et al. 1999a; Liu et al. 2000) and apoptotic pathways (Rampino et al. 1997). Inactivation of the *APC* gene results in overactivation of the Wnt signaling pathway, which leads to the expression of genes that favor cell growth. Mutations in *APC* are less frequent in HNPCC-CRCs than in sporadic tumors (Konishi et al. 1996; Akiyama et al. 2000). However, other genes (*AXIN2*, *TCF4*) of this pathway are reported to mutate due to MMR deficiency and thus alter the Wnt signaling pathway and contribute to the development of colon cancer (Duval et al. 1999a; Liu et al. 2000). During progression through the adenoma-carcinoma sequence, additional alterations accumulate in oncogenes including *K-ras* (Aaltonen et al. 1993; Tannergård et al. 1997; Fujiwara et al. 1998) and in tumor suppressor genes on chromosome 18q (*DCC*, *Smad2*, or *Smad4*) and *TP53* (Lynch and de la Chapelle 2003). Target gene mutations due to MMR deficiency appear to occur throughout the adenoma-carcinoma sequence. The timing of many of these events during tumor formation remains to be mapped, but preliminary studies have shown that they occur at distinct phases of tumor progression. This figure is modified from Lynch and de la Chapelle 2003.

In HNPCC-related CRC, *TGFβRII* is the most frequently targeted tumor suppressor gene with coding microsatellites (Konishi et al. 1996; Tannergård et al. 1997; Kuismanen et al. 2002; Paoloni-Giacobino et al. 2002). The *BAX* gene is mutated in 30% of HNPCC-CRCs (Kuismanen et al. 2002, Paoloni-Giacobino et al. 2002). It is a member of the *Bcl-2* gene family, which promotes apoptosis via the TP53-regulated pathway (Oltvai et al. 1993). Mutations in the tumor suppressor gene *TP53* are not so frequent in HNPCC-related colorectal cancers as in their sporadic counterparts, and it has been speculated that *BAX* may replace the role of mutant *TP53* in tumorigenesis of HNPCC-CRCs (Konishi et al. 1996; Eshleman et al. 1998). The frequency of *K-ras* mutations in HNPCC-CRCs is similar to that in MSS CRCs (Aaltonen et al. 1993; Tannergård et al. 1997; Fujiwara et al. 1998).

Alterations of the Wnt signaling pathway can be observed irrespective of the MSI status, indicating its general role in CRC development. The pathway has been shown to be overactivated in 65% of colorectal cancers related to HNPCC syndrome (Miyaki et al. 1999). Activating β-catenin mutations occur in about 40% of these tumors (Miyaki et al. 1999). Huang et al. (1996) have demonstrated that the mononucleotide tracts within the *APC* gene are frequently mutated in MSI colorectal cancers. However, other studies on HNPCC-related CRCs have not confirmed this result but suggest that the mutational spectrum in HNPCC-CRCs is similar to that in sporadic tumors (Konishi et al. 1996; Akiyama et al. 2000). Mutations in *APC* can be found only in 21% of MSI tumors (Konishi et al. 1996; Akiyama et al. 2000), which is significantly less than in sporadic colon carcinomas (Miyaki et al. 1994). Besides *APC*, other target genes in the Wnt signaling pathway which contain coding microsatellite repeats are *AXIN2* and *TCF4* (Duval et al.

1999a; Liu et al. 2000). *AXIN2* has been shown to be mutated in 25% of mismatch repair-deficient colon tumors (Liu et al. 2000). TCF4 is the predominant TCF family member expressed in the colonic epithelium (Korinek et al. 1997) and in MSI CRCs, mutations of the poly(A)9 coding repeat in *TCF4* gene are relative frequent (~40%) (Duval et al. 1999b).

Tumor progression in HNPCC-related endometrial cancer

Relatively little is known about the molecular events that lead to the development of HNPCC-related endometrial cancer. Interestingly, the risk for development of EC seems to vary according to the predisposing mutation. It has been suggested that the lifetime risk for EC is higher in *MSH2* mutation carriers than in *MLH1* mutation carriers, whereas the risk for CRC is similar (Vasen et al. 1996). Furthermore, *MSH6* mutation carriers remarkably often display endometrial carcinoma (Miyaki et al. 1997; Wijnen et al. 1999).

Complex atypical hyperplasia (CAH) is the premalignant lesion of uterine endometrioid carcinoma, the most prevalent type of endometrial cancer (Sherman and Brown 1979; Kurman et al. 1985). It appears to proceed in a stepwise fashion to the carcinoma stage (Cohn et al. 2001). This premalignant precursor, like adenoma in the colon, provides the opportunity to investigate the timing of specific genetic alterations that contribute to endometrial tumor progression.

MMR deficiency is an early event in HNPCC-related endometrial tumorigenesis, which has been demonstrated by immunohistochemical analyses using antibodies directed against the MMR proteins and by MSI studies in CAHs from HNPCC patients (de Leeuw et al. 2000). The variation in the extent and level of MSI accounts for the differences in the age of onset. For example, the high frequency of MSI in CAHs has been observed only in *MSH2* mutation carriers, which seems to correlate with an early age of cancer onset and thus indicates rapid tumor progression (de Leeuw et al. 2000). Slower tumor progression has been observed in *MSH6* mutation carriers (Miyaki et al. 1997; Wijnen et al. 1999). Their tumors show a low frequency of MSI affecting mainly mononucleotide repeat tracts, which is a possible explanation for the slow tumor progression and late age of onset (de Leeuw et al. 2000). Furthermore, many endometrial carcinomas from *MSH6* mutation carriers show instability at the $(C)_8$ repeat within the *MSH6* gene (Akiyama et al. 1997; Miyaki et al. 1997; Wijnen et al. 1999; de Leeuw et al. 2001), a site which is a rare target for *MSH2* and *MLH1* mutation carriers or sporadic EC

cases (Gurin et al. 1999). This significantly high frequency of instability at the $(C)_8$ repeat is suggested to contribute to bi-allelic inactivation of the *MSH6* gene (de Leeuw et al. 2001).

The most common target gene of the microsatellite mutator pathway in HNPCC-ECs is *PTEN* (Kuismanen et al. 2002; Zhou et al. 2002). The *BAX* gene is affected in 19% of HNPCC-ECs (de Leeuw et al. 2000; Kuismanen et al. 2002). Mutations in the *K-ras* oncogene and *PTEN* tumor suppressor gene are the only genetic alterations that have already been described in CAHs suggesting that their inactivation occurs early in endometrial tumorigenesis (Enomoto et al. 1991; Sasaki et al. 1993; Levine et al. 1998; Maxwell et al. 1998; Mutter et al. 2000). The mutation frequency of both genes is similar irrespective of the MSI status in sporadic endometrial cancers (Gurin et al. 1999; Swisher et al. 1999; Cohn et al. 2000; Mutter et al. 2000). In most MSI cases, MMR deficiency appears to precede alterations in *K-ras* (Cohn et al. 2001). All somatic *PTEN* alterations found from HNPCC patients with *MSH2* or *MLH1* germline mutations are proved to be frameshift mutations affecting two $(A)_6$ repeat tracts (Zhou et al. 2002). It is quite obvious that this instability is a consequence of MMR deficiency. Inactivation of *PTEN* appears to be involved in the initial stages of tumor development and also in endometrial tumor progression. Mouse studies also support the fundamental role for *PTEN* in the control of endometrial epithelial growth and proliferation (Wang et al. 2002).

The role of the Wnt signaling pathway is unclear in HNPCC-EC tumorigenesis. The combination of MMR deficiency and *APC* hypermethylation is observed in sporadic ECs (Zysman et al. 2002), and the activating β-catenin mutations occur in 10-30% of sporadic endometrial cancers (Fukuchi et al. 1998; Palacios and Gamallo 1998; Kobayashi et al. 1999; Schlosshauer et al. 2000; Machin et al. 2002). Interestingly, Persad et al. (2001) have described that PTEN also participates the regulation of β-catenin by inducing GSK-3β activity, which leads to increased phosphorylation of β-catenin and its subsequent degradation. PTEN inactivation and the occurrence of *APC* hypermethylation already in premalignant lesions suggest that aberrant Wnt signaling may present in the early stages of endometrial cancer development (Zysman et al. 2002).

AIMS OF THE PRESENT STUDY

1) To address the question of whether and how *MSH6* germline missense mutations cause susceptibility to HNPCC

2) To determine by functional assay which one of the two germline MMR gene mutations, one in *MSH6* and one in *MSH2*, found in an index patient could primarily underlie the cancer susceptibility in the family

3) To study the molecular basis of organ selection in HNPCC by comparing the protein expression patterns of APC and β-catenin, the two widely expressed components of the Wnt signaling pathway, in HNPCC-related CRC and EC

MATERIALS AND METHODS

Materials and methods are briefly described here. For details, see the original publications (**I**-**IV**). All investigations had approval from the appropriate institutional review boards.

STUDY MATERIAL

MSH6 and *MSH2* missense mutations in studies I, II and III

Altogether 11 *MSH6* and one *MSH2* germline missense mutations were included in the studies. Four analyzed *MSH6* mutations were selected from the HNPCC database maintained by ICG/InSiGHT (http://www.nfdht.nl) (**I**). The remaining seven *MSH6* mutations were novel and they were obtained from HNPCC researchers (**II**, **III**). The analyzed *MSH2* mutation was previously reported in the mutation database. Two probands carried missense mutations in both *MSH6* and *MSH2* genes (**II**). Other probands were verified not to have germline mutations in *MLH1* or *MSH2* (**I**, **III**). Studied mutations were also excluded in 53-200 healthy controls. Analyzed mutations are listed in Table 4 and the locations of the mutated codons in the MSH6 protein are represented in Figure 5.

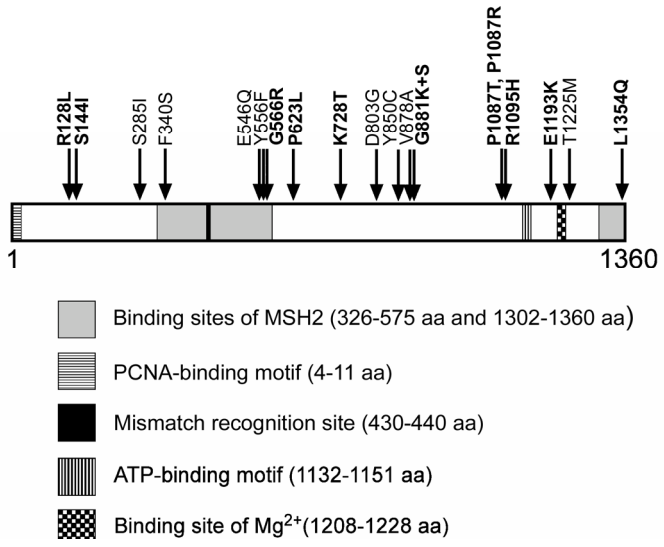

Figure 5 Schematic representation of the MSH6 protein showing *MSH6* missense alterations listed as pathogenic mutations in the mutation database (http://www.nfdht.nl), the mutations included in the present study (marked with bold letters) and functional domains in MSH6 (Guerrette et al. 1998; Iaccarino et al. 1998, 2000; Dufner et al. 2000; Kleczkowska et al. 2001).

Table 4 Studied missense mutations in *MSH6* and *MSH2*

Exon	Codon	Nucleotide change	Effect	Side chain changes of amino acid	Conserved codon[a]	Ref
***MSH6* mutation**						
2	128	CGT→CTT at 383	Arg→Leu	basic→nonpolar	No	III
2	144	AGC→ATC at 431	Ser→Ile	uncharged polar→nonpolar	Yes	Kolodner et al. 1999; Wu et al. 1999; Berends et al. 2002
4	566	GGA→AGA at 1696	Gly→Arg	nonpolar→basic	Yes	Kolodner et al. 1999
4	623	CCC→CTC at 1868	Pro→Leu	nonpolar→nonpolar	No	III
4	728	AAA→ACA at 2183	Lys→Thr	basic→uncharged polar	Yes	III
4	881	GGT→AAAAGT at 2641	Gly→Lys+Ser	nonpolar→basic+ uncharged polar	No	III
5	1087	CCC→ACC at 3259	Pro→Thr	nonpolar→uncharged polar	No	Kolodner et al. 1999
5	1087	CCC→CGC at 3260	Pro→Arg	nonpolar→basic	No	Wijnen et al. 1999
5	1095	CGC→CAC at 3284	Arg→His	basic→basic	Yes	II
7	1193	GAA→AAA at 3577	Glu→Lys	acidic→basic	Yes	III
10	1354	CTG→CAG at 4061	Leu→Gln	nonpolar→uncharged polar	No	II
***MSH2* mutation**						
3	145	ATT→ATG at 435	Ile→Met	nonpolar→nonpolar	Yes	II

[a]Conserved between human and yeast (*S. cerevisiae*)

Clinical data of the families and tumors included in studies I, II and III

None of the 15 families, in which the 11 *MSH6* mutations were found, fulfilled the Amsterdam criteria (I or II, Table 2) (Vasen et al. 1991, 1999) and they were classified as putative HNPCC families and named as Fam-MSH6-1-15. The mutation MSH6-S144I was detected in four different families (Fam-MSH6-2,3,4,5) (Kolodner et al. 1999, Wu et al. 1999, Berends et al. 2002)(**I**) and MSH6-E1193K was detected in two different families (Fam-MSH6-13,14) (**III**). Four families (Fam-MSH6-1,4,5,14) showed no family history of carcinomas. Seven probands had endometrial cancer (EC) and eight probands had colorectal cancer (CRC). The

probands' mean age at diagnosis was 59 years; the mean age of EC onset was 60 years and CRC onset 57 years. The number of affected family members ranged from 0 to 3 and their mean age at onset was 63 years. Nine of the ten first degree relatives had CRC and their mean age of onset was as high as 67 years. The pedigrees of the families are shown in Figure 6. Microsatellite instability and immunohistochemical data of the probands' tumors are shown in Table 5.

