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From the Department of Medical Biosciences, Medical and Clinical Genetics
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GENETIC AND EPIDEMIOLOGICAL STUDIES OF HEREDITARY COLORECTAL CANCER

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Akademisk avhandling

som med vederbörligt tillstånd av Rektorsämbetet vid Umeå universitet för avläggande av Medicine Doktorsexamen vid Medicinska fakulteten offentligen kommer att försvaras i sal Betula, byggnad 6M, Norrlands Universitetssjukhus, fredagen den 18 november 2005 kl. 9.00.

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To my family

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Publications

This thesis is based on the papers listed below, which will be referred to in the text by the corresponding roman numerals **(I-V)**.

I **Cederquist K**, Golovleva I, Emanuelsson M, Stenling R. and Grönberg H, "A population based cohort study of patients with multiple colon and endometrial cancer: correlation of microsatellite instability (MSI) status, age at diagnosis and cancer risk", Int. J. Cancer., 91(4), 486-491, 2001

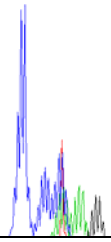
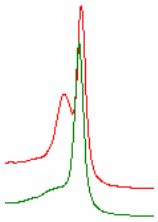
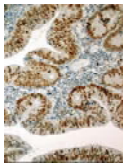
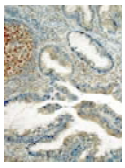
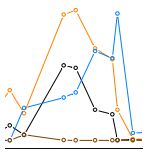
II **Cederquist K**, Emanuelsson M, Göransson I, Holinski-Feder E, Müller-Koch Y, Golovleva I. and Grönberg H, "Mutation analysis of the *MLH1*, *MSH2* and *MSH6* genes in patients with double primary cancers of the colorectum and the endometrium: a population-based study in northern Sweden", Int. J. Cancer., 109(3), 370-376, 2004

III **Cederquist K**, Palmqvist R, Emanuelsson M, Golovleva I. and Grönberg H, "Retained immunohistochemical staining in a large Swedish HNPCC family with a pathogenic *MLH1* missense mutation", Submitted to Genetic Testing.

IV **Cederquist K**, Emanuelsson M, Wiklund F, Golovleva I, Palmqvist R. and H Grönberg, "Two Swedish founder *MSH6* mutations, one nonsense and one missense, conferring high cumulative risk of Lynch syndrome", Accepted for publication in Clinical Genetics Sept. 20 2005.

V **Cederquist K**, Wiklund F, Emanuelsson M, Camp NJ, Thomas A, Farnham JM, Golovleva I, Cannon Albright L. and Grönberg H, "Genome-wide scan in a large Swedish family with hereditary colorectal cancer, suggestive evidence of linkage to chromosome 7", Manuscript.

Thesis at a glance

	Question	Material & Methods		Results	Conclusion
I	What are the cancer risks among relatives of probands with double primary colorectal and endometrial tumours?	78 probands and 649 first-degree relatives were identified on a population basis. MSI analysis of tumours. Statistical incidence ratios calculated.		SIR=1.69 among all probands, 2.67 with probands diagnosed before age 50 and 3.17 with probands diagnosed before age 50 with MSI tumours.	Early age at diagnosis and MSI in tumour of proband confer the highest cancer risks to relatives. Diagnosis after 50 years and MSS tumour confer no overall risk.
II	What is the MMR gene mutation spectrum in patients with MSI-positive double primary colorectal and endometrial tumours?	Mutation screening of *MLH1*, *MSH2* and *MSH6* in 25 patients by PCR+TMHA, and screening for large deletions by multiplex PCR-based methods.		Putative pathogenic mutations were found in 16 patients: five in *MLH1*, five in *MSH2* and six in *MSH6*.	Unexpectedly large impact of *MSH6*, possibly due to founder effects.
III	Is the novel *MLH1* sequence variation pathogenic?	Segregation analysis, MSI and MMR protein immunostaining in 10 tumours from family members.		Mutation segregates with MSI tumours with retained MLH1 staining.	Mutation pathogenic based on segregation, MSI, evolutionary conservation, non-conservative amino acid change, and absence in population.
IV	Is the novel *MSH6* sequence variation pathogenic? What is the cumulative risk conferred by *MSH6* mutations?	Segregation analysis, MSI and MMR protein immunostaining in 26+8 tumours from family members. Genealogical studies. Cumulative risk analyses.		Mutations segregate with MSI and lost MSH6 expression. Seven families merged into two. High cumulative cancer risks, significantly higher in women than in men.	Missense mutation pathogenic. *MSH6* founder mutations confer high cumulative risks despite late age of onset. Gender risk differences exist, due to high endometrial and ovarian cancer risks.
V	Is there a new locus for hereditary colorectal cancer?	Large family with non-FAP, non-Lynch syndrome hereditary colorectal cancer. Genome-wide scan and linkage analysis.		Suggested linkage to chromosome 7q21.	The chromosomal region with suggested linkage has been implicated in hereditary colorectal cancer previously and will be further analysed.

Tack Kajsa Ericson för idén "Thesis at a glance"!

Abstract

Lynch syndrome (Hereditary Nonpolyposis Colorectal Cancer, HNPCC) is the most common hereditary syndrome predisposing to colorectal cancer, accounting for 1-3% of all colorectal cancer. This multi-organ cancer predisposition syndrome is caused by mutations in the mismatch repair (MMR) genes, especially *MLH1* and *MSH2,* and to lesser extents *MSH6* and *PMS2*, which lead to widespread genetic instability and thus microsatellite instability (MSI). Hereditary cancer often manifests in two or more tumours in a single individual; 35-40% of Lynch syndrome patients have synchronous or metachronous tumours of the two major Lynch syndrome-related cancers: colorectal and endometrial.

The main purposes of the work underlying this thesis were to identify persons at risk of Lynch syndrome or other types of hereditary colorectal cancer, to estimate the cancer risks associated with these predispositions and to identify the underlying genetic causes.

A population-based cohort of 78 persons with double primary colorectal or colorectal and endometrial cancer was identified. Cancer risks in their 649 first-degree relatives were estimated in relation to tumour MSI status (positive or negative) and age at diagnosis (before or after 50 years of age) in the probands. The overall standardised incidence ratio was 1.69 (95% CI; 1.39-2.03). The highest risks for Lynch syndrome-associated cancers: (colorectal, endometrial, ovarian and gastric) were found in families with young MSI-positive probands, likely representing Lynch syndrome families. Importantly, no overall risk was found in families with old probands, irrespective of MSI status.

Blood samples were available from 24 MSI-positive patients for mutation screening of *MLH1*, *MSH2* and *MSH6*. Sequence variants or rearrangements predicted to affect protein function were found in 16 patients. Six novel variants were found: two large rearrangements, two truncating and two missense mutations. The missense mutations were found to segregate in the families. Studies of allele frequencies, MSI and loss of immunostaning in tumours from family members further supports the hypothesis that these missense changes play a role in Lynch syndrome, as do the non-conservative nature and evolutionary conservation of the amino acid exchanges. Five families had mutations in *MLH1*, five in *MSH2,* and six in *MSH6*. The unexpectedly large impact of *MSH6* was in genealogical studies shown to be due to a founder effect. Cumulative risk studies showed that the *MSH6* families, despite their late age of onset, have a high lifetime risk for all Lynch syndrome-related cancers, significantly higher in women (89% by age 80 years) than in men (69%). The gender differences are in part due to high endometrial (70%) and ovarian cancer risk (33%) in addition to the high colorectal cancer risk (60%). These findings are of great importance for counselling and surveillance of families with *MSH6* mutations.

Finally, in a large family with MSI-negative hereditary colorectal cancer for which the MMR genes and *APC* had been excluded as possible causes, a genome-wide linkage analysis was performed, resulting in a suggested linkage to chromosome 7.

Conclusions: Relatives of probands with MSI-positive, double primary colorectal and endometrial cancer diagnosed before the age of 50 years have significantly increased risks of Lynch syndrome-related cancers. *MSH6* mutations, which have unusually high impact in this study population due to a founder effect, confer high cumulative risks of cancer despite the generally late age of onset.

Key words: Lynch syndrome, HNPCC, colorectal cancer, endometrial cancer, cancer risk, MSI, MLH1, MSH2, MSH6, genome-wide scan

Abbreviations

AFAP	Attenuated Familial Adenomatous Polyposis
APC	Adenomatous Polyposis Coli
BMPR1A	Bone morphogenic protein receptor, type IA
BRAF	v-raf murine sarcoma viral oncogene homologue B1
CI	Confidence interval
CIN	Chromosomal instability
DHPLC	Denaturing high-pressure liquid chromatography
DNA	Deoxyribonucleic acid
ESE	Exonic splicing enhancer
EXO1	Exonuclease 1
FAP	Familial Adenomatous Polyposis
FDR	First degree relative
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
ICG-HNPCC	International collaboration group on HNPCC
IDL	Insertion-deletion loop
InSiGHT	International Society for Gastrointestinal Hereditary Tumours
IHC	Immunohistochemistry
KRAS	Kirsten rat sarcoma viral oncogene homologue
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinase
MGMT	O^6-methylguanine DNA methyltransferase
MIM	Mendelian Inheritance in Man
MLH	MutL homologue
MLPA	Multiplex ligation-dependent probe amplification
MMR	Mismatch repair
MSH	MutS homologue
MSI	Microsatellite instability
MSI-H	MSI-High
MSI-L	MSI-Low
MSS	Microsatellite stable
MTS	Muir-Torre Syndrome
MUTYH	MutY homologue
NCI	National Cancer Institute (USA)
NSAID	Nonsteroidal anti-inflammatory drugs
PCNA	Proliferating cell nuclear antigen
PIK3	Phosphatidylinositol-3-OH kinase
PMS	Post-meiotic segregation
PTCH	Patched homologue
PTEN	Phosphatase and tensin homologue
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SIR	Standardised incidence ratio
SMAD	Mothers against decapentaplegic homologue
SNP	Single nucleotide polymorphism
STK11	Serine/threonine kinase 11
TGFβ	Transforming growth factor beta
TMHA	Temperature-modulated heteroduplex analysis
TP53	Tumour protein 53
wt	wildtype, the normal (non-mutated) allele

Introduction

Why study cancer?

Because there are almost 11 million new cancer cases every year, causing the death of over 6 million people world-wide (1).

Why study colorectal cancer?

Because colorectal cancer is the second most prevalent cancer in the world and the third most commonly diagnosed (only lung and breast cancer are diagnosed more frequently). Worldwide, it had an incidence of over 1 million cases in 2002 and a mortality of about half that. Unlike most other cancers, the numbers are fairly equal among men and women, with a ratio of 1.2:1. (1).

Why study colorectal cancer as a geneticist?

Because genetics has a key role in the predisposition to colorectal cancer. The genetic contribution to colorectal cancer is estimated to be 35% (2). The relative ease with which the various stages of tumour development can be observed and the availability of biopsies has made colorectal cancer a useful model for other cancer. It is no exaggeration to say that colorectal cancer is one of the leading research fields in cancer genetics (3).

Colorectal anatomy and pathology

The normal colonic surface epithelium is composed of a single layer of columnar cells that are responsible for ion and water absorption, and occasional goblet cells, which synthesize and secrete mucin. It also has crypts, approximately 50 cells deep, that are lined with mostly goblet cells, except at the bases where a few undifferentiated progenitor cells are located. These cells undergo mitotic divisions and the mucosal cells migrate towards the most superficial regions of the crypts. Apoptosis, sloughing and extrusion from the mucosal surface balance proliferation in a self-renewing process that takes 4-6 days. The crypts most likely evolved to protect the crypt progenitor cells from the very mutagenic environment of the colonic lumen (4). Under normal circumstances, interactions between colonic contents and replicating cells are practically nonexistent. By the time the crypt cells reach the surface they are differentiated, non-replicating and on the verge of undergoing apoptosis. Thus any mutagenic event in these cells has little or no impact on the integrity of the cell population (4).

Colorectal polyps are growths that project from the lining of the colon or rectum. They can be sessile or pendunculate, single or multiple, benign or malign, but are seldom symptomatic. Their significance lies in their potential for malignant transformation. Histologically, they are subdivided into hamartomatous, serrated and adenomatous polyps.