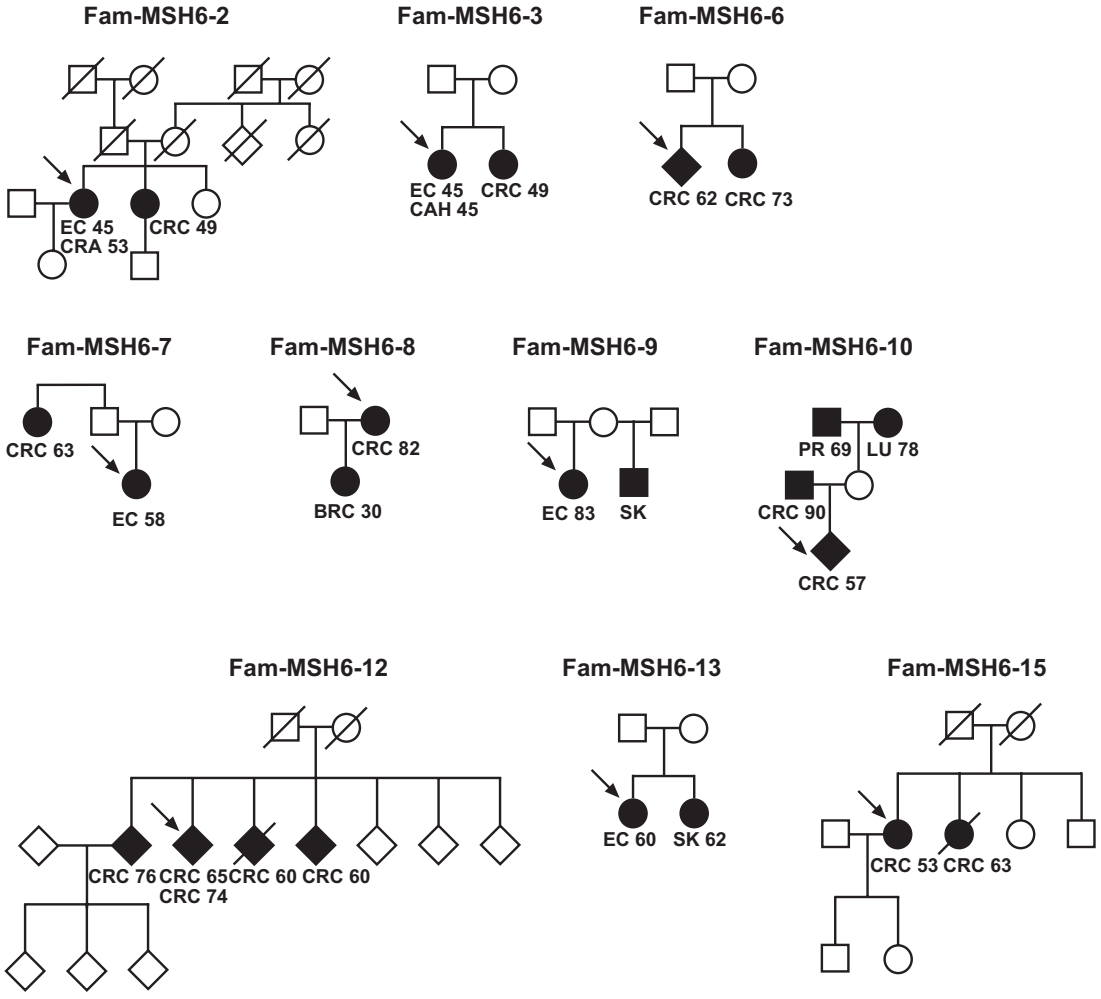

Figure 6 Pedigrees of the families with putative HNPCC and *MSH6* missense mutations. A square represents a male; circle, female; diamond, unspecified gender; diagonal stripe, deceased; white symbols, not affected; black symbols, affected; arrow, mutation carrier. The probands in Fam-MSH6-12 and Fam-MSH6-15 also carried *MSH2* mutation. Tumor types and ages at onset are marked. Abbreviations of the tumor types: BRC, breast cancer; CAH, complex atypical hyperplasia; CRA, colorectal adenoma; CRC, colorectal cancer; EC, endometrial cancer; LU, lung cancer; PR, prostate cancer; SK, skin cancer.

Table 5 Microsatellite instability status and immunohistochemical data of probands' tumors

Family ID	*MSH6* mutation	Mononucleotide repeat instability				Dinucleotide repeat instability						Immunohistochemistry (IHC)				References
		BAT17	BAT25[b]	BAT26[b]	BAT40	D1S128	D2S123[b]	D5S346[b]	D17S250[b]	D18S58	D18S69	MLH1	MSH2	MSH6	PMS2	
Fam-MSH6-1	R128L	ND			ND	ND						-	+	+	+	IV
Fam-MSH6-2	S144I					ND						ND	ND	ND	ND	Wu et al. 1999
Fam-MSH6-3	S144I	ND				ND		ND		ND	ND	+	+	+	ND	Berends et al. 2002
Fam-MSH6-4	S144I	ND				ND				ND	ND	+	+	+	ND	Berends et al. 2002
Fam-MSH6-5	S144I	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	Kolodner et al. 1999
Fam-MSH6-6	G566R	ND				ND				ND	ND	ND	ND	ND	ND	Kolodner et al. 1999
Fam-MSH6-7	P623L	ND			ND	ND				ND		+	+	-	+	IV
Fam-MSH6-8	K728T	ND			ND	ND				ND		-	+	+	+	IV
Fam-MSH6-9	G881K+S	ND			ND	ND				ND		-	+	+	+	IV
Fam-MSH6-10	P1087T	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	Kolodner et al. 1999
Fam-MSH6-11	P1087R	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	Wijnen et al.1999
Fam-MSH6-12	R1095H[a]	ND			ND					ND	ND	+	+	+	ND	II
Fam-MSH6-13	E1193K	ND			ND	ND				ND		+	-	-	+	IV
Fam-MSH6-14	E1193K	ND			ND	ND			ND	ND		+	+	- 5%	+	IV
Fam-MSH6-15	L1354Q[a]	ND	ND			ND		ND	ND		ND	+	-	-	ND	II

[a] The index patient carries also *MSH2* missense mutation (I145M)

[b] The markers included in Bethesda panel

MSI data: black, unstable; gray, stable

IHC data: +, expressed; -, not expressed

ND, not determined

Tissue samples in study IV

The colorectal and endometrial tumors were collected from a series of well-characterized HNPCC families with segregating germline mutations in the mismatch repair genes *MLH1* or *MSH2* (Nyström-Lahti et al. 1996; Holmberg et al. 1998). The analysis of APC and β-catenin expression patterns included 31 colorectal and 19 endometrial cancers from patients carrying the same predisposing mutations.

METHODS

All the methods used in studies **I-IV** are listed in Table 6. Cell lines used in the production of recombinant proteins and in an *in vitro* MMR assay are listed in Table 7 (**I**, **II**, **III**). Table 8 describes the antibodies used in Western blot, coimmunoprecipitation (**I**, **II**, **III**) and immunohistochemical analyses (**IV**). Table 9 represents experimental conditions for the site-directed mutagenesis used in studies **I**, **II** and **III**.

Table 6 Methods used in the studies

Methods	More information	Used in
Coimmunoprecipitation	Table 8, used antibodies	I, II, III
DNA cloning and subcloning		I, II, III
DNA heteroduplex preparation		I, II, III
Immunohistochemistry	Table 8, used antibodies	IV
In vitro MMR assay	Table 7, used cell lines	I, II, III
PCR	Table 9, used primers	I, II, III
Production of recombinant baculoviruses	Table 7, used cell lines	I, II, III
Production of recombinant proteins	Table 7, used cell lines	I, II, III
Site-directed mutagenesis	Table 9, experimental conditions	I, II, III
Transfection		I, II, III
Western blotting	Table 8, used antibodies	I, II, III

Table 7 Cell lines used in the studies

Cell line	Description	Source	Used in
HCT15 (DLD1)	human colorectal adenocarcinoma cells (*MSH6*$^{-/-}$)	ATCC	I, II, III
Lovo	human colorectal adenocarcinoma cells (*MSH2*$^{-/-}$)	ATCC	II
Sf9	Insect (*Spodoptera frugiperda*) cells	Gibco BRL	I, II, III
TK6	human lymphoblasts	ATCC	I, II, III

ATCC, American Type Culture Collection (USA)

Table 8 Antibodies used in the studies

Antigen	Description	Source	Used in
APC	mouse mAb (clone c-APC 28.9) against the C-terminal fragment of human APC	Abcam	IV
β-Catenin	mouse mAb (clone 14) against the C-terminal fragment (amino acids 571-781) of human β-catenin	BD Transduction Laboratories	IV
MSH6	mouse mAb (clone 44) against the N-terminal fragment (amino acids 225-333) of human MSH6	BD Transduction Laboratories	I, II, III
MSH2	mouse mAb (clone GB12, Ab-1) against the N-terminal fragment of human MSH2	Calbiochem	I, II, III

mAb, monoclonal antibody

Table 9 Experimental conditions for the site-directed mutagenesis (I, II, III)

Mutation	Oligos for fragments A and B in 1st PCR (5′→3′) (the mutated site is marked with bold letter and shaded background)	Size (bp)	Oligos for 2nd PCR	Size (bp)	Cloning site
MSH6-R128L	FA: CGGATTATTCATACCGTCCC	435	FA	1282	*BamHI*
	RA: CTGTACATGAACA**A**GGACTGATTTCCC		RB		*Eco81I*
	FB: GGGAAATCAGTCC**T**TGTTCATGTACAG	874			
	RB: GCCACCACTTCCTCATCC				
MSH6-S144I	FA: CGGATTATTCATACCGTCCC	487	FA	1282	*Bam*H1
	RA: GCTTTAAAAGCCTTTTG**A**TAACCCAGCCC		RB		*Eco*81I
	FA: GGGCTGGGTTA**T**CAAAAGGCTTTTAAAGC	824			
	RA: GCCACCACTTCCTCATCC				
MSH6-G566R	FA: GATGAGCACAGGAGGAGGC	574	FA	1599	*Eco*81I
	RA: CTATGAAAAACTTTC**T**CAGTGAAGTATC		RB		*Pst*1
	FB: GATACTTCACTG**A**GAAAGTTTTTCATAG	1053			
	RB: CCATCGGTTCAATTCTACAGTC				
MSH6-P623L	FA: GATGAGCACAGGAGGAGGC	746	FA	1599	*Eco81I*
	RA: CAAAACTGGGAGCCG**A**GTATCAGACCTTC		RB		*PstI*
	FB: GAAGGTCTGATAC**T**CGGCTCCCAGTTTTG	882			
	RB: CCATCGGTTCAATTCTACAGTC				
MSH6-K728T	FA: GATGAGCACAGGAGGAGGC	1058	FA	1599	*Eco81I*
	RA: CGTTGATAGGCT**G**TGGTGAAGATAGC		RB		*PstI*
	FB: GCTATCTTCACCA**C**AGCCTATCAACG	567			
	RB: CCATCGGTTCAATTCTACAGTC				
MSH6-G881K+S	FA: GATGAGCACAGGAGGAGGC	1518	FA	1602	*Eco81I*
	RA: AGACTTAAAAC**TTTT**ATCAGCAACTTC		RB		*PstI*
	FB: GAAGTTGCTGAT**AAAA**GTTTTAAGTCT	111			
	RB: CCATCGGTTCAATTCTACAGTC				
MSH6-P1087T	FA: GTCTAAAATCCTTAAGCAGG	623	FA	1612	*Pst*I
	RA: GCTCTAAGAAGG**T**GGGGGTATCTTCCG		RB		*Xho*I
	FB: CGGAAGATACCCCC**A**CCTTCTTAGAGC	1016			
	RB: CTGATTATGATCCTCTAGTAC				
MSH6-P1087R	FA: GTCTAAAATCCTTAAGCAGG	623	FA	1612	*Pst*I
	RA: GCTCTAAGAAG**C**GGGGGGTATCTTCCG		RB		*Xho*I
	FB: CGGAAGATACCCCCC**G**CTTCTTAGAGC	1016			
	RB: CTGATTATGATCCTCTAGTAC				

Table 9 continues on page 44

Table 9 continued from page 43

MSH6-R1095H	FA:GTCTAAAATCCTTAAGCAGG	646	FA	1612	*Pst1*
	RA: GCAAGGATGGTGTGATCCTTTAAGC		RB		*Xho1*
	FB: GCTTAAAGGATCACACCATCCTTGC	991			
	RB: CTGATTATGATCCTCTAGTAC				
MSH6-E1193K	FA: GTCTAAAATCCTTAAGCAGG	942	FA	1612	*PstI*
	RA: GTTTCACTTAATTTAACAAAAAATGTAC		RB		*XhoI*
	FB: GTACATTTTTTGTTAAATTAAGTGAAAC	698			
	RB: CTGATTATGATCCTCTAGTAC				
MSH6-L1354Q	FA: GTCTAAAATCCTTAAGCAGG	1427	FA	1612	*PstI*
	RA: CCTTAATCAAAGTCTTGCAATTTATGGAC		RB		XhoI
	FB: GTCCATAAATTGCAGACTTTGATTAAGG	213			
	RB: CTGATTATGATCCTCTAGTAC				
MSH2-I145M	FA: CGGATTATTCATACCGTCCC	534	FA	2024	*BamH1*
	RA: CACCCACAACACCCATGGAAGCTGAC		RB		*Nde1*
	FB: GTCAGCTTCCATGGGTGTTGTGGGTG	1516			
	RB: ATCTTGAACTTCAACACAAGC				

RESULTS

FUNCTIONALITY OF MSH6 AND MSH2 PROTEIN VARIANTS (I, II, III)

Missense mutations were introduced into *MSH6* or *MSH2* cDNAs using PCR-based site-directed mutagenesis and the products were cloned into the plasmid pFastBac1. The cloned mutant fragments were verified by DNA sequencing. The recombinant baculoviruses were used as expression vectors and they were transfected into *Sf9* insect cells. For protein production, insect cells were always co-infected with MSH2 and MSH6 recombinant baculoviruses, since both *in vivo* studies in mice (de Wind et al. 1999) and *in vitro* studies in human cells (Marra et al. 1998; Chang et al. 2000) have shown that the MSH6 protein is unstable in the absence of its partner MSH2. After 72 hours of culture, the total protein extracts, including the overexpressed MSH6 and MSH2 proteins, were prepared.