Colorectal cancers can be both polypoid vegetating masses and flat, infiltrating lesions, often ulcerated. They can reach large dimensions, especially when located in the caecum or ascending colon, but most colorectal malignancies are located distal to the splenic flexure, in the rectum and sigmoid colon. Colorectal malignancies grow both longitudinally and in depth, infiltrating surrounding organs. There is no consistent evidence that either the size or degree of differentiation of the tumours is associated with the clinical outcome (5). Instead, the invasiveness of the tumour is the main predictor of the prognosis. The traditionally used Dukes classification of the stages of carcinoma of the colon (6) is based on the degree of invasion of the primary tumour into the bowel wall, the presence of lymph nodes and distant metastasis. The TNM (tumour, nodes, metastasis) system recommended for classification nowadays

depends on the tumour size, depth of penetration into the bowel wall, presence of lymph node- and distant metastasis (7). At diagnosis, meta-stasis to regional lymph nodes is detected in 30-50% of patients, whereas metastasis to the liver (the main site of distant meta-stases) can be detected in 10-30% of patients (5). The malignant cells can also reach and colonize the lung, brain and bone marrow. The 5-year survival rate of patients with Dukes A, B and C tumours is almost 100%, 60-70% and 50% respectively. At more advanced stages of disease the survival rates are lower. Screening and surveillance lead to the detection of tumours at earlier stages, which improves survival rates.

Sporadic vs. familial colorectal cancer

Colorectal cancer has traditionally been classified as sporadic or familial, but these concepts are becoming less tenable with the growing accumulation of knowledge of the underlying mechanisms of colorectal cancer. A seemingly familial occurrence of cancer may be due to chance or a shared environment rather than a shared predisposition gene. Conversely, a cancer that appears to be sporadic may in fact be part of a familial syndrome, concealed by small family size, reduced penetrance, or poor diagnostics.

Hereditary factors undisputably contribute to colorectal cancer. In cohort studies, the risk ratio for colorectal cancer lies between 2 and 4 (8, 9) and 11% of all Swedish colorectal cancer patients have a first-degree relative with colorectal cancer (10). The proportion will be much higher if second- and third-degree relatives, and relatives with other cancers that may be caused by mutations in the same genes are also considered (3). A twin study has placed colorectal cancer among the most common cancers in terms of heritability, with a genetic contribution of 35% (2), but the fraction of cases attributable to high-penetrance genes is modest; Familial Adenomatous Polyposis (FAP) and Lynch syndrome jointly account for less than 5% of all colorectal cancers (3).

There are some very rare syndromes with an intermediate (up to about 50%) risk of intestinal cancer (Table 1) that account for small proportion of familial colorectal cancer cases. However, their contribution to states classified as "sporadic" colorectal cancer might be greater, given the variable and relatively low risk of cancer associated with these genes (3). The hamartomatous polyps of **Peutz-Jegher syndrome** are most common in the small bowel, followed by the large bowel and the stomach. These polyps do not display **STK11** (serine/ threonine kinase 11) staining, indicating that a deficiency in apoptosis is a key factor in their formation and subsequent development to malignant tumour (11). The lack of *STK11* mutations and absence of linkage to 19p13.3 in many individuals with clinical Peutz-Jegher syndrome plus reports of linkage to a second locus on chromosome 19q13.4 suggests genetic heterogeneity (12). Multiple juvenile polyps are present in a number of Mendelian disorders: either without associated features, as in **Juvenile Polyposis Syndrome** with an increased risk of colon and other gastrointestinal tumours, or in association with developmental abnormalities, dysmorphic features and other tumours, in syndromes such as **Cowden syndrome**, Macrocephaly, Multiple Lipomas and Hemangiomata (**Bannayan-Riley-Ruvalcaba syndrome**) and Basal Cell Nevus Syndrome (**Gorlin syndrome**) (Table 1). Most mutations in the two genes associated with Juvenile Polyposis are truncating (13-15). The 50-60% of patients with no germline mutations in ***SMAD4*** (*mothers against DPP homologue 4*) or ***BMPR1A*** (*bone morphogenic protein receptor, type IA*) might have large rearrangements or deletions of these genes. There may also be other susceptibility genes. Candidates include genes encoding other members of the TGFβ (transforming growth factor β) superfamily signalling pathway, although no

mutations have been found in the *SMAD1*, *SMAD2*, *SMAD3* or *SMAD5* genes in probands with Juvenile Polyposis (16). In patients with Cowden Disease and Bannayan-Riley-Ruvalcaba Syndrome, a majority of the germline mutations found in ***PTEN*** (*phosphatase and tensin homologue*) encoding a protein involved in the Phosphatidylinositol-3-OH kinase (PIK3) signalling pathway, results in truncated protein, lack of protein, or dysfunctional protein (17).

The contribution of **low-penetrance** alleles is probably larger. These alleles contribute to colorectal cancer in an interdependent way, involving interactions between genes and environmental factors. Thus, they likely contribute both to "hereditary" and the "sporadic" colorectal cancer cases. Many high-frequency, low penetrance alleles affecting colorectal cancer have been proposed and are being proposed, but few have so far been reported to be statistically significant in more than one study. Meta-analyses of the association of common alleles with colorectal cancer risk have shown significant associations for the polymorphisms ***TGFBR1**6A**, ***APC** I1307K**, ***HRAS**VNTR** and ***MTHFR** 677V** (18, 19).

High-penetrance genes

APC in FAP

Familial Adenomatous Polyposis, FAP, (MIM 175100) is a rare autosomal dominant disease with a prevalence of 1/8000 (12). It is characterised by the development of hundreds to thousands of adenomas throughout the entire colon and rectum. The average age of polyp appearance is 16 years and the average age of colorectal cancer diagnosis if the polyps are left untreated is 40 years, but inter- and intra-familial variation is common. If not removed, one or several adenomas will inevitably develop into carcinoma, and thus the penetrance of this syndrome is 100%. Apart from their number and age of onset, colorectal adenomas in FAP do not show any distinctive aspects or characteristics compared to common sporadic adenomas (5). Extracolonic manifestations may or may not be present and include adenomatous polyps in the upper gastrointestinal tract, osteomas, dental anomalies, congenital hypertrophy of the retina pigment epithelium (CHRPE), soft tissue tumours, and desmoid tumours. FAP is caused by germline mutations in the *APC* (*adenomatous polyposis coli*) gene on chromosome 5q21-q22. The mutation spectrum is very wide, more than 800 different germline mutations have

Table 1. Syndromes with intermediate risk of colorectal cancer

	Peutz-Jegher Syndrome	Juvenile Polyposis Syndrome		Cowden Syndrome	Bannayan-Riley-Ruvalcaba	Gorlin Syndrome
MIM	175200	174900		158350	153480	109400
Chromosome	19p13.3	18q21.1	10q22.3	10q23		9q22.1
Gene	*STK11*	*SMAD4*	*BMPR1A*	*PTEN*		*PTCH*
% mutations	30-80%	20%	20%	80%	60%	60-85%
Function	Protein kinase	Signal molecule	Surface receptor	Phosphatase		Surface receptor
Colon cancer risk	40%	70%		low		low
Pathway	p53-dependent apoptosis	TGF-superfamily signalling	TGF-superfamily signalling	PIK3-signalling pathway		Hedgehog signalling pathway

been found. A few recurrent mutations are known, but no hotspot accounting for more than 10% of the total. Approximately 20-25% of the mutations are *de novo*. The clinical features of FAP are associated with the location and type of mutation. The classic FAP seen in most patients is associated with mutations between codons 169 and 1393 (12). The severe form of APC, with thousands of polyps, young age of onset and extracolonic manifestations, is associated with central mutations. Mutations in the first or last third of the gene are associated with **Attenuated FAP** (AFAP), characterised by a significant risk for colorectal cancer, but fewer polyps (30 on average), that are more proximally located and a later age of onset (12). Another variant of FAP is **Gardner syndrome**: colonic polyps with extraintestinal tumours, especially osteomas and a characteristic retinal lesion.

The APC protein is central to colorectal tumorigenesis. It is mutated not only in FAP but also in the majority of sporadic colorectal cancers. Through its many functional domains it interacts with numerous other proteins and is involved in cell migration, adhesion, chromosome stability and cytoskeletal organization (12). A function that is central to colorectal tumorigenesis is the ability of normal APC to regulate intracellular β–catenin levels in the Wnt signalling pathway. Mutated APC proteins lack this ability, which is why intracellular β–catenin accumulates and the Wnt signalling pathway is constitutively active. Downstream targets of Wnt signalling include *MYC*, *CCND1*, *MMP7*, *CD44*, *PLAUR* and *PPARD* (20). The APC protein has also been suggested to play a role in chromosome stability, by stabilizing the ends of the kinetochore microtubules and facilitating their attachment to chromosomes, in complexes with the mitotic checkpoint proteins BUB1 and BUB3 (budding uninhibited by benzimidazoles homologues 1 and 3) (21).

The mismatch repair genes in Lynch syndrome

The most common hereditary high–penetrant syndrome predisposing to colorectal cancer is Lynch syndrome, due to mutations of the mismatch repair (MMR) genes. Lynch syndrome is discussed in the chapter by the same name.

APC, *MLH1* and *PMS2* in Turcot syndrome

The genetically heterogeneous Turcot syndrome (MIM 276300) is the rare association of colorectal cancer and CNS tumours. Colon polyposis and medulloblastomas are associated with *APC* mutations, while colon cancer and glioblastomas are associated with mutations in the MMR genes *MLH1* (*MutL homologue 1*) and *PMS2* (*post-meiotic segregation 2*). The molecular mechanisms underlying Turcot syndrome are poorly understood. There are no Turcot syndrome- specific mutations in either *APC* or the MMR genes. The mutations are diverse and affect the same regions or are even identical with mutations seen in patients without Turcot features (12).

MUTYH

The first high-penetrant recessive colorectal cancer-predisposition gene to be identified is the *MUTYH* (*mutY homologue*) gene located at chromosome 1p32-34.3, encoding an A/G-specific adenine DNA (deoxyribonucleic acid) glycosylase associated with the base-excision repair system. The role of base-excision repair in genomic stability maintenance is to counter oxidative DNA damage. Tumours from patients with *MUTYH*-associated polyposis have an excess of G→T and C→A mutations (22). The tumours are microsatellite stable (MSS), without chromosomal instability, have a near diploid karyotype and show a low level of loss of heterozygosity (LOH) (22). Somatic *APC* nonsense mutations are present in early adenomas (23). The *KRAS* gene is commonly mutated but not *BRAF*, *TP53*, *SMAD4* or *TGFB1*R (23).

The phenotype associated with *MUTYH* mutations, as reviewed by Chow *et al.* (22), with multiple colorectal adenomas, (typically five to hundreds) is difficult to differentiate clinically from AFAP, although *MUTYH*-associated polyposis tends to present later (i.e. around 50 years of age) and, being recessive, is commonly present as sporadic disease. The polyps are mainly small, mildly dysplastic tubular and tubulovillous adenomas with few hyperplastic polyps. About 70% of the colorectal cancers are left-sided and do not seem to differ in stage, grade or histology from sporadic cancers. Extracolonic features are uncommon but do occur. At least 30% of patients with 15-100 adenomas and 10% of patients with classic FAP, but lacking *APC* mutations, have biallelic *MUTYH* mutations.

Both nonsense and missense mutations have been detected in *MUTYH* (3). The two most common mutations, Tyr165Cys and Gly382Asp, together accounting for 80% of *MUTYH* mutations, cause 6-80 fold reductions in activity (22). By the age of 60 years, there is complete penetrance of biallelic *MUTYH* mutations (24). To date, there have been no reports of unaffected carriers of biallelic *MUTYH* mutations (22). Moreover, heterozygous *MUTYH* mutations confer a modest risk for colorectal cancer later in life (24).