Western blot and interaction analyses of the MSH6 variants with wildtype MSH2 (I, II, III)

The total protein extracts from *Sf9* insect cells were investigated by Western blot and coimmunoprecipitation analyses to study the effect of the mutations on the protein expression level and on the MSH2-MSH6 (MutSα) interaction. In Western blotting, the amount of the wildtype (WT) MSH2 protein was adjusted to be equal in different extracts in order to see a variation in the MSH6 expression level. In coimmunoprecipitation analysis, MSH6 protein was specifically immunoprecipitated from the total protein extract using the antibody against it. MSH6 and coprecipitated MSH2 proteins were resolved on a polyacrylamide gel and detected by Western blotting. The purified MutSα complex and the wildtype MutSα total protein extract were used as controls.

The MutSα-WT and ten out of eleven MutSα-variants contained similar amounts of MSH2 and MSH6 proteins in total protein extracts. In the extract MutSα-G566R, the amount of the MSH6 protein was clearly reduced (**I**: Figure 1). The reduction of this variant was observed even if twice the normal amount of the recombinant MSH6-G566R baculovirus was used for transfection and the amount of MSH2 kept normal. However, the mutation did not abolish the MSH2-MSH6

interaction. All the studied MSH6 variants interacted normally with MSH2-WT excluding MSH6-E1193K, which coprecipitated clearly less MSH2-WT than MSH6-WT (**III**: Figure 1A).

Western blot and interaction analyses of the MSH2 variant with wildtype MSH6 and with the MSH6 variants (II)

In study **II**, we described two colon cancer families in which the probands carried missense mutations in both *MSH2* and *MSH6* genes. The *MSH2* mutation, MSH2-I145M, was the same in both families, whereas the *MSH6* mutations were different (MSH6-R1095H and MSH6-L1354Q). The same functional analyses as in studies **I** and **III** were performed to determine which one of the mutations could primarily underlie the cancer susceptibility in these families.

The MSH2-I145M variant repeatedly showed a large amount of protein in co-expression with MSH6-WT in contrast to coexpression with either of the two mutated MSH6 proteins. Furthermore, when MSH2-I145M/MSH6-WT total protein extract was coimmunoprecipitated with anti-MSH6 antibody, the ratio of the amount of precipitated MSH2 to the amount of precipitated MSH6 was clearly increased when compared to the ratio of MSH2 to MSH6 in the wildtype control extract or in the mutated extracts. However, none of the mutations abolished the MSH2-MSH6 interaction (**II**: Figure 2).

Mismatch repair capability of MutSα heterodimers (I, II, III)

According to coimmunoprecipitation analysis, none of the studied mutant proteins entirely abolished the MSH2-MSH6 interaction. In order to find out if the mutations could still be responsible for the MMR-defect (**I**, **III**), and in study **II** to further clarify which one of the two mismatch repair gene mutations might contribute to cancer susceptibility, the recombinant MutSα variants were tested in an *in vitro* MMR assay. In the assay, the total protein extract from baculovirus-infected *Sf9* cells, which contained the overexpressed recombinant MutSα variant, was incubated together with nuclear extract (NE) from a mismatch repair-deficient cell line lacking functional MSH6 or MSH2 protein. The repair reaction was carried out using a nicked circular heteroduplex molecule, which contains a G•T mismatch, as the substrate (Lu et al. 1983; Lahue et al. 1989;

Holmes et al. 1990). If the MutSα variant can correct the mismatch a specific restriction enzyme is able to cut the heteroduplex, so that the repair efficiency can be estimated at the conclusion of the repair reaction. Since the heteroduplex DNA molecules are not all repairable, they are added in excess and the percentage of repaired molecules in the proficient wildtype controls are used as a reference level.

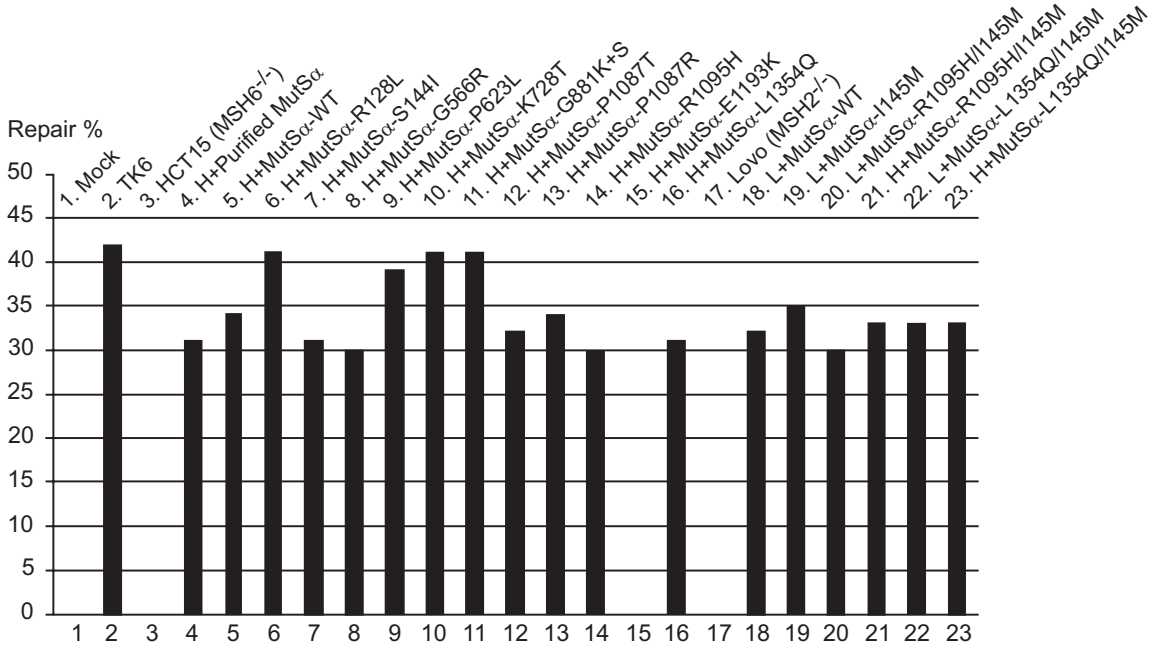

Figure 6 Mismatch repair activity of MMR-deficient HCT15 (MSH6$^{-/-}$) and Lovo (MSH2$^{-/-}$) nuclear extracts (NEs) complemented with MutSα variants (above). Mock (lane 1), heteroduplex DNA with no protein used as a negative control; TK6 (lane 2), MMR-proficient NE used as a positive control; HCT15 (H) (lane 3) and Lovo (L) (lane 17) are MMR-deficient NEs used as negative controls. The repair efficiencies are represented as repair percentages (the proportion of repaired DNA of the total DNA added to the reaction).

As represented in Figure 6, the negative controls, mock, and NEs from the HCT15 (MSH6$^{-/-}$) and Lovo (MSH2$^{-/-}$) cells, were not able to repair the G•T mismatch. The positive control, NE from MMR-proficient TK6 cells, repaired 42% of the heteroduplex DNA. Also the purified MutSα and MutSα-WT total extract were able to complement the MSH6-deficient NE of HCT15 and MSH2-deficient NE of Lovo cells and repaired approximately 30% of heteroduplex DNA. Interestingly, ten of eleven studied MSH6 mutant variants were proficient in the assay. MutSα-R128L, MutSα-S144I, MutSα-G566R, MutSα-P623L, MutSα-

K728T, MutSα-G881K+S, MutSα-P1087T, MutSα-P1087R, MutSα-R1095H and MutSα-L1354Q repaired 41%, 31%, 30%, 39%, 41%, 41%, 32%, 34%, 30% and 31% of G•T heteroduplexes, respectively. MutSα-E1193K failed to repair mismatches and displayed complete MMR deficiency (0%). The *Sf9* extracts including the mutated MSH2 with wildtype MSH6 (MutSα-I145M) or with the mutated MSH6 proteins (MutSα-R1095H/I145M and MutSα-L1354Q/I145M) were all proficient in the MMR assay. They were able to complement both MSH2-deficient and MSH6-deficient NEs, repairing approximately 32% of G•T molecules. The repair percentage values are an average of at least two independent experiments.

Table 10 summarizes the functionality of the studied MSH6 and MSH2 protein variants.

Table 10 The results of the functional tests.

	MSH6 variants									**MSH2 variant/ MSH6 variants**	
	R128L	**S144I**	**G566R**	**P623L**	**K728T**	**G881K+S**	**P1087T**	**P1087R**	**E1193K**	**MSH2-I145M/ MSH6-R1095H**	**MSH2-I145M/ MSH6-L1354Q**
Protein expression	Normal	Normal	Decreased	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
MutSα interaction	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Decreased	Normal	Normal
MMR capability	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Decreased	Normal	Normal

APC AND β-CATENIN EXPRESSION PATTERNS IN HNPCC-RELATED COLORECTAL AND ENDOMETRIAL CARCINOMAS (IV)

The molecular basis of organ selection in HNPCC is poorly understood. Overactivation of the Wnt signaling pathway has been shown to play an important role in tumorigenesis of CRCs, including HNPCC-related CRCs, as well as sporadic ECs, but HNPCC-ECs are poorly characterized in this regard. The overactivation of the pathway is mostly a consequence of mutations in the *APC* or *β-catenin* genes (Miyaki et al. 1999; Akiyama et al. 2000). The pathological impact of these mutations can be demonstrated by immunohistochemical staining of β-catenin protein, which is present in the nucleus only if the Wnt

signaling pathway has been activated due to the inactivation of *APC* or alterations in the *β-catenin* gene itself (Morin et al. 1997; Rubinfeld et al. 1997; Fukuchi et al. 1998; Palacios and Gamallo 1998; Sparks et al. 1998; Kobayashi et al. 1999; Mirabelli-Primdahl et al. 1999; Iwamoto et al. 2000). Thus, in tumor tissue, where APC immunoreactivity is absent, β-catenin staining can be seen in the nucleus. In normal colonic mucosa and in normal epithelium of the endometrium, β-catenin expression is seen at the plasmamembrane (Fukuchi et al. 1998; Iwamoto et al. 2000; Hao et al. 2002).

To clarify the molecular basis of organ selection in HNPCC, we immunohistochemically compared APC and β-catenin expression patterns in 31 HNPCC-CRCs and 19 HNPCC-ECs. Tumor samples were derived from patients carrying the same predisposing mismatch repair gene mutations in either *MLH1* or *MSH2*. Tumors were considered positive for APC if staining was present in $\geq$ 10% of tumor cells and negative if there was staining in < 10% of tumor cells accompanied by positive staining of the matching normal tissue. Tumors showing any nuclear staining for β-catenin were classified as nuclear.

APC and β-catenin protein expression in HNPCC-related CRCs and ECs

Loss of APC expression was observed in 39% of CRCs (12 /31) and in 16% of ECs (3/19). All negative CRC tumors were from HNPCC patients carrying *MLH1* mutations. Two negative EC tumors were from *MLH1* mutation carriers and one was from a *MSH2* mutation carrier. All APC negative tumors showed nuclear β-catenin staining. Overall, nuclear β-catenin expression was observed in 84% of CRCs (26/31) and in 53% of ECs (10/19). Our series comprised three tumors from *MSH2* mutation carriers, and two of them showed normal β-catenin expression. Immunohistochemical results from each HNPCC patient are described in detail in Table 11**.**

Table 11 APC and β-catenin expression in HNPCC-related colorectal and endometrial cancers

HNPCC-CRC					
ID	**Mut[a]**	**APC[b]**	**β-catenin[c]**		
			M	**C**	**N**
1:32	1	+	-	++	-
1:49	1	+	-	+++	+++
2:6	1	-	-	++	+++
2:67	1	-	-	+++	+++
3:9	1	+	-	+++	+++
10:6	1	-	-	-	+++
11:9	1	+	-	++	+
11:13	1	-	-	+++	+
19:48	1	+	-	++	++
43:23	1	+	+++	-	-
50:45	1	-	-	++	+++
54:84	1	+	++	-	-
59:11	1	+	+	+	-
60:5	1	+	-	+++	+++
66:47	1	+	-	+++	+++
82:10	1	-	-	+++	+++
99:9	1	-	+	+	+
26:9	2	-	-	++	+++
29:26	2	-	-	++	+++
61:44	2	+	+	+	-
90:15	2	+	-	++	++
91:20	2	+	-	+++	+++
90:29	2	+	+	-	-
7:20	3	-	+++	+++	+
67:11	5	+	-	++	+++
39:50	6	-	-	+	++
40:9	7	-	-	++	++
72:5	8	+	-	++	+++
38:8	9	+	++	-	-
93:4	9	+	-	++	+++
93:30	9	+	+++	-	-

HNPCC-EC					
ID	**Mut[a]**	**APC[b]**	**β-catenin[c]**		
			M	**C**	**N**
2:51	1	+	-	+	++
3:5	1	+	++	+	-
11:33	1	+	++	-	-
19:48	1	-	-	++	++
30:36	1	+	++	-	-
43:23	1	-	-	+++	+++
66:47	1	+	++	-	-
82:9	1	+	-	++	+++
99:13	1	+	++	++	-
7:18	3	+	+++	-	+
28:11	5	+	++	-	-
67:31	5	+	+	++	++
105:9	5	+	++	-	-
39:19	6	+	+++	-	-
39:50	6	+	-	+++	+++
40:9	7	+	+++	-	-
83:11	8	+	+++	-	-
93:7	9	-	+	+	+++
93:27	9	+	++	-	-

[a] Mutations 1-8 affect *MLH1* while mutation 9 affects *MSH2* (Holmberg et al. 1998; NystromLahti et al. 1996)

[b] Tumors were considered positive (+) for APC if staining was present in ≥ 10% of tumor cells and negative (-) for APC if there was staining in <10% of tumor cells accompanied by positive staining in ≥ 10% of the matching normal tissue

[c] Tumors showing any nuclear staining for β-catenin were classified as nuclear (N). C, cytoplasmic; M, membranous. The intensity of β-catenin staining was graded as weak (+), intermediate (++) or strong (+++).

DISCUSSION

PATHOGENICITY OF INHERITED MISSENSE ALTERATIONS IN THE MMR GENE, *MSH6* (I, III)

Germline mutations in four different MMR genes, *MSH2*, *MLH1*, *MSH6* and *PMS2*, are associated with HNPCC. A great proportion of all mutations identified in MMR genes are of the missense type, the consequences of which are difficult to interpret (http://www.nfdht.nl). Especially the pathogenicity of mutations in *MSH6*, the third most common suscebtibility gene in HNPCC syndrome, often remains questionable, because they are mainly linked to putative HNPCC families with atypical clinical characteristics such as a late age of onset, few affected family members, a tumor spectrum with an excess of endometrial cancers and a lack of or low MSI phenotype (Kolodner et al. 1999; Wijnen et al. 1999; Wu et al. 1999; Parc et al. 2000; Wagner et al. 2001).

The main aim of this thesis was to elucidate whether and how *MSH6* missense mutations cause cancer susceptibility (**I**, **III**). The pathogenicity of HNPCC germline mutations is associated with malfunction of the DNA mismatch repair reaction, which leads through the microsatellite mutator pathway to tumor formation. To clarify the role of *MSH6* missense mutations in this context, we selected to study alterations which fulfilled at least two of the following criteria supporting their assumed pathogenicity: (1) the mutated codon was located in an evolutionarily conserved residue (Table 4 in page 39); (2) the amino acid substitution was significant, for example changing its polarity (Table 4 in page 39); (3) the mutation was not present in healthy control individuals and (4) *MSH2* and *MLH1* germline mutations were excluded.

The nine *MSH6* missense mutations included in the studies were found in 13 putative HNPCC families (Figure 6 in page 40), none of which fulfilled the Amsterdam criteria I or II (Vasen et al. 1991, 1999). Five *MSH6* variations were derived from the series of consecutive EC and CRC patients selected for study after their tumors were determined to be microsatellite unstable. Irrespective of the MSI phenotype in their tumors, none of the mutation carriers had first-degree relatives with EC or CRC or any family history of cancer suggestive of HNPCC

(**III**). Four studied variants, listed as pathogenic mutations in the mutation database, were identified from non-HNPCC or suspected HNPCC families comprising only few affected family members with a late age of onset, which are uncharacteristic features of HNPCC (**I**) (Kolodner et al. 1999; Wijnen et al. 1999; Wu et al. 1999; Berends et al. 2002). Segregation studies to distinguish pathogenic missense alterations from nonpathogenic variation were not available from any families under scrutiny. The studies were hindered by insufficient family size or unavailability of specimens. Microsatellite instability data was available in 10 out of 13 studied tumors (Table 5 in page 41). Four tumors showed a MSI low phenotype and six showed a MSI high phenotype. This indicates that the majority of studied tumors were MMR-deficient. Three out of four MSI-low tumors and four out of six MSI-high tumors were endometrial cancers, suggesting that the MSI phenotype was not restricted to tumor type. Immunohistochemical staining results of the MMR proteins MLH1, MSH2, MSH6 and PMS2 were available in 8 out of 13 studied tumors (Table 5 in page 41). In immunohistochemical analysis, it is important to note that even if the amino acid substitution is pathogenic the mutation does not necessarily lead to loss of the protein. On the other hand, alterations in MMR proteins can prevent heterodimerization so that the unstable partner of the complex (MSH6, MSH3, PMS2) may be absent or present only in a low concentration in the tumor tissue (Marra et al. 1998; de Wind et al. 1999; Räschle et al. 1999; Chang et al. 2000).

Here, the pathogenicity of *MSH6* missense mutations was evaluated by studying their effect on the overall protein expression level, the MSH2-MSH6 protein interaction, and on the mismatch repair capability of MSH2-MSH6 (MutSα) heterodimers. The Baculovirus expression system was used to produce MutSα variants in cultured insect cells. The MSH2-MSH6 protein interaction was studied with a coimmunoprecipitation assay by using the anti-MSH6-antibody to coprecipitate MSH2 protein. The MMR capability of MutSα heterodimers was resolved in the human cell-based *in vitro* MMR assay (Lahue et al. 1989; Holmes et al. 1990; Nyström-Lahti et al. 2002). By using a cell line (HCT15) that has defective MutSα but functional MutSβ (MSH2-MSH3), and a heteroduplex with a base-base mismatch as a substrate, we were able to study the functionality of MSH6 variants in MMR.

Eight out of nine studied *MSH6* missense mutations showed no impairment in our functional tests, while one mutation, MSH6-E1193K, displayed marked impairment of both the MSH2-MSH6 interaction and mismatch repair function (**I**, **III**). Compared to MSH6 wildtype protein, the MSH6-E1193K variant coprecipitated clearly less MSH2, and the heterodimer MutSα-E1193K showed a defect in mismatch repair. This mutation occurred in two women with endometrial cancer (ages at onset 59 and 60 years). Both tumors showed a lack of mutated MSH6 protein. This observation was compatible with MMR deficiency and the MSI phenotype associated with this mutation. The substitution in the conserved codon 1193 was dramatic since amino acid alteration from negatively charged glutamate to positively charged lysine changes the polarity of the amino acid. This codon is further located in a highly conserved region between the ATP and Mg^{2+} binding sites (Iaccarino et al. 1998, 2000; Dufner et al. 2000) (Figure 5 in page 38) and could accordingly be expected to be sensitive to alterations. However, irrespective of the MMR deficiency associated with the MSH6-E1193K mutation, it occurred in probands who showed no family history of cancer suggestive of HNPCC (**III**).

Two mutations, MSH6-S144I and MSH6-G566R, had previously been studied in the *Saccharomyces cerevisiae* –based functional assay and suggested to be loss-of-function and partial loss-of-function mutations, respectively (Kolodner et al. 1999). In our assay, both protein variants MutSα-S144I and MutSα-G566R repaired mismatches as efficiently as wildtype MutSα and MSH6-S144I showed no impairment of expression level or heterodimerisation with MSH2 protein. However, MSH6-G566R was the only variant among the studied MSH6 variants that showed a markedly decreased expression level compared to the expression level of wildtype MSH6 protein, suggesting problems with its stability. The colorectal carcinoma from the MSH6-G566R mutation carrier was reported to have a high MSI status, but in that case the immunohistochemistry was not done to confirm that the high MSI phenotype was specifically linked to the inactivation of MSH6 protein. We cannot exclude the possibility that pathogenicity of this mutation is caused by lower levels of functional MSH6 protein, as has previously been shown to be the case with some *MLH1* mutations (Raevaara et al. 2003). The codon G566 is located in one of the two MSH2-MSH6 interaction regions (Figure 5 in page 38) (Guerrette et al. 1998). In

coimmunoprecipitation analysis the MSH6-G566R variant was still able to interact with MSH2 protein (**I**).

The codon 1087 was interesting, since it is located in the $(C)_8$ repetitive region in *MSH6*, frequently used as an MSI marker (Wijnen et al. 1999; Malkhosyan et al. 1996). Many endometrial carcinomas have shown instability in that repeat region (Akiyama et al. 1997; Miyaki et al. 1997; Wijnen et al. 1999; de Leeuw et al. 2001). The significant frequency of instability at the $(C)_8$ repeat is suggested to contribute to bi-allelic inactivation of the *MSH6* gene (de Leeuw et al. 2001). We studied two substitutions in that region, MSH6-P1087T and MSH6-P1087R, and interestingly both variants were functional in the *in vitro* MMR assay (**I**).

The three tumors, derived from the patients carrying the mutations MSH6-R128L, MSH6-K728T and MSH6-G881K+S, which were MMR-proficient in our assay, expressed MSH6 but lacked MLH1 protein. Promoter hypermethylation of the *MLH1* gene was observed in all these tumors, which might explain the inactivation of *MLH1*, and consequently the lack of MLH1 and high MSI phenotype in the tumors. Transcriptional silencing of MLH1 is frequently associated with somatic MMR defect and high MSI in sporadic colorectal carcinomas (Liu et al. 1995; Kane et al. 1997; Veigl et al. 1998; Esteller et al. 1999; Kuismanen et al. 2000). The mutation MSH6-P623L, which was also MMR-proficient in our functional assay, was found in a patient whose EC showed high MSI and lack of MSH6, but positive staining for the MMR proteins MSH2, MLH1 and PMS2, suggesting that loss of MSH6 was the reason for the MSI phenotype in the tumor (**III**).

In summary, the human cell-based *in vitro* MMR assay investigates the phenotypic consequences of HNPCC mutations in a homologous human MMR system. Our results showed that under stable *in vitro* circumstances, when the amounts of the proteins are sufficient for repair, eight out of nine mutated MSH6 proteins were as proficient as wildtype MSH6 protein in MMR reaction. However, the results do not exclude the possibility that the mutations can still have an effect, not directly on the repair function, but on earlier steps *in vivo*. Examples would be the instability of *MSH6* mutant mRNA transcripts, instability of mutated MSH6 proteins or problems in nuclear localization. The MMR-deficient missense variation, MSH6-E1193K, was derived from a family which

clinically resembles the other kindreds under study. Thus, this missense change seems to be clinically significant, but has low penetrance. Our results from the functional assay, together with the clinical data of the patients and their families, suggest that most missense changes in *MSH6* cause no or low cancer susceptibility. Despite the limitations concerning the functional tests, they are the only way to distinguish pathogenic alterations from nonpathogenic variations especially in cases where segregation studies are not possible. Thus, functional tests are needed to confirm that cancer susceptibility in a family is indeed linked to a detected mutation. This is important in genetic counselling since it has to be based on a reliable assessment of the pathogenicity of the mutation.

PATHOGENICITY OF TWO DIFFERENT MMR GENE MUTATIONS INHERITED IN A SINGLE COLON CANCER PATIENT (II)

In study **II**, we described two colon cancer families in which the probands carried missense mutations in both the *MSH2* and *MSH6* genes. The *MSH2* mutation, MSH2-I145M, was the same in both families, whereas the *MSH6* mutations, MSH6-R1095H and MSH6-L1354Q, were different. The families did not fulfill the Amsterdam criteria I or II (Vasen et al. 1991, 1999). The mean age at colon cancer onset was as high as 67 years in Fam-MSH6-12 and 58 years in Fam-MSH6-15. Colorectal tumors in both families displayed a MSI high phenotype. Despite the two susceptibility mutations in different MMR genes, these patients did not display a clinically more severe phenotype than heterozygous carriers in general, which is consistent with previous reports (Wu et al. 1999, 2001; Samowitz et al. 2001). Interestingly, all affected family members were in one generation in both families, which suggests a recessive rather than the dominant mode of inheritance characteristic of HNPCC (Figure 6 in page 40).

We used the same methods as in studies **I** and **III** to clarify which one of the MMR gene mutations could primarily underlie the cancer susceptibility in the families. Our results showed that none of the three mutations, MSH2-I145M, MSH6-R1095H or MSH6-L1354Q, individually lead to a deficiency in MMR, but the results did not exclude the possibility that the two mutations together result in compound pathogenicity. MSH2 and MSH6 proteins function together as a heterodimeric complex that recognizes mismatches and small insertion/deletion

loops. This is in agreement with the idea of their compound pathogenicity. Because no cell line in which both *MSH2* and *MSH6* are inactivated is available, and since it has been demonstrated that the MSH6 protein is unstable without its partner MSH2 (Marra et al. 1998; Chang et al. 2000), we used nuclear extracts of MSH2-deficient LoVo cells to study the compound effect of the two mutations. However, even a small amount of natural MSH6 protein, which might have been present in the LoVo extract, could have interacted with free MSH2-I145M molecules with the result that the complemented LoVo extract was functional in the MMR assay.

We also found that MSH2-I145M interacted more efficiently with wildtype MSH6 than with mutated MSH6 variants suggesting some kind of aggregation of MSH2-I145M molecules with MSH6-WT molecules. One of the three mutations, MSH6-L1354Q, is located in the MSH2-MSH6 interaction region in the extreme C-terminal part of the MSH6 polypeptide (Guerrette et al. 1998) (Figure 5 in page 38). This mutated protein was still able to interact with wildtype MSH2 protein and was functional in the MMR test.