Other possible genes and loci

Segregation analyses provide strong evidence that about 15% of all colorectal cancers might be attributable to dominantly acting predisposition genes (25). However, all the known syndromes associated with mutations in specific genes only account for about 2-6% of colorectal cancer cases (3), providing indirect evidence for the existence of additional loci. Direct evidence for this hypothesis is provided by families showing evidence against linkage to known loci (26) or linkage to new loci.

Other possible genes

A few other high-penetrant genes have been associated with colorectal cancer, each in one or a few cases. A mutation in the ***AXIN2*** gene (MIM 604025) was found in a Finnish family with severe oligodontia and colorectal cancer resembling AFAP (27). Axin2 acts as a scaffolding protein for the multiprotein complex organized by APC and involved in Wnt signalling. A missense mutation in the ***TGFBR2*** gene was found in a patient who did not fulfil the Amsterdam criteria (see below) with an MSS tumour lacking the wt (wildtype) allele (28). Functional analysis showed that the mutation caused a defect in growth inhibition in response to TGFβ. Another germline missense mutation was found in the ***POLD1*** (*DNA polymerase delta catalytic subunit*) gene, in a patient with microsatellite instability (MSI)-positive colorectal cancer at 70 years of age without family history of neoplasia (29). The gene ***EXO1*** (*exonuclease 1*) encodes the Exonuclease 1, which binds to both MLH1 and MSH2 (MutS homologue 2) and is believed to participate in DNA MMR. *EXO1* has been associated with Lynch syndrome (30), but according to later reports such association is unlikely, because the proposed mutations have also been found in controls (31, 32) and germline deletions of *EXOI* do not cause colorectal cancer (33).

15q15.3-q22.1

The **Hereditary Mixed Polyposis Syndrome** (MIM 601228) has been described in Ashkenazi pedigrees. This is an autosomal dominantly inherited predisposition to mixed polyps and early (mean age 40 years) onset colorectal cancer (34). The polyps, usually numbering fewer than 15 at initial examination, are distributed throughout the entire large bowel, as are the colorectal cancers. The polyp number and histology vary between patients; types found include tubular adenomas, villous adenomas, flat adenomas, juvenile adenomas, mixed juvenile adenomas mixed hyperplastic adenomas, serrated adenomas,

hyperplastic polyps and atypical juvenile polyps (34, 35). A genome-wide screen has provided evidence for a new colorectal cancer susceptibility gene on chromosome 15q14-q22, with a maximum two-point lod score of 2.16 at D15S118 (36). The gene was named *CRAC1*, for "*colorectal adenoma and carcinoma*". Candidate genes located in the region include e.g. *BUB1B* and *SMAD3*. More recently the region has been narrowed down to the interval between markers D15S1031 and D15S118 on chromosome 15q13-q14, with maximum two-point and multipoint lod scores of 5.3 and 7.2 respectively, at marker ACTC (37), but no gene responsible for the phenotype has yet been reported.

9q22.2-31.1

A whole genome scan using sibling pairs either concordant or discordant for colorectal cancer or advanced adenomas (>1 cm or high-grade dysplasia) before 65 years of age showed linkage to chromosome 9q22.2-32.2, consistent with autosomal dominant inheritance (38). Among the six loci showing evidence of linkage, the chromosome 9q locus did not give the strongest signal in the concordant sib pairs, but gave the strongest signal in discordant pairs, and thus had the strongest overall significance. This area has not previously been implicated in colorectal cancer, but contains numerous candidate genes including the tumour suppressor gene *PTCH* (*patched homologue*) involved in Basal Cell Nevus Syndrome, the DNA repair gene *XPA* (*xeroderma pigmentosum, complementation group A*) and the tyrosine kinase *SYK* (*spleen tyrosine kinase*) gene. This locus has very recently been confirmed: linkage of adenoma and colorectal cancer to chromosome 9q22.32-31.1 was reported in an extended Swedish family, with a multipoint LOD score of 2.4 (39). The region was narrowed down to about 8 cM between markers D9S280 and D9S277.

11q, 14q and 22q

A genome-wide linkage analysis of 18 Swedish families with hereditary non-FAP/non-HNPCC colorectal cancer provided evidence for genetic heterogeneity among Swedish colorectal cancer families (40). Three novel regions of interest were found in a proportion of families analysed: 11q, 14q and 22q.

Modifier genes

The mutations causing Lynch syndrome and FAP are highly penetrant, but there are large variations, both intra- and inter-familial, in factors such as age of onset, differences that can be due both to environmental factors and modifier genes (41, 42). Proof of the existence of modifier genes in colorectal cancer comes from studies of the *Min* mouse, which has symptoms similar to those in humans with FAP. In this mouse line, the number of polyps depends on the alleles at the *Mom1* (*Modifier of min*) locus containing a gene encoding a secretory phospholipase (Pla2g2a) (43). However, the human *Mom1* orthologue *PLA2G2A* (*phospholipase A2, group IIA*) does not greatly modify the penetrance or expressivity of *APC* mutations (44).

Environmental risk factors

Since the contribution of genetic factors to colorectal cancer is estimated to be 35%, the environmental contribution is 65% (2). There is a plenitude of information concerning the role of lifestyle and diet in human colorectal cancer, as reviewed for instance by Potter (4); increased colorectal cancer risk is associated with intake of processed red meat and animal fat, alcohol and smoking. Obesity may increase the risk of colon cancer, but does not appear to influence the risk of rectal cancer. Decreased risks are correlated with diets rich in vegetables and possibly fibre. Physical activity reduces the risk for colon cancer but there is little evidence that it modifies risks for rectal cancer risk. Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) have been consistently associated with reductions in colorectal cancer risks, as has hormone replacement therapy. In addition, patients

with inflammatory bowel disease, both ulcerative colitis and Crohn's disease, are at increased risk of developing colorectal cancer (45). The colorectal cancer risk in these cases appears to be related to the chronic inflammation.

Pathways to colorectal cancer

To maintain the tissues of a body, all cells are strictly regulated. There must be a perfect balance, homeostasis, between birth and death, proliferation and apoptosis, which is mediated by a complex system of signals. Tumorigenesis is a multistep process, which disturbs this homeostasis and allows cells to escape from the tight constraints controlling normal cells. The process provides one cell with survival advantages and thus leads to clonal expansion. The sequence of acquired mutations, the genetic pathway followed, is a reflection of the constraints that become rate limiting at different stages in the tumour evolution.

The genes implicated in tumorigenesis can be divided into three categories: oncogenes, tumour-suppressor genes and DNA stability genes. In a general sense, **oncogenes** promote cellular proliferation and growth. Mutations result in constitutively active gene products and thus mutation of one oncogene allele is sufficient to promote tumorigenesis. To date, no colorectal cancer syndrome has been attributed to an inherited mutation in an oncogene. **Tumour-suppressor genes** down-regulate growth-stimulatory pathways. Mutations of both alleles of tumour-suppressor genes are required to inactivate gene function. In sporadic cancers both of these "hits" are somatically acquired through a variety of mechanisms, e.g. promoter hypermethylation, point mutation, deletion or chromosomal rearrangement. In autosomal dominant hereditary cancer syndromes one allele is mutated in the germline and the second is somatically mutated. Tumour-suppressor genes mutated in hereditary colon cancer-syndromes include *APC* in FAP, *BMPR1A* and *SMAD4* in Juvenile Polyposis, and *STK11* in Peutz-Jeghers Syndrome. **DNA stability genes** help maintain the integrity of the genome by repairing DNA replication errors, inhibiting recombination between non-identical DNA sequences and participating in responses to DNA damage. When these systems are dysfunctional, deleterious mutations accumulate throughout the genome. DNA stability genes mutated in hereditary colon cancer syndromes include the MMR genes in Lynch syndrome and *MUTYH* in recessive adenomatous polyposis.

Colorectal cancer is not a single disease entity, but comprises subsets, all of which are characterized by specific genetic alterations and pathological features. A key molecular step in all types of cancer formation is loss of genomic stabilisation (46), which occurs in different ways in the two major pathways from normal cells to colorectal cancer. In the classic Adenoma-Carcinoma Sequence pathway, the main mechanism of neoplastic progression is chromosomal instability (CIN) leading to LOH. In the more recently described Microsatellite Instability (MSI) pathway, which shares some of the molecular machinery of the CIN pathway, the main neoplastic mechanism is loss of MMR.

The Chromosomal Instability (CIN) pathway

The vast majority of all colorectal carcinomas arise through the classic "Adenoma-Carcinoma sequence", describing the stepwise progression from normal to dysplastic epithelium, from adenoma to carcinoma, associated with the accumulation of multiple clonally selected genetic alterations (47). Chromosomal Instability (CIN), present in ~85% of all colorectal cancers, is characterised by aneuploidy, widespread gains and losses of chromosome material, and translocations (46). The mechanisms causing CIN are still

unknown but, seem to include inactivation of proteins regulating the mitotic spindle checkpoint and the DNA replication checkpoints (21).

Based on molecular characterisation of tumours at different histopathological stages, the frequencies of different genetic changes have been demonstrated to accumulate during tumorigenesis, and a preferred order of occurrence has been suggested (47). The most frequent early genetic change associated with this pathway is mutation and/or loss of the ***APC*** gene. *APC* mutations or allelic loss of chromosome 5q are observed in up to 80% of adenomas and carcinomas (21).

Another relatively early event, which correlates histologically with early to late adenomas, is an activating mutation in ***KRAS***. *KRAS* mutations are found in about 40% of large adenomas and carcinomas, but less frequently in small adenomas (21). The *KRAS* gene encodes the RAS kinase, which participates in the MAPK (Mitogen-activated protein kinase) signalling pathway. All known carcinogenic mutations of *KRAS* affect the GTP-binding domain and result in a constantly active protein (21). The MAPK signalling pathway mediates cellular responses to growth signals the RAS and RAF kinases.

One of the most common allelic losses in colorectal cancer is **18q**, seen in 10-30% of early adenomas, 60% of late adenomas, and 70% of carcinomas (21). Originally, the candidate tumour suppressor gene in the 18q region was the *DCC* (*deleted in colorectal cancer*) gene, but experiments have failed to support this hypothesis (21). Current potential tumour suppressor gene targets in this area are ***SMAD2*** and ***SMAD4***, encoding intracellular mediators of the TGFβ superfamily signalling pathway, which are inactivated in more than 80% of colorectal cancers (3).

Allelic loss of chromosome **17p** harbouring the ***TP53*** gene or mutations of the *TP53* gene itself have been reported in increasing proportions of adenomas, invasive foci within adenomatous polyps and carcinomas, suggesting that functional inactivation of the p53 protein marks the transition from adenoma to carcinoma (21). p53 has a central role in homeostasis due to its ability to block cell proliferation in the presence of DNA damage, to stimulate DNA repair and to promote apoptosis if repair is insufficient.

PIK3 represents a family of lipid kinases regulating signalling pathways involved in processes such as cell proliferation, survival, motility adhesion and differentiation. The PIK3 signalling pathway is up-regulated in nearly 40% of colorectal cancers but only a small fraction of pre-malignant colorectal tumours have alterations in different PIK3 pathway genes, suggesting they have a role just before or coincident with invasion (17, 48).

Microsatellite Instability (MSI) pathways

In contrast to CIN colorectal cancers, MSI-H (MSI-high) colorectal cancers are diploid and show normal rates of chromosomal aberrations. However, they demonstrate at least two-fold higher higher mutation rates than normal cells (49). Their high mutation rate is attriutable to MMR deficiency, which may be due to a number of mechanisms. Regardless of the mechanism involved, most of the target genes have been found to be mutated at comparable frequencies (50). Due to their repetitive nature, microsatellites are particularly prone to replication errors, so MSI is a hallmark of MMR deficiency.

The MSI pathway to sporadic MSI-H colorectal cancer

About 10% of all sporadic colorectal cancer show the MSI-H phenotype (51). Many, if not all, of those cancers are believed to arise via the recently proposed "Serrated Polyp

Neoplasia Pathway"(52). This pathway describes the development of proximal, MSI-H cancer from serrated polyps via a two-step process of dysregulated apoptosis due to *BRAF* mutation followed by loss of DNA repair proficiency by hypermethylation of *MLH1* (51). The *BRAF* gene encodes the RAF kinase involved in the MAPK signalling pathway. Sporadic MSI-H colorectal cancers are more frequent in elderly people, among females and with a right-sided location. Mucin secretion, poor differentiation, tumour heterogeneity, and coexisting serrated polyps are more evident in sporadic than in the hereditary types (53).