To summarize, the mutations alone did not cause MMR deficiency in the families. In addition, the human cell-based *in vitro* MMR assay did not permit the clarification of the possible compound pathogenicity of the two mutations or the biochemical events preceding the MMR reaction (see above). Hence, the results of the test should always be interpreted together with clinical and genetic data of the families under study.

APC/β-CATENIN INVOLVEMENT IN HNPCC-RELATED COLORECTAL AND ENDOMETRIAL CARCINOMAS (IV)

The molecular basis of organ selection in HNPCC is poorly understood. It is known that the target gene pattern is more heterogenous and involves a lower proportion of unstable markers per tumor in HNPCC-ECs than HNPCC-CRCs despite the same predisposing MMR mutation in the patients (Kuismanen et al. 2002). Some target genes of the microsatellite mutator pathway, such as *IGFIIR*, *MSH3* and *MSH6*, seems to be fairly evenly involved in these tumors (de Leeuw et al. 2000; Kuismanen et al. 2002), whereas *TGFβRII* and *PTEN* show

considerable tissue specificity. (Konishi et al. 1996; Tannergård et al. 1997; Kuismanen et al. 2002; Paoloni-Giacobino et al. 2002; Zhou et al. 2002). The differences between affected target genes and their inactivation frequencies support the suggestion that tumor progression occurs via distinct mechanisms in these two organs (de Leeuw et al. 2000; Schweizer et al. 2001; Kuismanen et al. 2002). We concentrated on comparing the involvement of APC and β-catenin, the two major components of the Wnt signaling pathway, in the tumorigenesis of HNPCC-related CRCs and ECs. The Wnt pathway is overactivated in 65% of HNPCC-CRCs (Miyaki et al. 1999), in about 90% of sporadic CRCs (Iwamoto et al. 2000; Hao et al. 2002) and in about 30% of sporadic ECs (Fukuchi et al. 1998; Palacios et al. 2001; Schlosshauer et al. 2002). However, in HNPCC-related ECs its role has not been determined.

The overactivation of the Wnt pathway is mainly a consequence of mutations in the *APC* or *β-catenin* genes, but alterations in *AXIN2* and *TCF4*, target genes of the microsatellite mutator pathway, have also been found in mismatch repair defective colon tumors (Miyaki et al. 1999; Akiyama et al. 2000; Duval et al. 1999a; Liu et al. 2000). The pathological impact of these mutations can be demonstrated by immunohistochemical staining of β-catenin protein. In normal colonic mucosa and in normal epithelium of the endometrium, β-catenin expression can be seen at the plasmamembrane (Fukuchi et al. 1998; Iwamoto et al. 2000; Hao et al. 2002), where it is bound to E-cadherin and α-actin, mediating the connection of cadherin cell adhesion molecules to the actin cytoskeleton (McCrea et al. 1991; Hulsken et al. 1994). The cytoplasmic concentration of free β-catenin is very low due to its degradation by proteosomes in normal epithelial cells. Inactivation of *APC* leads to the accumulation of β-catenin in the cytoplasm and nucleus. In tumor tissue, where APC immunoreactivity is absent, β-catenin staining can be seen in the cytoplasm and nucleus (Iwamoto et al. 2000). Alterations in the *β-catenin* gene itself can also lead to its stabilization. In that case, mutations often occur in the regulatory domain (codons 29-48) in exon 3 of the *β-catenin* gene, which prevents its phosphorylation and degradation (Morin et al. 1997; Rubinfeld et al. 1997; Fukuchi et al. 1998; Palacios and Gamallo 1998; Sparks et al. 1998; Kobayashi et al. 1999; Mirabelli-Primdahl et al. 1999).

Comparison between 31 CRCs and 19 ECs from HNPCC patients with similar predisposing germline mutations revealed differences in APC and β-catenin

expression. Tumor-specific loss of APC expression was always associated with nuclear β-catenin localization, suggesting deregulation of the Wnt signaling pathway. Our APC and β-catenin expression results in HNPCC-CRCs were consistent with studies of sporadic CRCs (Huang et al. 1996; Miyaki et al. 1999; Sparks et al. 1998; Mirabelli-Primdahl et al. 1999; Iwamoto et al. 2000; Hao et al. 2002). This study did not aim to analyze the exact inactivation mechanism of APC. The frequency of somatic *APC* mutation is observed to be lower in HNPCC-CRCs (~20%) than in sporadic MSS colon tumors (>70%) (Miyaki et al. 1994; Huang et al. 1996; Konishi et al. 1996), and no somatic *APC* mutations have so far been found in endometrial cancers (Schlosshauer et al. 2000; Moreno-Bueno et al. 2002). We identified 16% (3/19) of EC tumors with loss of APC. This might be the first clear evidence of Wnt pathway involvement in endometrial tumorigenesis, since APC loss was associated with nuclear β-catenin localization. Previous studies of sporadic ECs that detected APC promotor hypermethylation (47%) and LOH at the APC locus (26%) showed that neither of these was associated with nuclear β-catenin (Moreno-Bueno et al. 2002). The frequency of nuclear β-catenin in HNPCC-ECs (53%) was consistent with sporadic ECs (Fukuchi et al. 1998; Palacios et al. 2001; Schlosshauer et al. 2002), but it was lower than what we observed in HNPCC-related CRCs (84%). We did not find any significant correlation between deregulation of the Wnt signaling pathway and a specific predisposing MMR gene mutation.

Taken together, we showed that activation of the Wnt pathway, as judged by nuclear β-catenin expression, plays an important role in both colorectal and endometrial tumorigenesis. APC and β-catenin abnormalities in endometrial tumors are less frequent than in colon tumors even when the same mismatch repair gene mutation predisposed to both tumor types.

CONCLUSIONS

1) In stable *in vitro* circumstances, when the amounts of the proteins are sufficient for repair, most *MSH6* missense mutations are MMR-proficient
2) *In vivo MSH6* missense mutations may affect biochemical events preceding the MMR function, for example the pathogenicity of some *MSH6* missense mutations can be linked to the shortage of functional protein
3) Some missense changes in *MSH6*, which lead to impaired mismatch repair, are likely to be clinically significant, but can still have low penetrance
4) Results of the functional assay together with the clinical data of the patients and their families suggest that most missense changes in *MSH6* cause no or low cancer susceptibility
5) Especially in situations where cosegregation of a missense mutation and disease phenotype cannot be studied, a functional test is needed to confirm that cancer susceptibility in a family is linked to a detected mutation
6) The human cell-based *in vitro* MMR assay can be used to resolve which one of the two MMR gene mutations found in the same patient is pathogenic, but compound pathogenicity of MSH2 and MSH6 mutations can not be studied by it because of the lack of a suitable cell line
7) HNPCC-related endometrial cancers also progress through deregulated Wnt signaling. APC/β-catenin abnormalities in HNPCC-related ECs, although significant, are less frequent than in HNPCC-related CRCs even when the same mismatch repair gene mutation predisposed to both tumor types

FUTURE PROSPECTS

To further explore how *MSH6* missense mutations can cause cancer susceptibility it would be necessary

1) To study the expression level and stability of the mutated MSH6 proteins in a homologous human expression system
2) To study whether the *MSH6* mutant mRNA transcripts are unstable
3) To clarify if MSH6 variants have problems in nuclear transfer and localization

To further clarify the molecular basis of organ selection and tumor progression in HNPCC, it would be required

4) To clarify whether there are other gene defects in the Wnt signaling pathway that might substitute for β-catenin abnormalities, e.g. AXIN2
5) To make larger-scale expression studies of the target tumor suppressor genes of the mutator pathway

ACKNOWLEDGEMENTS

This study has been carried out at the Department of Biological and Environmental Sciences, Division of Genetics, University of Helsinki, under the supervision of docent Minna Nyström, during the years 2000-2004. The work has been financially supported by the Sigrid Juselius Foundation, the European Commission and the Research Foundation of the University of Helsinki.

My warmest thanks go to my supervisor Minna. I thank her for giving me the opportunity to work with such an interesting research project and for great supervision. It has been safe and productive to work with her. I thank her for constant support, encouragement, kindness and friendship.

I wish to acknowledge Professor Jim Schröder and Professor Hannu Saarilahti as heads of the Division of Genetics for providing excellent research facilities. I warmly thank my teacher-colleagues docent Pekka Heino and docent Liisa Halkka. Teaching with you has been fun and instructive.

I am grateful to the reviewers of this thesis, docents Nina Horelli-Kuitunen and Jukka Partanen for their comments and careful examination. I thank Jack Leo for the skillful revision of the language of this thesis.

I thank all the former and present members of our research group, Tiina Raevaara, Saara Ollila, Mari Siitonen, Karin Lönnqvist, Jukka Kantelinen and Tommi Timoharju. We have had a great and relaxed atmosphere both in the lab and outside the lab. Thank you for always being optimistic, even when I wasn't. Thanks for understanding me and my noisy work in the lab, especially my repeatedly calling out "ei hätää" when it sounded as if all our glassware had crashed on the floor. I especially thank Tiina Raevaara for all technical tips, help and support.

I warmly acknowledge Professor Päivi Peltomäki for interest in my work, helpfulness and important conversations. I also thank the members of her research group, Miina Ollikainen, Wael Abdel-Rahman, Elise Renkonen, Hannes Lohi, Annette Lindross, Shannon Kuismanen and Saila Saarinen. In particular I

express my deep gratitude to my excellent co-authors Wael and Miina. I warmly thank both of you your professional contribution to our immunohistochemical studies and encouraging attitude to my experiments.

I am very grateful to all friendly persons on the 5th floor lab, Anne Kujanpää, Elina Helenius, Tuula Puhakainen, Liisa Silvennoinen, Anne Leino, Nina von Nummers, Kalle A. Ojala, Merja Valtonen and Katarina Pelin; and on the 4th floor office, Arja Välimäki and Arja Ikävalko. It has been great to work with you and to discuss non-scientific but extremely important things with you. I would especially like to thank Arja Välimäki for secretarial help and Arja Ikävalko for all kind of helpful organizing during courses. I acknowledge Jim Schröder, Outi Kilpivaara, Outi Hallikas, Riitta Sallinen and Maija Wessman for their support and interest in my work.

I warmly thank my dear friends Miina Ollikainen, Katja Sääjärvi, Roosa Laitinen, Tarja Kariola and Leena Ahonen. Thanks that you have been interested what I have been doing. Thanks for listening to me when I exhausted you with my boring experimental problems. Thanks for understanding me when I have been living on an other planet.

I express my warmest thanks to my loving family; my mom Titta, my dad Matti, my sister Riikka and my little brother Markus (the professor of all sciences), for their understanding and never-ending support. Most of all, I want to thank my loving husband Jukka and my cheerful son Elias. You are my life.

Helsinki, October 2004

Reetta Kariola

REFERENCES

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

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

Aaltonen LA, Salovaara R, Kristo P, Canzian F, Hemminki A, Peltomäki P, Chadwick RB, Kääriäinen H, Eskelinen M, Järvinen H, Mecklin J-P, de la Chapelle A (1998) Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. N Engl J Med 338:1481-1487

Aarnio M, Sankila R, Pukkala E, Salovaara R, Aaltonen LA, de la Chapelle A, Peltomäki P, Mecklin J-P, Järvinen HJ (1999) Cancer risk in mutation carriers of DNA-mismatch-repair genes. Int J Cancer 81:214-218

Aberle H, Bauer A, Stappert J, Kispert A, Kemler R (1997) β-catenin is a target for the ubiquitin-proteosome pathway. EMBO J 16:3797-3804

Acharya S, Wilson T, Gradia S, Kane MF, Guerrette S, Marsischky GT, Kolodner R, Fishel R (1996) hMSH2 forms specific mispair-binding complexes with hMSH3 and hMSH6. Proc Natl Acad Sci USA 93:13629-13634

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

Akiyama Y, Nagasaki H, Yagi KO, Nomizu T, Yuasa Y (2000) β-Catenin and adenomatous polyposis coli (APC) mutations in adenomas from hereditary non-polyposis colorectal cancer patients. Cancer Lett 157:185-191

Arber N, Shapira I, Ratan J, Stern B, Hibshoosh H, Moshkowitz M, Gammon M, Fabian I, Halpern Z (2000) Activation of c-K-*ras* mutations in human gastrointestinal tumors. Gastroenterology 118:1045-1050

Bac-to-Bac® baculovirus expression system, Instruction Manual (2003) Invitrogen™ life technologies

Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, Grosscheld R, Birchmeier W (1996) Functional interaction of β-catenin with the transcription factor LEF-1. Nature 382:638-642

Berends MJW, Wu Y, Sijmons RH, Mensink RGJ, van der Sluis T, Hordijk-Hos JM, de Vries EGE, Hollema H, Karrenbeld A, Buys CHCM, van der Zee AGJ, Hofstra RMW, Kleibeuker JH (2002) Molecular and clinical characteristics of *MSH6* variants: an analysis of 25 index carriers of a germline variant. Am J Hum Genet 70: 26-37

Bhattacharyya NP, Skandalis A, Ganesh A, Groden J, Meuth M (1994) Mutator phenotypes in human colorectal carcinoma cell lines. Proc Natl Acad Sci USA 91:6319-6323

Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, Srivastava S (1998) A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 58:5248-5257

Bos JL, Fearon ER, Hamilton SR, Verlaan-de Vries M, van Boom JH, van der Eb AJ, Vogelstein B (1987) Prevalence of ras gene mutations in human colorectal cancers. Nature 327:293-297

Brieger A, Trojan J, Raedle J, Plotz G, Zeuzem S (2002) Transient mismatch repair transfection for functional analysis of genetic hMLH1 and hMSH2 variants. Gut 51:677-684

Cederquist K, Emanuelsson M, Göransson I, Holinski-Feder E, Müller-Koch Y, Golovleva I, Grönberg H (2004) Mutation analysis of the *MLH1*, *MSH2* and *MSH6* genes in patients with double primary cancers of the colorectum and the endometrium: a population-based study in northern Sweden. Int J Cancer 109:370-376