The MSI pathway to Lynch syndrome-related colorectal cancer

MSI is present in 90-95% of Lynch syndrome-related cancers. In Lynch syndrome, the loss of MMR is due to a germline mutation in one of the MMR gene alleles and a somatic mutation in the other. The hereditary subtype arises in conventional adenomas (as do most sporadic MSS tumours) with *KRAS, APC* and/or *CTNNB1* mutations (53). The Lynch syndrome-related cancers have an earlier age of onset, more lymphocytic infiltration, and more coexisting adenomas than the sporadic MSI-H tumours (53).

Due to their different origins (53), the two subsets of MSI-H colorectal cancer should be distinguished and considered separately. As MMR gene carriers account for only a small proportion of all cases in unselected case series (54), most of the observed differences between MSI and MSS tumours, e.g. the more favourable survival outcome, are attributable to sporadic rather than hereditary cases (55). The data currently available do not support the assumption that the prognosis of hereditary MSI tumours is equivalent to that of sporadic MSI tumours (55). The distinction is also very important since the identification of Lynch syndrome warrants specific management strategies with respect to genetic counselling, screening and cancer prevention for both the patients and their relatives.

Mismatch repair

DNA repair pathways

Genomic DNA is constantly modified by both exogenous and endogenous agents. In addition, some pathways of DNA metabolism, e.g. DNA replication, can modify the genetic material. The integrity of genetic information depends on the fidelity of DNA replication and the efficiency of DNA repair. If the DNA repair systems fail, other responses such as cell cycle arrest and apoptosis are triggered, which stop cell proliferation and remove damaged cells from the organ concerned. Eukaryotic cells have several different DNA repair pathways, which have partially overlapping functions. Reduced capacity of these systems is linked to several human syndromes leading to cancer predisposition, developmental abnormalities, neurological disorders and premature aging syndromes. Inactivation of the ***MGMT*** (*O^6-methylguanine DNA methyltransferase*) gene by promoter hypermethylation has been reported in various tumours, such as gliomas, lymphomas, breast tumours, retinoblastomas, and colorectal tumours (56). MGMT removes methyl adducts from guanine nucleotides. Failure of this process leads to G to A transitions or strand breaks. **Nucleotide excision repair** excises oligonucleotide fragments surrounding abnormal bases, and defects cause predisposition to the skin-cancer prone disease Xeroderma Pigmentosum and Cockayne syndrome, which is characterized by severe developmental and neurological disorders (56). MUTYH is one of the proteins involved in **Base excision repair** is a DNA-repair pathway for single-base abnormalities. Bi-allelic mutations in *MUTYH* predisposes to colorectal cancer. **Non-homologous DNA end-joining** is the main repair pathway for double–stranded DNA breaks (56). Defects result in marked sensitivity to ionising radia-

tion and immunodeficiency, e.g. severe combined immunodeficiency (SCID), because lymphocyte diversity cannot be generated. **Homologous recombination** repairs double strand breaks, but can also lead to misalignment, deletions and re-arrangements. Deficiencies are associated with several genetic disorders that cause progressive illnesses and premature ageing, e.g. Ataxia Telangiectasia, Nijmegen Breakage Syndrome, Werner Syndrome, Bloom syndrome and *BRCA1*-and *BRCA2*-related breast cancer (56). Finally, the **mismatch repair (MMR)** system associated with both Lynch syndrome and a subset of sporadic colorectal cancers is described below.

Mismatch repair, MMR

The DNA MMR system is responsible for correcting base substitution mismatches and insertion-deletion loop mismatches (IDLs) that are misincorporated into the newly synthesized strand by replicative DNA polymerase and avoid the proofreading activity of this enzyme complex. The first step in the correction of replication errors via the MMR system is efficient recognition of the mismatch. Next, the newly synthesized DNA strand containing the incorrect information must be selectively removed and re-synthesized. Strand discrimination is an essential feature of all MMR systems; in its absence a sequence with a replication error is just as likely to be used as a template for repair as it is to be repaired. Whereas the latter steps in MMR makes use of proteins involved in general DNA metabolic processes, the initial mismatch recognition and removal steps require specialised Mut proteins, which are highly conserved in evolution. The Mut proteins were originally identified in prokaryotic organisms, and much of the knowledge of MMR derives from studies of prokaryotes.

MutS is the ATPase that performs the mismatch recognition. The prokaryotic MutS homodimer binds asymmetrically to DNA. Most of the MutS-DNA contacts are to the DNA backbone and thus sequence non-specific, but two residues from one of the MutS subunits make mismatched base-specific contacts. The specific interactions involve a Phe-X-Glu motif that is conserved in human MSH6 (MutS homologue 6), but not in MSH2 or MSH3 (MutS homologue 3). Eukaryotic MutS proteins have five domains homologous to prokaryotic MutS domains plus an N-terminal region containing a motif for interaction with PCNA (proliferating cell nuclear antigen) (57). Humans have five known homologues of MutS, not all of which participate in MMR. MutSα (the MSH2-MSH6 heterodimer) is primarily responsible for repairing single base-base and IDL mismatches while MutSβ (MSH2-MSH3) is primarily responsible for repairing IDL mismatches containing up to 16 extra nucleotides in one strand. The two complexes can share responsibility for repairing some IDL mismatches, especially those with just one extra base (57). MSH4 (MutS homologue 4) and MSH5 (MutS homologue 5) form a heterodimer that is active in meiosis (58).

MutL is the ATPase that couples mismatch recognition by MutS to downstream processing events. The MutL dimers interact with other MMR proteins and modulate their activities, bind and hydrolyse ATP and bind DNA, but unlike MutS in a mismatch-independent manner. Similarly to MutS proteins, the MutL proteins are functionally asymmetric and likely bind and hydrolyse ATP in a sequential or alternating manner during MMR. DNA binding by MutL may facilitate the search for the strand discrimination signal or the initiation or progression of nascent strand excision (57). Humans have four known homologues of MutL, not all of which participate in MMR. MutLα (MLH1-PMS2) is active in repairing a wide variety of mismatches. Human MutLβ (MLH1-PMS1) (post-meiotic segregation 1) has no known function, but yeast MutLβ is thought to participate in repairing a subset of IDLs. MutLγ (MLH1-MLH3) (MutL

homologue 3) suppresses some IDL mutagenesis and participates in meiosis (57).

MutH is the methylation-sensitive endonuclease that targets repair to the right DNA strand, nicking the nascent unmethylated DNA strand at hemimethylated GATC sites. Eukaryotes have no known homologue of MutH.

MMR in prokaryotes

MMR in prokaryotes is initiated when MutS binds mismatched DNA (57). MutS interacts with the β-clamp accessory protein, which is required for processive DNA replication and may help deliver MutS to mismatches. Targeting of the newly synthesized DNA strand for excision is accomplished in an ATP-dependent manner when the MutS-MutL interaction activates the latent endonuclease activity of MutH. MutH cleaves the newly synthesized, temporarily unmethylated strand at hemi-methylated GATC-sites located about 1 kb either 3' or 5' to the mismatch. The resulting nick is the entry point for the MutL-dependent loading of DNA helicase II and the binding of single-strand DNA (ssDNA) binding protein. Working together, these proteins unwind the DNA to generate ssDNA that is digested by either 3'- or 5'-exonucleases. The excision removes the error and the highly accurate DNA polymerase II correctly resynthesizes the DNA strand. Finally, DNA ligase seals the nick to complete the MMR.

MMR in eukaryotes

The eukaryotic mismatch repair machinery is highly conserved with respect to the bacterial one, but the level of complexity is enhanced by the addition of several MutS and MutL homologues. For a thorough review and references on MMR in eukaryotes, see Kunkel and Erie (2005) (57). Eukaryotic MMR is initiated when either MutSα or MutSβ binds to a mismatch. The M'utS complexes interact with PCNA, the eukaryotic homologue of the *Escherichia coli* β–clamp. PCNA in turn interacts with the matchmaking MutLα complex. As eukaryotes have no known homologue of *E. coli* MutH, the strand discrimination mechanism is currently less certain than in *E. coli*. The strand discontinuities associated with replication might serve this purpose, as nicks and gaps can direct strand-specific MMR activity *in vitro*. Recent data also suggest that PCNA may not only act as polymerase processivity factors but also participate in directing MMR to mismatches in the newly replicated DNA. PCNA is loaded onto DNA by the RFC (replication factor C) clamp-loader complex at junctions between ssDNA and dsDNA in a specific orientation. PCNA can assist in the delivery of MutSα to mismatched DNA and increase its mismatch-binding specificity. To date, no eukaryotic helicase has been shown to participate in the repair of replication errors, but more than one eukaryotic exonuclease has been implicated in MMR. Currently, EXO1 is the most likely candidate, due to its interactions with MutS, MutL and PCNA, and its catalytical and structural role in MMR. Accurate DNA resynthesis is likely catalysed by DNA polymerase δ with the participation of PCNA. After completion of DNA synthesis the nick is sealed by DNA ligase.

Consequences of defective MMR

The most well studied consequence of a defect MMR system is the highly increased rate of genome-wide point mutations and small IDLs, due to loss of the ability to repair base-pairing errors. Most of these somatic mutations are background events, but mutations also occur in coding or regulatory sequences and thus affect protein function or expression. When it affects genes with growth-related and care-taking functions, MSI may facilitate malignant transformation. This **Mutator hypothesis** (59) is consistent with the accelerated tumorigenesis in Lynch syndrome, where polyps arise at the same frequency as in the general population, but develop into tumours much more rapidly. Whereas the

development of sporadic colorectal cancer from an adenoma takes approximately 8-10 years, a Lynch syndrome carcinoma may evolve in just 2-3 years (60).

If a mutation provides a growth advantage to affected cells, there will be positive selection and increased mutation frequencies for these sequences. Conversely, if a mutation disrupts essential metabolic or signalling pathways, the cells will stop growing and eventually die, leading to counter-selection and a bias toward decreased mutation frequencies. If the mutation does not exert any tumorigenic effect, genetic alterations are believed to occur randomly. Thus, attempts to identify the real target genes for MSI in tumorigenesis have mostly relied on mutation frequency data (61). However, genes with infrequent mutations cannot be ignored. Tumorigenesis in different tumours may be caused by mutations in different genes of the same signalling pathways, which may lead to a lower mutation incidence of any particular gene in that pathway. The relevance of microsatellite-specific mutations in MSI-positive tumours can thus be proven only when there is supporting evidence for functionality, regardless of mutation incidence (62).

To date, over 500 putative MSI target genes have been proposed (61, 63). The target genes found most frequently mutated in a meta-analysis (50) and/or selected on the basis of a statistical model (61, 64) are presented in table 2. At least for *TGFB2R*, *BAX*, *MSH6*, *AXIN2, PRDM2* and *IGF2R* there is evidence for the functionality of the mutations (61, 62). However, no functional evidence has been obtined for *ACVR2A*, *PTHLH*, *HT100*, *AC1*, *SLC23A1, MARCKS, TAF1B* or *NDUFC2* (61, 64).

Once an MMR defective cell arises, the conditions required to acquire multiple mutations that can lead to tumorigenesis have been established. However, such conditions would not provide an immediate selective advantage for the cell *per se*. The selection for MMR mutations may be explained by recent findings linking the MMR machinery to resistance to DNA damage-induced apoptosis. This new paradigm, the **Revised Mutator hypothesis** (65) is based on the ability of some of the MMR proteins to operate as sensors of DNA damage and subsequently activate cell cycle checkpoints and signal apoptosis. Loss of these functions decreases apoptosis, increases cell survival and results in resistance to chemotherapy (57). The MMR proteins involved in this signal function are MLH1-PMS2 and MSH2-MSH6, which is consistent both with published Lynch syndrome mutation frequencies (table 11) and new theories regarding the contribution of PMS2 (66, 67).