Chadwick RB, Jiang G-L, Bennington GA, Yuan B, Johnson CK, Stevens MW, Niemann TH, Peltomaki P, Huang S, de la Chapelle A (2000) Candidate tumor suppressor RIZ is frequently involved in colorectal carcinogenesis. Proc Natl Acad Sci USA 97:2662-2667

Chang DK, Ricciardiello L, Goel A, Chang CL, Boland, R (2000) Steady-state regulation of the human DNA mismatch repair system. J Biol Chem 275:18424-18431

Cohn DE, Basil JB, Venegoni AR, Mutch DG, Rader JS, Herzog TJ, Gersell DJ, Goodfellow PJ (2000) Absence of PTEN repeat tract mutation in endometrial cancers with microsatellite instability. Gynecol Oncol 79:101-106

Cohn DE, Mutch DG, Herzog TJ, Rader JS, Dintzis SM, Gersell DJ, Todd CR, Goodfellow PJ (2001) Genotypic and phenotypic progression in endometrial tumorigenesis: determining when defects in DNA mismatch repair and *KRAS2* occur. Genes Chromosomes Cancer 32:295-301

Das Gupta R, Kolodner RD (2000) Novel dominant mutations in Saccharomyces cerevisiae MSH6. Nat Genet 24:53-56

Davis TW, Wilson-Van Pattern C, Meyers M, Kunugi KA, Cuthill S, Reznikoff C, Garces C, Boland CR, Kinsella TJ, Fishel R, Boothman DA (1998) Defective expression of the DNA mismatch repair protein, MLH1, alters G2-M cell cycle checkpoint arrest following ionizing radiation. Cancer Res 58:767-778

de Leeuw WJF, Dierssen JW, Vasen HFA, Wijnen JT, Kenter GG, Meijers-Heijboer H, Brocker-Vriends A, Stormorken A, Moller P, Menko F, Cornelisse CJ, Morreau H (2000) Prediction of a mismatch repair gene defect by microsatellite instability and immunohistochemical analysis in endometrial tumours from HNPCC patients. J Pathol 192:328-335

de Leeuw WJF, van Puijenbroek M, Merx R, Wijnen JTh, Bröcker-Vriends AHJT, Tops C, Vasen H, Cornelisse CJ, Morreau H (2001) Bias in detection of instability of the (C)8 mononucleotide repeat of MSH6 in tumours from HNPCC patients. Oncogene 20:6241-6244

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

de Wind N, Dekker M, Claij N, Jansen L, van Klink Y, Radman M, Riggins G, van der Valk M, van't Wout K, te Riele H (1999) HNPCC-like cancer predisposition in mice through simultaneous loss of Msh3 and Msh6 mismatch-repair protein functions. Nat Genet 23:359-362

Duckett DR, Bronstein SM, Taya Y, Modrich P (1999) hMutSα- and hMutLα-dependent phosphorylation of p53 in response to DNA methylator damage. Proc Natl Acad Sci USA 96:12384-12388

Dufner P, Marra G, Räschle M, Jiricny J (2000) Mismatch recognition and DNA-dependent stimulation of the ATPase activity of hMutSα is abolished by a single mutation in the hMSH6 subunit. J Biol Chem 275:36550-36555

Duval A, Gayet J, Zhou X-P, Iacopetta B, Thomas G, Hamelin R (1999a) Frequent frameshift mutations of the *TCF-4* gene in colorectal cancers with microsatellite instability. Cancer Res 59:4213-4215

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

Drummond JT, Genschel J, Wolf E, Modrich P (1997) *DHFR/MSH3* amplification in methotrexate-resistant cells alters the hMutSα/hMutSβ ratio and reduced the efficiency of base-base mismatch repair. Proc Natl Acad Sci USA 94:10144-10149

Edelmann W, Yang K, Umar A, Heyer J, Lau K, Fan K, Liedtke W, Cohen PE, Kane MF, Lipford JR, Yu N, Crouse GF, Pollard JW, Kunkel T, Lipkin M, Kolodner R, Kucherlapati R (1997) Mutation in the mismatch repair gene *Msh6* causes cancer susceptibility. Cell 91:467-477

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

Ellison AR, Lofing J, Bitter GA (2001) Functional analysis of human MLH1 and MSH2 missense variants and hybrid human-yeast MLH1 proteins in *Saccharomyces cerevisiae*. Hum Mol Genet 10:1889-1900

Enomoto T, Inoue M, Perantoni AO, Buzard GS, Miki H, Tanizawa O, Rice JM (1991) K-ras activation in premalignant and malignant epithelial lesions of the human uterus. Cancer Res 51:5308-5314

Eshleman JR, Casey G, Kochera ME, Sedwick WD, Swinler SE, Veigl ML, Willson JKV, Schwartz S, Markowitz SD (1998) Chromosome number and structure both are markedly stable in RER colorectal cancers and are not destabilized by mutations of p53. Oncogene 17:719-725

Esteller M, Catasus L, Matias-Guiu X, Mutter GL, Prat J, Baylin SB, Herman JG (1999) *hMLH1* promoter hypermethylation is an early event in human endometrial tumorigenesis. Am J Pathol 155:1767-1772

Fang W, Modrich P (1993) Human strand-specific mismatch repair occurs by a bidirectional mechanism similar to that of bacterial reaction. J Biol Chem 268:11838-11844

Fearon ER, Cho KR, Nigro JM, Kern SE, Simons JW, Ruppert JM, Hamilton SR, Preisinger AC, Thomas G, Kinzler KW, Vogelstein B (1990) Identification of a chromosome 18q gene that is altered in colorectal cancers. Science 247:49-56

Fujiwara T, Stolker JM, Watanabe T, Rashid A, Longo P, Eshleman JR, Booker S, Lynch HT, Jass JR, Green JS, Kim H, Jen J, Vogelstein B, Hamilton SR (1998) Accumulated clonal genetic alterations in familial and sporadic colorectal carcinomas with widespread instability in microsatellite sequences. Am J Pathol 153:1063-1078

Fukuchi T, Sakamoto M, Tsuda H, Maruyama K, Nozawa S, Hirohashi S (1998) β-Catenin mutation in carcinoma of the uterine endometrium. Cancer Res 58:3526-3528

Genschel J, Littman SJ, Drummond JT, Modrich P (1998) Isolation of MutSβ from human cells and comparison of the mismatch repair specificities of MutSβ and MutSα. J Biol Chem 273:19895-19901

Glickman BW, Radman M (1980) *Escherichia coli* mutator mutants deficient in methylation-instructed DNA mismatch correction. Proc Natl Acad Sci USA 77:1063-1067

Goodfellow PJ, Buttin BM, Herzog TJ, Rader JS, Gibb RK, Swisher E, Look K, Walls KC, Fan M-Y, Mutch DG (2003) Prevalence of defective DNA mismatch repair and MSH6 mutation in an unselected series of endometrial cancers. Proc Natl Acad Sci USA 100:5908-5913

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

Guanti G, Resta N, Simone C, Cariola F, Demma I, Fiorente P, Gentile M (2000) Involvement of *PTEN* mutations in the genetic pathways of colorectal cancerogenesis. Hum Mol Genet 9:283-287

Guerrette S, Wilson T, Gradia S, Fishel R (1998) Interactions of human hMSH2 with hMSH3 and hMSH2 with hMSH6: examination of mutations found in hereditary nonpolyposis colorectal cancer. Mol Cell Biol 18:6616-6623

Guerrette S, Acharya S, Fishel R (1999) The interaction of the human MutL homologues in hereditary nonpolyposis colon cancer. J Biol Chem 274:6336-6341

Gurin CC, Federici MG, Kang L, Boyd J (1999) Causes and consequences of microsatellite instability in endometrial carcinoma. Cancer Res 59:426-466

Hackman P, Tannergård P, Osei-Mensa S, Chen J, Kane MF, Kolodner R, Lambert B, Hellgren D, Lindblom A (1997) A human compound heterozygote for two *MLH1* missense mutations. Nat Genet 17:135-136

Hao X, Frayling IM, Willcocks TC, Han W, Tomlinson IPM, Pignatelli MN, Pretlow TP, Talbot IC (2002) β-Catenin expression and allelic loss at *APC* in sporadic colorectal carcinogenesis. Virchows Arch 440:362-366

He T-C, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW (1998) Identification of *c-MYC* as a target of the APC pathway. Science 281:1509-1512

Helland Å, Børresen-Dale A-L, Peltomäki P, Hektoen M, Kristensen GB, Nesland JM, de la Chapelle A, Lothe RA (1997) Microsatellite instability in cervical and endometrial carcinomas. Int J Cancer 70:499-501

Hemminki A, Peltomäki P, Mecklin JP, Järvinen H, Salovaara R, Nyström-Lahti M, de la Chapelle A, Aaltonen LA (1994) Loss of the wild type MLH1 gene is a feature of hereditary nonpolyposis colorectal cancer. Nat Genet 8:405-410

Holmberg M, Kristo P, Chadwick RB, Mecklin J-P, Järvinen H, de la Chapelle A, Nyström-Lahti M, Peltomäki P (1998) Mutation sharing, predominant involvement of *MLH1* gene, and description of four novel mutations in hereditary nonpolyposis colorectal cancer. Hum Mutat 11: 482

Holmes JJ, Clark S, Modrich P (1990) Strand-specific mismatch correction in nuclear extracts of human and *Drosophila melanogaster* cell lines. Proc Natl Acad Sci USA 87:5837-5841

Huang J, Papadopoulos N, McKinley AJ, Farrington SM, Curtis LJ, Wyllie AH, Zheng S, Willson JKV, Markowitz SD, Morin P, Kinzler KW, Vogelstein B, Dunlop MG (1996) *APC* mutations in colorectal tumors with mismatch repair deficiency. Proc Natl Acad Sci USA 93:9049-9054

Hulsken J, Birchmeier W, Behrens J (1994) E-cadherin and APC compete for the interaction with β-catenin and the cytoskeleton. J Cell Biol 127:2061-2069

Iaccarino I, Marra G, Palombo F, Jiricny J (1998) hMSH2 and hMSH6 play distinct roles in mismatch binding and contribute differently to the ATPase activity of hMutSα. EMBO J 17:2677-2686

Iaccarino I, Marra G, Dufner P, Jiricny J (2000) Mutation in the magnesium binding site of hMSH6 disables the hMutSα sliding clamp from translocating along DNA. J Biol Chem 275:2080-2086

Ichii S, Takeda S, Horii A, Nakatsuru S, Miyoshi Y, Emi M, Fujiwara Y, Koyama K, Furuyama J, Utsunomiya J, Nakamura Y (1993) Detailed analysis of genetic alterations in colorectal

tumors from patients with and without familial adenomatous polyposis (FAP). Oncogene 8:2399-2405

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

Iwamoto M, Ahnen DJ, Franklin WA, Maltzman TH (2000) Expression of β-catenin and full-length APC protein in normal and neoplastic colonic tissues. Carcinogenesis 21:1935-1940

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

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

Kishida S, Yamamoto H, Ikeda S, Kishida M, Sakamoto I, Koyama S, Kikuchi A (1998) Axin, a negative regulator of the Wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates and the stabilization of β-catenin. J Biol Chem 273:10823-10826

Kleczkowska HE, Marra G, Lettieri T, Jiricny J (2001) hMSH3 and hMSH6 interact with PCNA and colocalize with it to replication foci. Genes Dev 15:724-736

Knudson AG (1971) Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci USA 68:820-823

Kobayashi K, Sagae S, Nishioka Y, Tokino T, Kudo R (1999) Mutations of the *β-catenin* gene in endometrial carcinomas. Jpn J Cancer Res 90:55-59

Kolodner RD, Tytell JD, Schmeits JL, Kane MF, Das Gupta R, Weger J, Wahlberg S, Fox EA, Peel D, Ziogas A, Garber JE, Syngal S, Anton-Culver H, Li FP (1999) Germ-line *msh6* mutations in colorectal cancer families. Cancer Res 59:5068-5074

Kondo E, Horii A, Fukushige S (2001) The interacting domains of three MutL heterodimers in man: hMLH1 interacts with 36 homologous amino acid residues within hMLH3, hPMS1 and hPMS2. Nucleic Acids Res 29:1695-1702

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

Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B, Clevers H (1997) Constitutive transcriptional activation by a β-catenin-Tcf complex in APC($^{-/-}$) colon carcinoma. Science 275:1784-1787

Kuerbitz SJ, Plunkett BS, Walsh WV, Kastan MB (1992) Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc Natl Acad Sci USA 89:7491-7495

Kuismanen SA, Holmberg MT, Salovaara R, de la Chapelle A, Peltomäki P (2000) Genetic and epigenetic modification of *MLH1* accounts for a major share of microsatellite-unstable colorectal cancers. Am J Pathol 156:1773-1779

Kuismanen SA, Moisio A-L, Schweizer P, Truninger K, Salovaara R, Arola J, Butzow R, Jiricny J, Nyström-Lahti M, Peltomäki P (2002) Endometrial and colorectal tumors from patients with hereditary nonpolyposis colon cancer display different patterns of microsatellite instability. Am J Pathol 160:1953-1958

Kurman RJ, Kaminski PF, Norris HJ (1985) The behavior of endometrial hyperplasia. A Long-term study of "untreated" hyperplasia in 170 patients. Cancer 56:403-412

Lahue RS, Au KG, Modrich P (1989) DNA mismatch correction in a defined system. Science 245:160-164

Lau PJ, Kolodner RD (2003) Transfer of the MSH2•MSH6 complex from proliferating cell nuclear antigen to mispaired bases in DNA. J Biol Chem 278:14-17