Table 2. Somatic targets for MSI in colorectal cancer

Cellular process	Target gene	References
Signal transduction	*TGFBR2*	(50, 61, 64)
	AXIN2	(61)
	IGF2R	(61)
	PTHLH	(61)
	ACVR2A	(50, 61)
Apoptosis	*BAX*	(50, 61, 64)
	PTEN	(61)
	CASP5	(50)
	NDUFC2	(64)
DNA repair	*MSH6*	(61)
	MSH3	(61)
Transcriptional regulation	*PRDM2 (RIZ)*	(61)
	TCF4	(61)
	TAF1B	(64)
Posttranslational modification	*SEC63*	(50)
Unknown	*AIM2*	(50)
	HT100	(61)
	AC1	(61)
	SLC23A1	(50, 61)
	MARCKS	(64)

Animal models of MMR gene deficiencies

Mouse lines with targeted inactivation mutations in all of the known MMR genes have been developed. As reviewed by

Edelmann and Edelmann (2004), the phenotypes of mice with inactivated Mlh1, Msh2, Msh6 or Pms2 generally correlate well with observations in Lynch syndrome patients (68). However, in contrast to Lynch syndrome patients the heterozygous mice do not develop early onset tumours. This most likely reflects the smaller size and shorter lifespan of the mice compared to humans, making somatic loss of the wt allele less likely. Nevertheless, the homozygous mutant mice are cancer prone. Their tumour spectrum includes, among others, skin and gastrointestinal cancers, mostly of the small intestine, but most of the mice die of aggressive lymphomas. Although rare, human patients with homozygous mutations in *MLH1*, *MSH2*, *PMS2* and *MSH6* have haematological malignancies and a severely reduced lifespan (69-79).

There is currently no evidence for the involvement of the *PMS1*, *MSH3*, *MSH4* or *MSH5* genes in Lynch syndrome (80). Mouse deficient in Pms1 are viable, fertile and are not tumour prone (81). Msh3 deficiency in mouse causes very low cancer predisposition, but Msh3 deficiency in an Msh6-deficient background accelerates intestinal tumorigenesis (82). Mice deficient in Msh4 or Msh5 are viable and not prone to tumours but they are unfertile (83). Human MSH4 and MSH5 form a heterodimer that is active in meiosis (58). The human missense mutations detected in *MLH3* are of uncertain pathogenic significance (84-86) and mice deficient in Mlh3 are viable but infertile and do not show MSI (87). Tumours from patients with *MLH3* sequence changes also do not display MSI and most *MLH3* mutations are found in low-risk patients, suggesting that it is a low risk colorectal cancer gene (86). More data are needed for a reliable evaluation of the significance of *MLH3* in Lynch syndrome (66).

Lynch syndrome

Lynch syndrome, or Hereditary Non-Polyposis Colorectal Cancer (HNPCC) is a multi-organ cancer predisposition syndrome caused by mutations in the MMR genes. It is the most common hereditary syndrome predisposing to colorectal cancer, accounting for 1-3% of all colorectal cancers (88). Clinically, Lynch syndrome is heterogeneous, which complicates both diagnostics and surveillance strategies. Besides colorectal cancer, endometrial cancer is most common, followed by cancer of the ovary, stomach, small bowel, hepatobiliary tract, pancreas, upper uroepithelial tract, and brain (3). Mutations in the MMR genes *MLH1*, *MSH2*, *MSH6* and *PMS2* have been convincingly linked to Lynch syndrome.

History of Lynch syndrome research

The first recordings of Lynch syndrome date back to 1895 when pathologist Aldred Warthin at the University of Michigan described the cancer prone family of his seamstress, "Family G" (89). The next step was taken in 1966 when Dr. Henry Lynch and colleagues reported the observation of two families from Nebraska and Michigan with an autosomal dominant genetic predisposition for early onset colorectal cancer in absence of multiple colonic polyps, and called the condition "Cancer Family Syndrome" (90). In 1984 the terms Lynch syndrome I was suggested for families with colon cancer at an early age and Lynch syndrome II for families with both colonic and extracolonic carcinoma (91). In 1985 the term HNPCC for both Lynch I and II was coined, to clarify the lack of multiple colonic polyps and thus distinguish it from the polyposis syndrome FAP (92). In 1989 the International Collaborative Group on HNPCC (ICG-HNPCC) was established (93). Apart from several multicenter studies, the ICG-HNPCC has developed a number of criteria and guidelines for various aspects of the Lynch syndrome. In 2001, the ICG-

HNPCC and the Leeds Castle Polyposis Group (LCPG) formed a new international society for the study of hereditary gastrointestinal tumours, the International Society for Gastrointestinal Hereditary Tumours (InSiGHT) (94). At the 2002 NCI (National Cancer Institute) HNPCC workshop the notion of adopting the name "Lynch syndrome" (again) was discussed (95). This is because HNPCC is a misnomer, given the facts that the phenotype of the syndrome is not completely without polyps and in addition to colorectal cancer involves at least seven extracolonic cancer types, of which the most common is endometrial cancer.

The molecular genetic era of Lynch syndrome research began in 1993, when a locus on chromosome 2p was identified through linkage analysis in two large kindreds with early onset colorectal cancer (96). Shortly thereafter, a second locus on chromosome 3p was found (97). Simultaneously with the linkage analyses, three independent groups reported the phenomenon of MSI (49, 98, 99). The first proof that MSI was due to mutations in MMR genes came from studies of yeast, showing that mutations in y*PMS1*, y*MLH1* and y*MSH2* lead to increased tract instability (100). Subsequently, the human MMR gene *MSH2* on chromosome 2p22 was cloned and germline mutations were identified in Lynch syndrome kindreds (101, 102). A second human MMR gene, *MLH1* on chromosome 3p21, was also cloned and shown to harbour mutations (103, 104). Since then, several other MMR genes have been implicated in Lynch syndrome. Germline mutations in *PMS1* on chromosome 2q31-q33 and *PMS2* on chromosome 7p22 were reported in 1994 (105). However, when the family with the only *PMS1* germline mutation reported was re-examined a large deletion of exons 1-7 of *MSH2* co-segregating with the disease was detected, and thus there is presently no evidence for the involvement of *PMS1* in Lynch syndrome (80). In 1995, the MSH6 protein (then called G/T binding protein, GTBP) was identified as one of two subunits of MutSα (106, 107). The corresponding gene was localised to within 1 Mb of the *MSH2* gene, on chromosome 2p16 and found to be mutated in three hypermutable cell lines (108). In 1997 the first reports of germline mutations in the *MSH6* gene in Lynch syndrome patients were published (109, 110). The *MLH3* gene was cloned in 2000 (111) and its possible involvement in colorectal cancer tumorigenesis has been studied since then, with conflicting results.

Diagnosing Lynch syndrome

An early diagnosis of patients at risk for Lynch syndrome is critical, but the diagnosis is often not straightforward. There is usually no premorbid phenotype: the first manifestations are often symptoms of cancer (112). The ICG-HNPCC definition of Lynch syndrome was aimed at helping clinicians to identify affected families (Table 3). (113). It contains a detailed description of specific Lynch syndrome features. As a general rule of thumb, the number of suggestive features in a kindred correlates with the likelihood of Lynch syndrome (113). The diagnosis is confirmed by the identification of a pathogenic mutation in one of the MMR genes, but can also be made on clinical grounds if specific criteria are met, together with the presence of MSI and/or abnormal immunohistochemistry (IHC). As the molecular detection of MMR gene mutations is laborious and expensive, there are also a number of pre-screening methods and criteria for whom to be tested.

The Amsterdam criteria

In August 1990, at the meeting of the ICG-HNPCC in Amsterdam, the Netherlands, the original Amsterdam criteria (Table 4) were developed in an attempt to standardise diagnostic criteria for recruiting Lynch syndrome patients to collaborative studies (93). Before these criteria, the descriptions of the syndrome in the literature varied widely. Although crucial for identification of the molecular basis of Lynch syndrome,

Table 3. ICG-HNPCC definition of HNPCC (Lynch syndrome)

Familial clustering of colorectal and/or endometrial cancer.
Associated cancers: cancer of the stomach, ovary, ureter/renal pelvis, brain, small bowel, hepatobiliary tract, and skin (sebaceous tumours).
Development of cancer at an early age.
Development of multiple cancers.
Features of colorectal cancer:
(1) predilection for proximal colon;
(2) improved survival;
(3) multiple colorectal cancer;
(4) increased proportion of mucinous tumours, poorly differentiated tumours, and tumours with marked host-lymphocytic infiltration and lymphoid aggregation at the tumour margin.
Features of colorectal adenoma:
(1) the numbers vary from one to a few;
(2) increased proportion of adenomas with a villous growth pattern and
(3) a high degree of dysplasia;
(4) probably rapid progression from adenoma to carcinoma.
High frequency of MSI (MSH-H).
Immunohistochemistry: loss of MLH1, MSH2, or MSH6 protein expression.
Germline mutation in MMR genes (*MSH2*, *MLH1*, *MSH6*, *PMS1*, *PMS2*).

Table 4. Amsterdam I criteria for HNPCC

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

the criteria were soon criticised for being too stringent, since they did not account for extracolonic cancers or small kindreds. Furthermore, some FAP or AFAP families with multiple polyps but without profuse polyposis could be incorrectly classified as having Lynch syndrome. The extended Amsterdam II criteria (Table 5), including associated extracolonic cancers, were proposed and accepted in 1998 (113). Originally, none of the Amsterdam criteria were intended for clinical use. As criteria for the selection of families for research they were aimed at specificity rather than sensitivity. As a consequence of the low sensitivity[1], about 80% (114), many true Lynch syndrome families are missed if the criteria are applied to clinical diagnosis and might be falsely reassured and excluded from genetic counselling, DNA testing or surveillance. The low sensitivity can be explained in part by the criteria heavily relying on the patient's recollection of family cancer history, which is often incomplete and in need of verification (115, 116). Furthermore, the Amsterdam criteria also have low specificity[2], ranging from 46 to 68% (114). Thus, many families meeting the Amsterdam criteria do not have an MMR gene mutation (117)

The Bethesda guidelines

The finding of a pathogenic MMR gene mutation is the most definitive way of diagnosing Lynch syndrome, but searching for mutations in the MMR genes is difficult, time-consuming and expensive, due not only to laboratory expenses but also to the need for laborious interpretation of the sequence tracings and findings. For these reasons, and since MSI occurs in 90-95% of Lynch syndrome-related cancers, MSI analysis of the patient's tumour has been

[1]The sensitivity of the Amsterdam criteria is the proportion of *MLH1* and *MSH2* mutation carriers complying with the Amsterdam criteria of all *MLH1* and *MSH2* mutation carriers.

[2]The specificity of the Amsterdam criteria is the proportion of mutation negative patients not complying with the Amsterdam criteria of all mutation negative patients.

Table 5. Amsterdam II criteria for HNPCC (revised)

There should be at least three relatives with an HNPCC-associated cancer (colorectal cancer, cancer of the endometrium, small bowel, ureter or renal pelvis).
All the following criteria should be present:
1. One should be a first degree relative of the other two.
2. At least two successive generations should be affected.
3. At least one cancer should be diagnosed before age 50.
4. FAP should be excluded in the colorectal cancer case (if present).
5. Tumours should be verified by pathological examination.

adopted as a useful pre-screening tool (118). However, the MSI analysis lacks specificity, since ~15% of sporadic colorectal cancers are also MSI-H, so the total cost will be high. Therefore, at the 1996 NCI International Workshop on HNPCC in Bethesda, USA, guidelines (dubbed the Bethesda guidelines, Table 6) were designed to limit MSI testing to cancers that are likely to be Lynch syndrome-related if proven MSI-H, and patients hence would benefit from being tested for MMR gene mutations (119). The sensitivity[1] of the Bethesda guidelines, 89% in a familial colorectal cancer study population (114), is somewhat higher than the sensitivity of the Amsterdam criteria, but at the cost of specificity[2] being only 53%, suggesting that these guidelines still select too many patients for MSI analysis. The Bethesda guidelines have also been criticised for being too complex and too cumbersome for use in daily clinical practice. A better understanding of the clinical and histological manifestations of Lynch syndrome led to revision and improvement of the Bethesda guidelines at another NCI HNPCC workshop in Bethesda in 2002 (Table 7) (95). The wording of the guidelines was altered for clarification (120) and the benefit of Lynch syndrome testing for an entire family was emphasised. The sensitivity of the Bethesda II guidelines in identifying *MLH1* and *MSH2* gene mutation carriers has been shown to be 91% and the specificity to be 77% (121).

Table 6. Bethesda guidelines for testing of colorectal tumours for microsatellite instability

1. Individuals with cancer in families that meet the Amsterdam criteria.
2. Individuals with two HNPCC-related cancers or associated extracolonic cancers[1].
3. Individuals with colorectal cancer and a first-degree relative with colorectal cancer and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma; one of the cancers diagnosed at age <45 years, and the adenoma diagnosed at age <40 years.
4. Individuals with colorectal cancer or endometrial cancer diagnosed at age <45years.
5. Individuals with right-sided colorectal cancer with an undifferentiated pattern (solid/cribform) on histopathology diagnosed at age <45years[2].
6. Individuals with signet-ring-cell-type colorectal cancer diagnosed at age <45years[3].
7. Individuals with adenomas diagnosed at age <40years.

[1]Endometrial, ovarian, gastric, hepatobiliary, or small-bowel cancer or transitional cell carcinoma of the renal pelvis or ureter.
[2]Solid/cribform defined as poorly differentiated or undifferentiated carcinoma composed of irregular, solid sheets of large eosinophilic cells and containing small gland-like spaces.
[3]Composed of >50% signet ring cells.

Other selection methods

A quantitative improvement of the Amsterdam criteria and the Bethesda guidelines by a logistic regression model to calculate the likelihood of detecting *MLH1* or *MSH2* mutations in a kindred using various clinical criteria as predictive variables has been developed (122). The performance of the Wijnen model and two other models as predictors of Lynch syndrome was tested and verified in an in-

[1]The sensitivity of the Bethesda guidelines defined as the proportion of MSI-positive patients complying with the Bethesda guidelines of all MSI-positive patients.
[2]The specificity of the Bethesda guidelines defined as the proportion of MSI-negative patients not complying with the Bethesda guidelines of all MSI-negative patients.

dependent set of families (123). The Wijnen model held true not only when Lynch syndrome was detected by *MLH1* and *MSH2* mutation screening but also by MSI or IHC analysis. The sensitivity (85-95%) and specificity (20-65%) of the models depend on the chosen Lynch syndrome probability cut-off. The conclusion was that all three quantitative models provide better predictions of Lynch syndrome than the Amsterdam II criteria or the Bethesda guidelines, but need additional evaluation to assess their relative merits.

Table 7. The Revised Bethesda Guidelines for testing colorectal tumours for MSI.

Tumours from individuals should be tested for MSI in the following situations:
1. Colorectal cancer diagnosed in a patient who is less than 50 years of age.
2. Presence of synchronous, metachronous colorectal, or other HNPCC-associated tumours[1], regardless of age.
3. Colorectal cancer with the MSI-H[2] histology[3] diagnosed in a patient who is less than 60 years of age[4].
4. Colorectal cancer or HNPCC-related tumour[1] diagnosed under age 50 years in at least one first-degree relative.
5. Colorectal cancer or HNPCC-related tumour[1] diagnosed at any age in two first- or second-degree relatives.

[1]Hereditary nonpolyposis colorectal cancer (HNPCC)-related tumours include: colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, and brain (usually glioblastomas as seen in Turcot syndrome) tumours, sebaceous gland adenomas and keratoacanthomas in Muir-Torre syndrome, and carcinoma of the small bowel.
[2]MSI-H=microsatellite instability-high tumours refers to changes in two or more of the five NCI-recommended panels of microsatellite markers.
[3]Presence of tumour infiltrating lymphocytes. Crohn disease-like lymphocytic reaction, mucinous/signet-ring differentiation, or medullary growth pattern.
[4]There was no consensus among the workshop participants on whether to include the age criteria in guideline 3 above; participants voted to keep less than 60 years of age in the guidelines.

There is no universally accepted strategy to identify Lynch syndrome patients and there might never be one, as Lynch syndrome is manifested in different ways worldwide and the medical practice also varies between different parts of the world. The common goal, however, is to find the most specific, sensitive and cost-effective way to predict who would benefit from MMR gene testing and both the Bethesda II guidelines and the quantitative models come close to fulfilling those objectives. However, none of the above mentioned guidelines take the increasing frequency of *MSH6* and *PMS2* mutations into account and evaluation of the guidelines in such study groups are needed.

Pre-screening tools

Uniform criteria for the definition and detection of MSI in colorectal cancer were developed at an international workshop (Table 8) (118). This international consensus meeting proposed the use of two mono-nucleotide repeats and three dinucleotide repeats in tumour DNA and matching normal DNA to determine the MSI status of the tumours. MSI analysis using the NCI panel has been used as the gold standard to detect tumours with an abnormal MMR function (124), so independent control of its sensitivity to detect MMR deficiency has not been possible. However, it is clearly subject to both biological and technical variability. One potential source of false-negative results is intralesional heterogeneity of the tumour, which can be overcome by microdissection to enrich for areas rich in tumour cells (125). At least 70% of the cells examined should be tumour cells and at least 100 tumour cells should be analysed (125). The choice of markers also affects the sensitivity of the analysis. For the detection of tumours with MMR defects, mononucleotides are most sensitive and specific (126, 127). This is also in line with the recommendations from the 2002 NCI international workshop on HNPCC (95). Several suggestions regarding the mono-nucleotide markers that should be used have recently been published, which gives sensitivities of 92-99% (126-128).

Table 8. International guidelines for evaluation of MSI in colorectal cancer (Boland, Thibodeau et al. 1998).

Reference panel:	
Marker:	Repeating unit:
BAT25	Mononucleotide
BAT26	Mononucleotide
D5S346	Dinucleotide
D2S123	Dinucleotide
D17S250	Dinucleotide
Criteria for interpretation:	
MSI-H: 2 markers unstable	
MSI-L: 1 marker unstable	
MSS or MSI-L: 0 markers unstable[1]	

[1]A distinction between MSI-L and MSS can only be accomplished if a greater number of markers are utilized.

Since MSI testing is labour intensive and has a relatively long turnaround time, the development of monoclonal antibodies against the MMR proteins was welcome (129). Tumours in Lynch syndrome generally show LOH of the MMR gene responsible for the phenotype and loss of the corresponding protein in tumour tissue, which can be readily demonstrated with the use of IHC. MSH2 IHC loss is often accompanied with loss of MSH6, due to instability of the MSH6 protein in the absence of its heterodimeric partner (125). Similarly, loss of MLH1 is often associated with loss of PMS2. Loss of MSH6 or PMS2 alone indicates that an alteration in these genes is the primary defect. IHC has an indisputable advantage over MSI in that it directs the mutation screening towards a specific gene, but is IHC as effective as MSI in identifying MMR-deficient colorectal cancers? Some studies suggest that they are equally effective (130) while others suggest that MSI is more sensitive (129, 131). The lower sensitivity of IHC is explained mainly by its low sensitivity in detecting missense mutations; tumours with an MSI-H phenotype may not entirely lose expression of an MMR protein, due to missense mutations or even truncating mutations that do not affect the epitope of the protein (129). A false negative result, i.e. loss of MMR gene expression in the absence of a mutation, may also be caused by over-fixation of the tissue (125). Thus, only samples with positively stained internal normal control tissue should be evaluated. In a meta-analysis, IHC had an overall sensitivity[1] of 74% in predicting *MLH1* mutations, 91% in predicting *MSH2* mutations and 78% in predicting *MSH6* mutations (129). The lower sensitivity for *MLH1* and *MSH6* may be attributable to the large proportions of missense mutations in these genes. The undefined specific epitope of the commonly used MLH1 antibody may be the reason for discordant results in some cases (129). As long as the role of other putative MMR genes in Lynch syndrome has not been elucidated, IHC analysis cannot completely replace MSI, but due to its simplicity it should be incorporated as a screening measure. A suggested approach is to vary the order of IHC and MSI depending on the probability of finding a mutation (132). In high-risk cases, fulfilling the Amsterdam criteria, IHC might be the first step, followed by MSI in cases with retained staining. In patients meeting the Bethesda guidelines the first step might be MSI followed by IHC of MSI-H tumours. MSI or IHC analysis of endometrial cancer or adenomas has been found to be less sensitive than the same analyses of colorectal cancer, and thus both techniques may be needed to assess them (133).

BRAF V600E screening

Since epigenetic inactivation of *MLH1* is a common event - about 80% of all MSI-H colorectal cancers show loss of MLH1 by IHC - neither MSI nor MLH1 loss detected by IHC are perfect predictors of a germline *MLH1* mutation (125). However, a new tool that could possibly be used to distinguish Lynch syndrome-related MSI-H tumours from sporadic MSI-H tumours has been discovered recently. An oncogenic *BRAF* p.Val600Glu mutation (V600E,

[1]The sensitivity indicates how often the staining of a tumour is truly negative for MMR proteins in case of proven MSI-H or mutation (false positive samples occur)

previously known as V599E) has been found in about 40% of all sporadic MSI-H colorectal cancers, but in no colorectal cancers associated with *MLH1*, *MSH2* and *MSH6* mutations (134-136). To exclude sporadic tumours, it has been suggested that *BRAF* testing should be included prior to germline testing of *MLH1* in MLH1-negative, MSI-H tumours and prior to any MMR gene screening if IHC is not being performed (134).

Mutation screening

Regardless of which criteria are used, MMR gene mutations are not found in all families. The frequency of mutations found varies widely depending on the inclusion criteria applied in the studies and, thus, the varying inclusion of other forms of familial colorectal cancer cases and sporadic cases. It is also important to bear in mind the imperfection of the current mutation screening methods. Mutation screening techniques such as Single-Strand Conformation Polymorphism (SSCP) assays, Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature-Mediated Heteroduplex Analysis (TMHA) do not detect all sequence changes. Techniques based on mRNA analysis are not reliable when the mutations cause instability of the mRNA (129). Haploid conversion technology may increase the sensitivity of mutation detection, but is laborious and expensive (137). Screening of large deletions is generally done by Multiplex Ligation-dependent Probe Amplification (MLPA). However there are concerns of this procedure due to the occurrence of false negatives and positives because of positioning of probes and DNA variants near the probe ligation sites (138). Mutations may be present in regions that are not commonly examined, such as promoters or introns. Interpreting sequence changes can also be problematic, especially for missense mutations.

Assessing pathogenicity of missense mutations

The pathogenicity of missense mutations is often unclear, as illustrated by changes that have been reported both as pathogenic mutations and innocent sequent variants, e.g. *MLH1* p.Lys618Ala, *MLH1* p.Val326Ala, *MSH2* p.Gly322Asp and *MSH6* p.Val878Ala (66). Several pathogenicity assessment criteria have been suggested (66, 139-141). One important criterion is ab-sence of the genetic variant in the normal population, as indicated by studies of control individuals and its absence in public disease-specific mutation databases (e.g. the InSiGHT mutation database at www.insight-group.org/ and the Human Gene Mutation Database at www.hgmd.cf.ac.uk/) and public databases of single nucleotide polymorphisms (SNPs) (e.g. the NCBI SNP database dbSNP at www.ncbi.nlm.nih.gov/SNP/, the NCI SNP500Cancer database at snp500cancer.nci.nih.gov/ and the SNP database of CHIP Bioinformatics Tools at snpper.chip.org/). The genetic change should segregate with the disease in the pedigree analysed. The amino acid change should be of non-conservative nature, e.g. a change of polarity, alpha-helix propensity or size of the encoded amino acid. The amino acid variation can be viewed e.g. by CHIP Bioinformatics Tools (142). The risk for the amino acid change being deleterious also increases if it is in a domain that is highly evolutionary conserved or conserved between proteins belonging to the same family. Given a protein sequence, the amino acid substitutions that will affect protein function can be predicted by the SIFT tools at blocks.fhcrc.org/sift/SIFT.html (143). Another tool for predicting the possible impact of an amino acid substitution on the structure and function of a human protein using physical and comparative considerations is PolyPhen at www.bork.embl-heidelberg.de/PolyPhen/ (144). Interspecies sequence comparisons can be done using ClustalW at www.ebi.ac.uk/clustalw/index.html (145). Some genetic changes (nonsense, missense

or even silent) can alter pre-mRNA splicing by disrupting exonic splicing enhancers (ESEs) and thus promote exon skipping (146). Evidence in support of this mechanism is available for some Lynch syndrome-related mutations (147, 148). There are prediction programs for ESEs, e.g. at rulai.cshl.edu/tools/ESE/ (149) and genes.mit.edu/burgelab/rescue-ese/ (150), but they cannot identify all actual ESEs, so RNA studies are warranted (148). The tumour material of patients should have the MSI phenotype and and the protein of interest should not be detected in appropriate IHC staining assays. The proposed mutation should affect the MMR capacity in a functional assay.