Levine RL, Cargile CB, Blazes MS, van Rees B, Kurman RJ, Ellenson LH (1998) PTEN mutations and microsatellite instability in complex atypical hyperplasia, a precursor lesion to uterine endometrioid carcinoma. Cancer Res 58:3254-3258

Levy DB, Smith KJ, Beazer-Barclay Y, Hamilton SR, Vogelstein B, Kinzler KW (1994) Inactivation of both APC alleles in human and mouse tumors. Cancer Res 54:5953-5958

Li G-M, Modrich P (1995) Restoration of mismatch repair to nuclear extracts of H6 colorectal tumor cells by a heterodimer of human MutL homologs. Proc Natl Acad Sci USA 92:1950-1954

Li J, Simpson L, Takahashi M, Miliaresis C, Myers MP, Tonks N, Parsons R (1998) The PTEN/MMAC1 tumor suppressor induces cell death that is rescued by the AKT/protein kinase B oncogene. Cancer Res 58:5667-5672

Lin YL, Shivji MK, Chen C, Kolodner R, Wood RD, Dutta A (1998) The evolutionary conserved zinc finger motif in the largest subunit of human replication protein A is required for DNA replication and mismatch repair but not for nucleotide excision repair. J Biol Chem 273:1453-1461

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

Liu B, Nicolaides NC, Markowitz S, Willson JKV, Parsons RE, Jen J, Papadopolous N, Peltomäki P, de la Chapelle A, Hamilton SR, Kinzler KW, Vogelstein B (1995) Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability. Nat Genet 9:48-55

Liu B, Parsons R, Papadopoulos N, Nicolaides NC, Lynch HT, Watson P, Jass JR, Dunlop M, Wyllie A, Peltomäki P, de la Chapelle A, Hamilton SR, Vogelstein B, Kinzler KW (1996) Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer petients. Nat Med 2:169-174

Liu W, Dong X, Mai M, Seelan RS, Taniguchi K, Krishnadath KK, Halling KC, Cunningham JM, Qian C, Christensen E, Roche PC, Smith DI, Thibodeau SN (2000) Mutations in *AXIN2* cause colorectal cancer with defective mismatch repair by activating β-catenin/TCF signalling. Nat Genet 26:146-147

Longerich S, Galloway AM, Harris RS, Wong C, Rosenberg SM (1995) Adaptive mutation sequences reproduced by mismatch repair deficiency. Proc Natl Acad Sci USA 92:12017-12020

Lu AL, Clark S, Modrich P (1983) Methyl-directed repair of DNA base-pair mismatches *in vitro*. Proc Natl Acad Sci USA 80:4639-4643

Luckow VA, Lee SC, Barry GF, Olins PO (1993) Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in Escherichia coli. J Virol 67:4566-4579

Lustig B, Behrens J (2003) The Wnt signaling pathway and its role in tumor development. J Cancer Res Clin Oncol 129:199-221

Lynch HT, de la Chapelle A (1999) Genetic suscebtibility to non-polyposis colorectal cancer. J Med Genet 36:801-818

Lynch HT, de la Chapelle A (2003) Hereditary colorectal cancer. N Engl J Med 348:919-932

Machin P, Catasus L, Pons C, Muñoz J, Matias-Guiu X, Prat J (2002) CTNNB1 mutations and β-catenin expression in endometrial carcinomas. Hum Pathol 33:206-212

Malkhosyan S, Rampino N, Yamamoto H, Perucho M (1996) Frameshift mutator mutations. Nature 382:499-500

Mann B, Gelos M, Siedow A, Hanski ML, Gratchev A, Ilyas M, Bodmer WF, Moyer MP, Riecken EO, Buhr HJ, Hanski C (1999) Target genes of β-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. Proc Natl Acad Sci USA 96:1603-1608

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

Markowitz SD, Roberts AB (1996) Tumor suppressor activity of the TGF-β pathway in human cancers. Cytokine Growth Factor Rev 7:93-102

Marra G, Iaccarino I, Lettieri T, Roscilli G, Delmastro P, Jiricny J (1998) Mismatch repair deficiency associated with overexpression of the *MSH3* gene. Proc Natl Acad Sci USA 95:8568-8573

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

Marsischky GT, Lee S, Griffith J, Kolodner RD (1999) *Saccharomyces cerevisiae* MSH2/6 complex interacts with Holliday junctions and facilitates their cleavage by phage resolution enzymes. J Biol Chem 274:7200-7206

Matton N, Simonetti J, Williams K (2000) Identification of mismatch repair protein complexes in HeLa nuclear extracts and their interaction with heteroduplex DNA. J Biol Chem 275:17808-17813

Maxwell GL, Risinger JI, Gumbs C, Shaw H, Bentley RC, Barrett JC, Berchuck A, Futreal PA (1998) Mutations of the PTEN tumor suppressor gene in endometrial hyperplasias. Cancer Res 58:2500-2503

McCrea PD, Turck CW, Gumbiner B (1991) A homolog of the armadillo protein in Drosophila (plakoglobin) associated with E-cadherin. Science 254:1359-1361

Mellon I, Rajpal DK, Koi M, Boland CR, Champe GN (1996) Transcription-coupled repair deficiency and mutations in human mismatch repair genes. Science 272:557-560

Mellon I, Champe GN (1996) Products of DNA mismatch repair genes *mutS* and *mutL* are required for transcription- coupled nucleotide-excision repair of the lactose operon in *Escherichia coli*. Proc Natl Acad Sci USA 93:1292-1297

Miller JR (2001) The Wnts. Genome Biol 3:3001.1-3001.15

Mirabelli-Primdahl L, Gryfe R, Kim H, Millar A, Luceri C, Dale D, Holowaty E, Bapat B, Gallinger S, Redston M (1999) β-Catenin mutations are specific for colorectal carcinomas with microsatellite instability but occur in endometrial carcinomas irrespective of mutator pathway. Cancer Res 59:3346-3351

Miyaki M, Konishi M, Kikuchi-Yanoshita R, Enomoto M, Igari T, Tanaka K, Muraoka M, Takahashi H, Amada Y, Fukayama M (1994) Characteristics of somatic mutation of the adenomatous polyposis coli gene in colorectal tumors. Cancer Res 54:3011-3020

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

Miyaki M, Iijima T, Kimura J, Yasuno M, Mori T, Hayashi Y, Koike M, Shitara N, Iwama T, Kuroki T (1999) Frequent mutation of *β-catenin* and *APC* genes in primary colorectal tumors from patients with hereditary nonpolyposis colorectal cancer. Cancer Res 59:4506-4509

Miyoshi Y, Nagase H, Ando H, Horii A, Ichii S, Nakatsuru S, Aoki T, Miki Y, Mori T, Nakamura Y (1992) Somatic mutations of the *APC* gene in colorectal tumors: mutation cluster region in the *APC* gene. Hum Mol Genet 1:229-233

Modrich P (1997) Strand-specific mismatch repair in mammalian cells. J Bio Chem 272:24727-24730

Moreno-Bueno G, Hardisson D, Sanchez C, Sarrio D, Cassia R, Garcia-Rostan G, Prat J, Guo M, Herman JG, Matias-Guiu X, Esteller M, Palacios J (2002) Abnormalities of the APC/β-catenin pathway in endometrial cancer. Oncogene 21:7981-7990

Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW (1997) Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. Science 275:1787-1790

Mutter GL, Lin M-C, Fitzgerald JT, Kum JB, Baak JPA, Lees JA, Weng L-P, Eng C (2000) Altered PTEN expression as a diagnostic marker for the earliest endometrial precancers. J Natl Cancer Inst 92:924-930

Nishisho I, Nakamura Y, Miyoshi Y, Miki Y, Ando H, Horii A, Koyama K, Utsunomiya J, Baba S, Hedge P (1991) Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science 253:665-669

Nyström-Lahti M, Wu Y, Moisio A-L, Hofstra RMW, Osinga J, Mecklin J-P, Järvinen HJ, Leisti J, Buys CHCM, de la Chapelle A, Peltomäki P (1996) DNA mismatch repair gene mutations in 55 kindreds with verified or putative hereditary non-polyposis colorectal cancer. Hum Mol Genet 5:763-769

Nyström-Lahti M, Perrera C, Räschle M, Panyushkina-Seiler E, Marra G, Curci A, Quaresima B, Costanzo F, D'Urso M, Venuta S, Jiricny J (2002) Functional analysis of *MLH1* mutations linked to hereditary nonpolyposis colon cancer. Genes Chromosomes Cancer 33:160-167

Oltvai ZN, Milliman CL, Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74:609-619

Palacios J, Gamallo C (1998) Mutations in the β-catenin gene (*CTNNB1*) in endometrioid ovarian carcinomas. Cancer Res 58:1344-1347

Palacios J, Catasús L, Moreno-Bueno G, Matias-Guiu X, Prat J, Gamallo C (2001) β- And γ-catenin expression in endometrial carcinoma. Relationship with clinicopathological features and microsatellite instability. Virchows Arch 438:464-469

Palombo F, Iaccarino I, Nakajima E, Ikejima M, Shimada T, Jiricny J (1996) hMutSβ, a heterodimer of hMSH2 and hMSH3, binds to insertion/deletion loops in DNA. Curr Biol 6:1181-1184

Paoloni-Giacobino A, Rey-Berthod C, Couturier A, Antonarakis SE, Hutter P (2002) Differential rates of frameshift alterations in four repeat sequences of hereditary nonpolyposis colorectal cancer tumors. Hum Genet 111:284-289

Parc YR, Halling KC, Wang L, Christensen ER, Cunningham JM, French AJ, Burgart LJ, Price-Troska TL, Roche PC, Thibodeau SN (2000) *hMSH6* alterations in patients with microsatellite instability-low colorectal cancer. Cancer Res 60:2225-2231

Parsons R, Li GM, Longley MJ, Fang WH, Papadopoulos N, Jen J, de la Chapelle A, Kinzler KW, Vogelstein B, Modrich P (1993) Hypermutability and mismatch repair deficiency in RER+ tumor cells. Cell 75:1227-1236

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

Peltomäki P, Aaltonen LA, Sistonen P, Pylkkänen L, Mecklin J-P, Järvinen H, Green JS, Jass JR, Weber JL, Leach FS, Petersen GM, Hamilton SR, de la Chapelle A, Vogelstein B (1993) Genetic mapping of a locus predisposing to human colorectal cancer. Science 260:810-812

Peltomäki P, Vasen HFA, the International Collaborative Group on HNPCC (1997) Mutations predisposing to hereditary nonpolyposis colorectal cancer: a database and results of a collaborative study. Gastroenterology 113:1146-1158

Peltomäki P (2001) Deficient DNA mismatch repair: a common etiologic factor for colon cancer. Hum Mol Gen 10:735-740

Peltomäki P, Vasen H (2004) Mutations associated with HNPCC predisposition – Update of ICG-HNPCC/insight mutation database. Disease Markers, in press

Persad S, Troussard AA, McPhee TR, Mulholland DJ, Dedhar S (2001) Tumor suppressor PTEN inhibits nuclear accumulation of β-catenin and T cell/lymphoid enhancer factor 1-mediated transcriptional activation. J Cell Biol 153:1161-1173

Piao Z, Fang W, Malkhosyan S, Kim H, Horii A, Perucho M, Huang S (2000) Frequent frameshift mutations of *RIZ* in sporadic gastrointestinal and endometrial carcinomas with microsatellite instability. Cancer Res 60:4701-4704

Plaschke J, Kruppa C, Tischler R, Bocker T, Pistorius S, Dralle H, Rüschoff J, Saeger HD, Fishel R, Schackert HK (2000) Sequence analysis of the mismatch repair gene *hMSH6* in the germline of patients with familial and sporadic colorectal cancer. Int J Cancer 85:606-613

Plaschke J, Krüger S, Dietmaier W, Gebert J, Sutter C, Mangold E, Pagenstecher C, Holinski-Feder E, Schulmann K, Möslein G, Rüschoff J, Engel C, Evans G, Schackert HK and the German HNPCC Consortium (2004) Eight novel *MSH6* mutations in patients with familial and nonfamilial colorectal cancer selected by loss of protein expression in tumor tissue. Hum Mutat 23:285-285

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

Powell SM, Petersen GM, Krush AJ, Booker S, Jen J, Giardiello FM, Hamilton SR, Vogelstein B, Kinzler KW (1993) Molecular diagnosis of familial adenomatous polyposis. N Engl J Med 329:1982-1987

Quaresima B, Alifano P, Tassone P, Avvedimento EV, Costanzo FS, Venuta S (2003) Human mismatch-repair protein MutL homologue 1 (MLH1) interacts with *Escherichia coli* MutL and MutS *in vivo* and *in vitro*: a simple genetic system to assay MLH1 function. Biochem J 371:183-189

Raevaara TE, Timoharju T, Lönnqvist KE, Kariola R, Steinhoff M, Hofstra RMW, Mangold E, Vos YJ, Nyström-Lahti M (2002) Description and functional analysis of a novel in frame mutation linked to hereditary non-polyposis colorectal cancer. J Med Genet 39:747-750

Raevaara TE, Vaccaro C, Abdel-Rahman WM, Mocetti E, Bala S, Lönnqvist KE, Kariola R, Lynch HT, Peltomäki P, Nyström-Lahti M (2003) Pathogenicity of the hereditary colorectal cancer mutation *hMLH1* del616 linked to shortage of the functional protein. Gastroenterology 125:501-509

Raevaara TE, Gerdes AM, Lönnqvist KE, Tybjaerg-Hansen A, Abdel-Rahman WM, Kariola R, Peltomäki P, Nyström-Lahti M (2004) HNPCC mutation MLH1 P648S makes the functional protein unstable, and homozygosity predisposes to mild neurofibromatosis type 1. Genes Chromosomes Cancer 40:261-265