Some of these criteria might be difficult to apply for various reasons. Tumour material for MSI, LOH and IHC analyses is not always available. The degree of MSI may vary with the type of markers used (95). Proteins with missense mutations may still have intact epitopes for the antibodies used for IHC. The number of affected and/or available family members might be too low for segregation analysis. Previous inclusions in mutation databases might be misleading, as most variants have not been tested properly (139). Not all SNPs included in the SNP databases are validated. Functional assays are not readily available in all laboratories and not flawless. Differences in the design of functional assays, such as the model system used or the way MMR function is monitored can lead to differences in the results of the tests (139).

Several different functional assays for measuring the MMR capacity of the mutated proteins have been developed. Many systems depend on measuring the protein-protein binding capacity, often in combination with other tests (151-159). A limitation of these studies for determining pathogenicity is that some mutations abolish repair capacity without altering the dimer formation ability (160). The yeast-based assays are based on the assumption that the MMR system is evolutionary conserved between humans and yeast, but as this homology is only moderate, a mutant human allele in the yeast environment may have different effects than the same allele in a human environment (139). This limitation can be overcome by using *in vitro* analyses on human cells (151, 153, 155, 157-159, 161, 162) or human expression systems with human cell lines lacking intrinsic MMR (155, 161-163).

Surveillance of Lynch syndrome patients

Surveillance

For patients with a definite or suspected diagnosis of Lynch syndrome, interval surveillance is recommended. The major aim of surveillance is to diagnose malignant or premalignant lesions at the asymptomatic stage. According to a controlled 15-year trial, colonoscopic screening at three-year intervals more than halves the risk of colorectal cancer, prevents colorectal cancer deaths and decreases overall mortality by 65% in Lynch syndrome families (164). There are no data on the benefit of the endometrial screening programmes, but it has been considered justifiable even if this tumour is an infrequent cause of death in Lynch syndrome patients (165). Screening for other associated tumours is recommended only in families in which they have developed, as there is no evidence for their benefit (165). The current Swedish surveillance recommendations are the same as those recommended by the InSiGHT group (www.insight-group.org/) (Table 9).

Surveillance of Lynch syndrome patients has been shown to be cost-effective in several studies (114, 166-168). The costs of surveillance are less than the costs of treatment of tumours. The identification of mutation and true mutation carriers is more cost-effective than life-long screening of all family members at risk (165).

Table 9. Current Swedish surveillance recommendations

- ✓ Colorectal cancer screening with full colonoscopy every one to two years, beginning age 20-25
- ✓ Endometrial and ovarian cancer screening with gynaecological examination including transvaginal ultrasound and CA-125 annually, beginning age 30-35

If cancer of the urinary tract and/or stomach has been found in the family also:

- ✓ Screening for urinary tract cancer by urinary analysis, annually or biannually beginning age 30-35
- ✓ Screening for gastric cancer by gastroscopy, annually or biannually beginning age 30-35

Chemoprevention

Chemoprevention with NSAIDs has reduced adenoma development and progression in patients with FAP as well as with colorectal cancer in the general population (169). Exposure of colon cancer cells harbouring defects in the MMR genes to NSAIDs has promoted apoptosis in MSI-positive cells (170) and clinical trials in Lynch syndrome patients are being conducted; preliminary data from the large international collaboration study, CAPP2, are encouraging (165).

Predictive testing and genetic counselling

When a pathogenic MMR mutation has been identified in a Lynch syndrome patient, family members can be presymtomatically tested. The advantages of this testing are manifold (171). Individuals at risk can be identified before they develop the disease and invited to participate in surveillance programs. The uncertainty of being a mutation carrier is removed. Non-carriers are relieved of the burdensome surveillance, which also halves the surveillance costs. Nevertheless, many uncertainties remain (171): Carriers may experience feelings of anxiety regarding the increased risk, feelings of guilt about transmitting the mutation and uncertainty about the future health situation. They will also have to face regular surveillance for a long time and often the detection of polyps, premalignant or malignant. Non-carriers are confronted with the residual cancer risk and survivor guilt feelings. However, genetic testing for Lynch syndrome does not appear to induce major psychological problems, perhaps because the focus lies on the benefit of regular cancer screening and thus the options of preventing cancer (171).

Cancer risk

The total lifetime risk of cancer associated with Lynch syndrome is estimated to be 80-90% (172-176). However, the commonly reported risk estimates might be too high, since most of the studies did not adjust for ascertainment bias. Ascertainment-corrected estimates are lower, but not to an extent that calls for changes in the screening practice (177). If no preventive control programs are followed, the 10-year incidence of a second primary cancer occurring is 40% (172). Lifetime cancer risks of Lynch syndrome patients are shown in Table 10 together with mean age of onset, which is generally 15-20 years earlier than in sporadic cases.

Some **differences in risk between genes** have been reported, where *MSH2* mutation carriers are at higher risk of developing colorectal cancer than *MLH1* mutation carriers (178). *MSH2* mutation carriers are also at a higher risk for extracolonic cancers than *MLH1* mutation carriers. This holds true for endometrial, ovarian, gastric, upper urothelial and brain cancers (174, 178). *MSH6* mutation carriers have a lower risk of colorectal cancer than both *MLH1* and *MSH2* mutations carriers, but higher risk for endometrial cancer (179, 180).

Gender differences have also been found; male mutation carriers of *MLH1* and *MSH2* are reported to have a higher lifetime **colorectal cancer** risk than women (176, 178, 181, 182). Most of the colorectal

cancer tumours associated with Lynch syndrome occur proximal to the splenic flexure (53).

Table 10. Cancer risks in individuals with Lynch syndrome

Cancer	Risk	Mean age of onset
Colorectal	54-100%	46
Endometrial	24-62%	48
Gastric	10-20%	56
Ovarian	5-15%	43
Upper urothelial	2-5%	55
Hepatobiliary tract	2-7%	None reported
Small bowel	1-4%	49
Brain	1-4%	50

(172-174, 177, 187, 188, 190, 192)

Endometrial cancer is the second most common cancer type of Lynch syndrome, with cumulative incidences often exceeding that of colorectal cancer in women (173, 181). Families with an MMR mutation and exclusively endometrial cancer have been reported (113). About 75% of endometrial carcinoma associated with Lynch syndrome and 17-32% of sporadic endometrial cancers display the MSI phenotype (183).

The most commonly reported type of **gastric cancer** in Lynch syndrome is adenocarcinoma (184). The MSI phenotype is common in Lynch syndrome, occurring in 80-90% of cases compared to 14-47% of sporadic cancers, but *Helicobacter pylori* infection is rare (183, 184). Although gastric cancer has declined in Lynch syndrome families since 1900, paralleling its decline in the general western population (185), it is still an important feature in geographic areas where gastric cancer is endemic, most notably Japan and Korea (186). Gastric cancer is not included in the Amsterdam II criteria because of its high prevalence in the general population in some Asian countries and thus the risk of familial aggregation due to chance (113).

Ovarian cancer in Lynch syndrome differs from ovarian cancer in the general population in several clinically important respects (187): it occurs at a markedly earlier age, it is more likely to be epithelial, and more likely to be well or moderately differentiated. Lynch syndrome patients with ovarian cancer are more likely to have a synchronous endometrial cancer than other ovarian cancer patients and are more likely to be diagnosed at an early stage. The frequency of MSI in ovarian cancer is about 3-17% in sporadic and up to 100% in Lynch syndrome-associated cases (183).

The **upper urinary tract cancers** associated with Lynch syndrome are transitional cell carcinomas of the ureter and renal pelvis, with a female predominance (172, 173, 188). MMR defects are found in 2-5% of upper urothelial tumours, mostly affecting *MSH2* or *MSH6,* and thus indicating Lynch syndrome association (189).

The majority of the reported **small bowel cancers** are adenocarcinomas, and the main locations are the duodenum and jejunum (190). Defective MMR is found in 18% of small intestine adenocarcinomas. In half of these cases it is due to *MLH1* promoter hypermethylation and in the other half presumably due to Lynch syndrome mutations (191). The Lynch syndrome-related small bowel tumours appear to have a better prognosis than those occurring in the general population (190).

The most common type of **central nervous system tumour** associated with Lynch syndrome is glioblastoma (173, 192).

Lynch syndrome also confers an increased risk of **skin tumours**. The variant of Lynch syndrome associated with autosomal dominant predisposition to skin tumours and various internal malignancies is named **Muir-Torre syndrome** (MTS, MIM 158320). The characteristic cutaneous manifestation of MTS, a sebaceous gland neoplasm, is rare in the general population and thus MTS should always be suspected when it presents (193). MTS is caused by the same

genes, and even the same mutations, as those involved in general Lynch syndrome (194), with a predominance of *MSH2* mutations (193).

Double primary tumours

Hereditary cancer often manifests not only in the presence of many tumours in a single family, but also in two or more tumours in a single individual. Double primary tumours within the above tumour spectrum are characteristic of Lynch syndrome: MSI is more common in patients with multiple primary cancers than in patients with a single lesion, and more frequent in double primary tumours of Lynch syndrome patients than in patients with sporadic tumours (195, 196). In one study, 38% of patients with four primary malignancies, at least two of which were colorectal cancers, had MSI and concordant loss of either MLH1 or MSH2 by IHC, strongly suggestive of Lynch syndrome (197). Up to 45% of Lynch syndrome patients have double primary tumours (113, 198), compared to 5-10% of all cancer patients (199).

The two principal Lynch syndrome-associated tumour sites are the colorectum and the endometrium. In one study of women with double primary colorectal and endometrial cancer, 44% had MSI and concordant loss of MLH1 or MSH2 according to IHC (200) and in another study 18% had a mutation in either *MLH1* or *MSH2* (201). First degree relatives (FDR) of women with double primary colorectal and endometrial cancers have been shown to have an increased risk of colorectal, endometrial and a variety of other Lynch syndrome-related tumours (202); a risk that is higher in mutation-positive cases (201).

Mutation spectrum and genotype-phenotype correlations

The latest (July 31st, 2003) published compilation at the time of writing of the ICG-HNPCC/InSiGHT mutation database (www.insight-group.org/) lists 448 sequence alterations that are likely to be pathogenic (66). The distribution of these mutations is presented in Table 11. The mutations are scattered throughout the genes, with some hotspot areas, e.g. exons 3 and 12 of *MSH2*, exon 4 (which is the largest exon, comprising almost 60% of the coding sequence) in *MSH6*, and exons 1 and 16 in *MLH1*. Most of the *MSH2*, *MSH6* and *MLH1* missense mutations meet several pathogenicity criteria and several have been shown to be pathogenic in functional assays (66, 151, 152, 159, 161, 203-205).

Families with ***MLH1*** and ***MSH2*** mutations mainly display typical Lynch syndrome with MSI-H tumours. Compared to *MLH1* mutation carriers, *MSH2* mutation carriers are at higher lifetime risk for cancer and at higher risk for extracolonic cancers (174, 178). Of MTS patients with an MMR gene mutation, 93% have mutations in *MSH2* (193). ***MSH6*** mutations are preferentially, but not exclusively, found in families with atypical Lynch syndrome: later age of onset; lower colorectal and higher endometrial cancer incidence; and tumours with a variable microsatellite pattern, ranging from stable to highly unstable (110, 179, 206-211).