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

Ricciardone MD, Özçelik T, Cevher B, Özdağ H, Tuncer M, Gürgey A, Uzunalimoğlu Ö, Çetinkaya H, Tanyeli A, Erken E, Öztürk M (1999) Human *MLH1* deficiency predisposes to hematological malignancy and neurofibromatosis type 1. Cancer Res 59:290-293

Rodriguez-Bigas MA, Boland CR, Hamilton SR, Henson DE, Jass JR, Khan PM, Lynch H, Perucho M, Smyrk T, Sobin L, Srivastava S (1997) A National Cancer Institute Workshop on

Hereditary Nonpolyposis Colorectal Cancer Syndrome: Meeting Highlights and Bethesda Guidelines. J Natl Cancer Inst 89:1758-1762

Rubinfeld B, Albert I, Porfiri E, Fiol C, Munemitsu S, Polakis P (1996) Binding of GSK3β to the APC-β-catenin complex and regulation of complex assembly. Science 272:1023-1026

Rubinfeld B, Albert I, Porfiri E, Munemitsu S, Polakis P (1997) Loss of β-catenin regulation by the APC tumor suppressor protein correlates with loss of structure due to common somatic mutations of the gene. Cancer Res 57:4624-4630

Räschle M, Dufner P, Marra G, Jiricny J (2002) Mutations within the hMLH1 and hPMS2 subunits of the human MutLα mismatch repair factor affect its ATPase activity, but not its ability to interact with hMutSα. J Biol Chem 277:21810-21820

Räschle M, Marra G, Nyström-Lahti M, Schär P, Jiricny J (1999) Identification of hMutLβ, a heterodimer of hMLH1 and hPMS1. J Biol Chem 274:32368-32375

Samowitz WS, Curtin K, Lin HH, Robertson MA, Schaffer D, Nichols M, Gruenthal K, Leppert MF, Slattery ML (2001) The colon cancer burden of genetically defined hereditary nonpolyposis colon cancer. Gastroenterology 121:830-838

Sasaki H, Nishii H, Takahashi H, Tada A, Furusato M, Terashima Y, Siegal GP, Parker SL, Kohler MF, Berchuck A (1993) Mutation of the Ki-ras protooncogene in human endometrial hyperplasia and carcinoma. Cancer Res 53:1906-1910

Schar P, Baur M, Schneider C, Kohli J (1997) Mismatch repair in *Schizosaccharomyces pombe* requires *mutL* homologous gene *pms1*: molecular cloning and functional analysis. Genetics 146:1275-1286

Schlosshauer PW, Pirog EC, Levine RL, Ellenson LH (2000) Mutation analysis of the *CTNNB1* and *APC* genes in uterine endometrioid carcinoma. Mod Pathol 13:1066-1071

Schlosshauer PW, Ellenson LH, Soslow RA (2002) β-Catenin and E-cadherin expression patterns in high-grade endometrial carcinoma are associated with histological subtype. Mod Pathol 15:1032-1037

Schweizer P, Moisio A-L, Kuismanen SA, Truninger K, Vierumäki R, Salovaara R, Arola J, Butzow R, Jiricny J, Peltomäki P, Nyström-Lahti M (2001) Lack of MSH2 and MSH6 characterizes endometrial but not colon carcinomas in hereditary nonpolyposis colorectal cancer. Cancer Res 61:2813-2815

Shcherbakova PV, Kunkel TA (1999) Mutator phenotypes conferred by *MLH1* overexpression and by heterozygosity for *mlh1* mutations. Mol Cell Biol 19:3177-3183

Sherman AI, Brown S (1979) The precursors of endometrial carcinoma. Am J Obstet Gynecol 135:947-956

Shibata D, Peinado MA, Ionov Y, Malkhosyan S, Perucho M (1994) Genomic instability in repeated sequences is an early somatic event in colorectal tumorigenesis that persists after transformation. Nat Genet 6:273-281

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

Sia EA, Kokoska RJ, Dominska M, Greenwell P, Petes TD (1997) Microsatellite instability in yeast: dependence on repeat unit size and DNA mismatch repair genes. Mol Cell Biol 17:2851-2858

Soussi T, Dehouche K, Béroud C (2000) p53 Website and analysis of p53 gene mutations in human cancer: Forging a link between epidemiology and carcinogenesis. Hum Mutat 15:105-113

Souza RF, Appel R, Yin J, Wang S, Smolinski KN, Abraham JM, Zou TT, Shi YQ, Lei J, Cottrell J, Cymes K, Biden K, Simms L, Leggett B, Lynch PM, Frazier M, Powell SM, Harpaz N,

Sugimura H, Young J, Meltzer SJ (1996) Microsatellite instability in the insulin-like growth factor II receptor gene in gastrointestinal tumours. Nat Genet 14:255-257

Sparks AB, Morin PJ, Vogelstein B, Kinzler KW (1998) Mutational analysis of the APC/β-Catenin/Tcf pathway in colorectal cancer. Cancer Res 58:1130-1134

Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW (1998) Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell 95:29-39

Sugawara N, Paques F, Colaiacovo M, Haber JE (1997) Role of *Saccharomyces cerevisiae* Msh2 and Msh3 repair proteins in double-strand break-induced recombination. Proc Natl Acad Sci USA 94:9214-9219

Swisher EM, Peiffer-Schneider S, Mutch DG, Herzog TJ, Rader JS, Elbendary A, Goodfellow PJ (1999) Differences in patterns of *TP53* and *KRAS2* mutations in a large series of endometrial carcinomas with or without microsatellite instability. Cancer 85:119-126

Takagi Y, Kohmura H, Futamura M, Kida H, Tanemura H, Shimokawa K, Saji S (1996) Somatic alterations of the DPC4 gene in human colorectal cancers in vivo. Gastroenterology 111:1369-1372

Tannergård P, Liu T, Weger A, Nordenskjöld M, Lindblom A (1997) Tumorigenesis in colorectal tumors from patients with hereditary non-polyposis colorectal cancer. Hum Genet 101:51-55

Tetsu O, McCormick F (1999) β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398:422-426

Thibodeau SN, Bren G, Schaid D (1993) Microsatellite instability in cancer of the proximal colon. Science 260:816-819

Thomas DC, Roberts JD, Kunkel TA (1991) Heteroduplex repair in extracts of human HeLa cells. J Biol Chem 266:3744-3751

Trojan J, Zeuzem S, Randolph A, Hemmerle C, Brieger A, Raedle J, Plotz G, Jiricny J, Marra G (2002) Functional analysis of *hMLH1* variants and HNPCC-related mutations using a human expression system. Gastroenterology 122:211-219

Umar A, Buermeyer AB, Simon JA, Thomas DC, Clark AB, Liskay M, Kunkel TA (1996) Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis. Cell 87:65-73

Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Rüschoff J, Fishel R, Lindor NM, Burgart LJ, Hamelin R, Hamilton SR, Hiatt RA, Jass J, Lindblom A, Lynch HT, Peltomäki P, Ramsey SD, Rodriguez-Bigas MA, Vasen HFA, Hawk ET, Barrett JC, Freedman AN, Srivastava S (2004) Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst 96:261-268

Vasen HFA, Mecklin J-P, Khan PM, Lynch HT (1991) The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC). Dis Colon Rectum 34:424-425

Vasen HF, Wijnen JT, Menko FH, Kleibeuker JH, Taal BG, Griffioen G, Nagengast FM, Meijers-Heijboer EH, Bertario L, Varesco L, Bisgaard ML, Mohr J, Fodde R, Khan PM (1996) Cancer risk in families with hereditary nonpolyposis colorectal cancer diagnosed by mutation analysis. Gastroenterology 110:1020-1027

Vasen HFA, Watson P, Mecklin J-P, Lynch HT, the ICG-HNPCC (1999) New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the international collaborative group on HNPCC. Gastroenterology 116:1453-1456

Veigl ML, Kasturi L, Olechnowicz J, Ma AH, Lutterbaugh JD, Periyasamy S, Li G-M, Drummond J, Modrich P, Sedwick WD, Markowitz SD (1998) Biallelic inactivation of *hMLH1* by

epigenetic silencing, a novel mechanism causing human MSI cancers. Proc Natl Acad Sci USA 95:8698-8702

Verma L, Kane MF, Brassett C, Schmeits J, Evans DGR, Kolodner RD, Maher ER (1999) Mononucleotide microsatellite instability and germline *MSH6* mutation analysis in early onset colorectal cancer. J Med Genet 36:678-682

Vilkki S, Tsao J-L, Loukola A, Pöyhönen M, Vierimaa O, Herva R, Aaltonen LA, Shibata D (2001) Extensive somatic microsatellite mutations in normal human tissue. Cancer Res 61:4541-4544

Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL (1988) Genetic alterations during colorectal-tumor development. N Engl J Med 319:525-532

Wagner A, Hendriks Y, Meijers-Heijboer EJ, de Leeuw WJF, Morreau H, Hofstra R, Tops C, Bik E, Bröcker-Vriends AHJT, van der Meer C, Lindhout D, Vasen HFA, Breuning MH, Cornelisse CJ, van Krimpen C, Niermeijer MF, Zwinderman AH, Wijnen J, Fodde R (2001) Atypical HNPCC owing to *MSH6* germline mutations: analysis of a large Dutch pedigree. J Med Genet 38:318-322

Wang T-F, Kleckner N, Hunter N (1999a) Functional specificity of MutL homologs in yeast: evidence for three Mlh1-based heterocomplexes with distinct roles during meiosis in recombination and mismatch correction. Proc Natl Acad Sci USA 96:13914-13919

Wang Q, Lasset C, Desseigne F, Frappaz D, Bergeron C, Navarro C, Ruano E, Puisieux A (1999b) Neurofibromatosis and early onset of cancers in h*MLH1* –deficient children. Cancer Res 59:294-297

Wang H, Douglas W, Lia M, Edelmann W, Kucherlapati R, Podsypanina K, Parsons R, Hedrick Ellenson L (2002) DNA mismatch repair deficiency accelerates endometrial tumorigenesis in *Pten* heterozygous mice. Am J Pathol 160:1481-1486

Watson P, Lynch HT (1994) The tumor spectrum in HNPCC. Anticancer Res 14:1635-1639

Weng L-P, Smith WM, Dahia PLM, Ziebold U, Gil E, Lees JA, Eng C (1999) PTEN suppresses breast cancer cell growth by phosphatase activity-dependent G_1 arrest followed by cell death. Cancer Res 59:5808-5814

Whiteside D, McLeod R, Graham G, Steckley JL, Booth K, Somerville MJ, Andrew SE (2002) A homozygous germ-line mutation in the human *MSH2* gene predisposes to hematological malignancy and multiple café-au-lait spots. Cancer Res 62:359-362

Wijnen JT, Vasen HFA, Khan PM, Zwinderman AH, van der Klift H, Mulder A, Tops C, Moller P, Fodde R, Menko F, Taal B, Nagengast F, Brunner H, Kleibeuker J, Sijmons R, Griffioen G, Brocker-Vriends A, Bakker E, van Leeuwen-Cornelisse I, Meijers-Heijboer A, Lindhout D, Breuning M, Post J, Schaap C, Apold J, Heimdal K, Bertario L, Bisgaard ML, Goetz P (1998) Clinical findings with implications for genetic testing in families with clustering of colorectal cancer. N Engl J Med 339:511-518

Wijnen J, de Leeuw W, Vasen H, van der Klift H, Møller P, Stormorken A, Meijers-Heijboer H, Lindhout D, Menko F, Vossen S, Möslein G, Tops C, Bröcker-Vriends A, Wu Y, Hofstra R, Sijmons R, Cornelisse C, Morreau H, Fodde R (1999) Familial endometrial cancer in female carriers of *MSH6* germline mutations. Nat Genet 23:142-144

Wu SP, Theodorescu D, Kerbel RS, Willson JK, Mulder KM, Humphrey LE, Brattain MG (1992) TGFβ 1 is an autocrine-negative growth regulator of human colon carcinoma FET cells in vivo as revealed by transfection of an antisense expression vector. J Cell Biol 116:187-196

Wu Y, Berends MJW, Mensink RGJ, Kempinga C, Sijmons RH, van der Zee AGJ, Hollema H, Kleibeuker JH, Buys CHCM, Hofstra RMW (1999) Association of hereditary nonpolyposis

colorectal cancer-related tumors displaying low microsatellite instability with *MSH6* germline mutations. Am J Hum Genet 65:1291-1298

Wu Y, Berends MJW, Sijmons RH, Mensink RGJ, Verlind E, Kooi KA, van der Sluis T, Kempinga C, van der Zee AGJ, Hollema H, Buys CHCM, Kleibeuker JH, Hofstra RMW (2001) A role for *MLH3* in hereditary nonpolyposis colorectal cancer. Nat Genet 29:137-138

Yamamoto H, Sawai H, Weber TK, Rodriguez-Bigas MA, Perucho M (1998) Somatic frameshift mutations in DNA mismatch repair and proapoptosis genes in hereditary nonpolyposis colorectal cancer. Cancer Res 58:997-1003

Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A, Oren M (1991) Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature 352:345-347

Zhou X-P, Kuismanen S, Nyström-Lahti M, Peltomäki P, Eng C (2002) Distinct PTEN mutational spectra in hereditary non-polyposis colon cancer syndrome-related endometrial carcinomas compared to sporadic microsatellite unstable tumors. Hum Mol Gen 11:445-450

Zysman M, Saka A, Millar A, Knight J, Chapman W, Bapat B (2002) Methylation of *adenomatous polyposis coli* in endometrial cancer occurs more frequently in tumors with microsatellite istability phenotype. Cancer Res 62:3663-3666