Recent data indicate that previous mutation analyses of ***PMS2*** have been complicated by a large number of pseudogenes with significant homology to *PMS2* (77, 212). Mutation analyses using strategies accounting for the pseudogenes implies a larger role for *PMS2* than previously considered (212-214). This is also in agreement with the expected mutation frequencies according to the revised mutator hypothesis (65). As there are Lynch syndrome families with MSI-H tumours but no MMR gene mutation, the question arises whether other genes linked to this phenotype remain to be identified. Many of the other proteins involved in MMR play key roles in DNA replication and mutations that inactivate them would be lethal (215). Furthermore, the hypothesis that mutations must both

provide a selective advantage and act as mutators provide a theoretical basis to explain the lack of mutations in the majority of other genes implicated in maintaining genomic stability (65).

Homozygous mutations

Homozygous or compound heterozygous mutation carriers of the *MLH1* (69-72, 162), *MSH2* (73, 74), *MSH6* (75, 76) or *PMS2* (77-79) genes have a phenotype resembling Neurofibromatosis type 1 (NF1, MIM 162200). There seems to be a co-dominant model of cancer risk, where the heterozygous carriers are predisposed to Lynch syndrome, while homozygous or compound heterozygous carriers of the same mutations develop very early onset and aggressive haematological and brain cancers, in addition to café au lait spots and colorectal neoplasms before the second decade of life. The colorectal cancers are relatively rare, perhaps due to very early premature death from haematological cancers (67). The tumour spectrum is consistent with that of homozygous MMR deficient mice (68). The normal tissues of the homozygous individuals display MSI (69-72).

Table 11. MMR gene mutation spectrum in Lynch syndrome

Gene	*MSH2*	*MSH6*	*MLH1*	*PMS2*
Chromosome	2p22-p21	2p21	3p21-23	7p22
No. of pathogenic mutations	175	32	225	5
Proportion of all mutations	39%	7%	50%	1%
No. of non-pathogenic variants	28	43	27	5
Frame-shift mutations	49%	37%	44%	40%
Nonsense mutations	19%	22%	10%	20%
Missense mutations	18%	37%	32%	20%
In-frame insertions/deletions	9%	-	11%	20%
Other mutations/large deletions	5%	5%	3%	20%

Aims of the studies

The main aims of the work underlying this thesis were to identify persons at risk of hereditary colorectal cancer, especially Lynch syndrome, to estimate the cancer risks associated with these predispositions, and to identify the underlying genetic causes.
The specific aims were:

Study I.

To identify persons at a high risk for hereditary colorectal cancer and to estimate the risk for colorectal cancer and other Lynch syndrome-associated tumours in their families.

Study II.

To investigate the *MLH1, MSH2* and *MSH6* mutation spectra in patients with MSI-positive double primary colorectal cancer or colorectal/endometrial cancer tumours.

Study III.

To determine the clinical significance of the *MLH1* c.62C>T, p.Ala21Val sequence variant found in a large family with Lynch syndrome.

Study IV.

To describe the clinical and genetic characteristics of *MSH6*-related Lynch syndrome families. Also, to determine the clinical significance of the *MSH6* c.1346T>C, p.Leu449Pro sequence variant found in a large family with Lynch syndrome and to investigate if it is a founder mutation.

Study V.

To identify identify a novel locus for predisposition to hereditary colorectal cancer and adenomas.

Material and Methods

All studies were granted ethical approval by the Umeå University ethical committee for scientific research: Um dnr 97-289 (Studies I, II, III and IV), Um dnr 02-211 (Studies III and IV), and Um dnr 00-270 (Study V).

Patient and tumour material

The Swedish Cancer Registry, founded in 1958, is based on compulsory reports from every health care provider for all cancer cases diagnosed during clinical, morphological, or other laboratory examinations, and cases diagnosed at autopsy. The registry refers to patients using unique personal identification numbers and contains information on their sex, domicile, the hospital and department(s) where examinations took place, the specimen number, date, tumour site and diagnostic basis. The National registry is divided into six regional registries maintained at the oncological centres in each medical region of Sweden. The Regional Cancer Register at the University Hospital of Umeå contains information on all persons in the northern health care region of Sweden: Norrbotten, Västerbotten, Västernorrland and Jämtland counties (900 000 persons). The quality of the Swedish Cancer Registry has been studied and found to be very accurate (216, 217). All cancer diagnoses in the studies have been verified in the Swedish Cancer Registry.

Study I

In **study I** 89 probands, with either multiple colorectal cancers born after 1922, or women with both colorectal cancer and endometrial cancer born after 1917, were identified in the Regional Cancer Register at the University Hospital of Umeå. The cut-off years 1917 and 1922 were chosen since they seemed to be optimal for maximising the amount of family data that could be acquired. In ten of the cases it was not possible to locate the person involved, to verify the cancer diagnosis, or to obtain approval to contact the family. Among the remaining 79 probands, one had both multiple colorectal and endometrial cancers and thus was detected twice. Two probands were found to be from the same family. Thus, 78 persons from 77 families were included: 35 colorectal/endometrial cancer cases, 42 double primary colorectal cancer cases, and one double primary colorectal and endometrial cancer case. Tumour material was available for MSI analysis from all of these patients; an endometrial cancer in three cases and colorectal cancer in the remaining 75 cases.

The physician who threated each patient in the study was contacted and approved contact with the patient or a close relative. A questionnaire explaining the nature of the study and seeeking consent for inclusion in it was sent to each proband, surviving next of kin, or spouse. Requested information included the patient's current age or age at death, cause of death, cancer diagnoses, cancer site and age at diagnosis of all FDRs, second degree relatives, and more distant relatives where possible. Since patients' recollection of family history can be incomplete (115, 116), completion of the personal identification numbers of all FDR and verification of all diagnoses were made through parish records and the Swedish Cancer Registry. The 78 probands had 649 FDR in total (328 males and 321 females).

Study II.

Of the probands with MSI tumours included in **study I**, blood samples were available for mutation screening from the proband or an FDR with Lynch-syndrome-related cancer in 20 MSI-H families and one MSI-L family, which was reclassified as MSI-H after the completion of **study II**. An additional MSI-H patient, diagnosed shortly after **study I** was closed, was also included. This patient had four different primary tumours of the colon, one tumour of the small bowel and a gastric tumour at the age of 62 years. Two of the MSS families fulfilling the Amsterdam criteria, and the family in which the MSI analysis failed, were also included in the mutation screening. The family with inconclusive MSI data and one of the MSS families have also been reclassified as MSI-H since the completion of **study II**. Hence, mutation analysis was done on 24 patients with MSI-positive tumours and one patient with an MSS tumour.

Study III.

The family of one of the probands included in **study I**, in whom the sequence variant of unknown significance *MLH1* c.62C>T, p.A21V was found in **study II**, was further examined. Tumour material was available for MSI, IHC and mutation analysis from ten tumours of the proband and seven relatives with Lynch-syndrome related cancers. Of these tumours, five were colonic, four were rectal and one was located in the small intestine.

Study IV.

Four probands from **study I**, who were found in **study II** to have the same sequence variant of unknown significance (*MSH6* c.1346T>C, p.Leu449Pro) and to originate from the same geographical area, were further examined. An additional patient with an MSI-positive tumour who was included in **study I,** but not in **study II** due to lack of blood sample, originated from the same region. She was shown to carry the same sequence variant and was thus also included in **study IV**. Tumour material was available for MSI, IHC and mutation analysis from 27 tumours of the five probands and 17 of their relatives with Lynch-syndrome related cancers and breast cancer. Of these tumours, eleven were colonic, four rectal, four endometrial, two ovarian and six were breast cancers. The breast cancers were included since there were many breast cancer cases in the families and breast cancer has been reported in *MSH6* families (179, 180) (179, 180, 211, 218). Peripheral blood was available for mutation analysis of three asymtomatic family members.

Two probands from **study I**, who were found in **study II** to have the same sequence variant (*MSH6* c.2931C>G, p.Tyr977X), which is predicted to create a truncated protein, were likewise further examined. Tumour material was available for MSI, IHC and mutation analysis from the two probands and six relatives with Lynch-syndrome related cancers. Of these tumours, five were colonic, one rectal and two endometrial. Peripheral blood was available for mutation analysis of two asymtomatic family members.

Study V.

"Family 19" was identified in a cancer screening and counselling program run by the Cancer-genetic counselling clinic at the De-partments of Clinical Genetics and Onco-logy of the University Hospital of Umeå, Sweden. This large family has an autosomal dominant susceptibility to colorectal cancer and adenomas. The proband, who pre-sented with polyps at the age of 64 years, was referred to the clinic because his brother, father, two paternal uncles and a paternal aunt had developed colorectal cancer. In this large family, ten family members have been affected with colorectal cancer, and colorectal polyps of several types have been detected by colonoscopy in at least 21. The *APC*, *MLH1*, *MSH2*, *MSH6* and *CDH1* genes have been screened for mutations with various techniques but no evidence for any

mutation was found (data not shown). Three different tumours have been analysed and shown to be MSS (data not shown). Cytogenetic analysis of one family member with colorectal cancer revealed no large rearrangements (data not shown). The family was investigated and details of their cancer status were derived from histopathology reports and medical records. Cancer diagnoses were confirmed in the Swedish National Cancer Register. For the genome-wide scan, blood was available from two family members with colorectal cancer, 13 with polyps and four unaffected.

Genealogical studies

In **study IV**, the ancestry of the families was traced by examining parish registers available in the Demographic Database hosted by Umeå University (www.ddb.umu.se/index_eng.html), which contains detailed information of the Swedish population classified according to variables such as age, sex, civil status, fertility, and mortality back to the 17th century. Since family relationships are stated this provides extensive genealogic information, from which detailed pedigrees were constructed.

MSI analysis

MSI analysis was performed in **studies I, III and IV** using five different microsatellite markers - two $(CA)_n$ repeats (D2S123 and D17S250) and three quasimonomorphic markers (BAT25, BAT26 and BAT34C4) - all of which are among those recommended by the National Cancer Institute (118). Mononucleotide markers have both higher sensitivity[1] and specificity[2] than dinucleotide markers for detecting MSI-H tumours (126, 127), and hence the dinucleotide D5S346 from the original Bethesda panel was exchanged for the mononucleotide BAT34C4, also among those recommended by the National Cancer Institute.

The PCR reactions were performed in 15 µl mixtures containing 120 ng DNA, 5 pmol of each primer, 0.25 mM dNTP, 2.5 mM $MgCl_2$, 1x GeneAmp® PCR-buffert II (Applied Biosystems, Foster City, CA, USA) and 0.6 U Amplitaq Gold ® DNA polymerase (Applied Bioisystems, Foster City, CA, USA). After initial denaturation at 95°C for 12 min, 15 cycles of denaturation were performed at 94°C for 15 sec and annealing at 51°C or 58°C for 15 sec, then 25 cycles of 89°C for 15 sec and 51°C or 58°C for 15 sec, followed by a final extension step of 10 min at 72°C. The annealing temperature of 58°C was used for BAT25, BAT26, BAT34C4 and D2S123 and the annealing temperature of 51°C for D17S250. The five sets of primers, one of which in each pair was fluorescently labelled, are listed in Table 12.

The PCR products were analysed as follows:
In Study I, 3 µl of both the BAT25 and BAT 26 PCR product mixtures were pooled with 4.5 µl of each of the BAT34C4, D2S123 mixtures and 4.5 µl of the D17S250 mixtures. Portions (1.5 µl) of the pooled products were then mixed with 0.5 µl GeneScan®-350 [TAMRA]™ size standard (Applied Biosystems, Foster City, CA, USA), 2.5 µl deionised formamide and 0.5 µl EDTA/dextran blue and analysed on 6.5% PAGE, 7.5 M urea gels in an ABI PRISM ® 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) with GeneScan™ software.
In studies III and IV, 15.0 µl of both the BAT25 and BAT34C4 PCR product mixtures were pooled with 3.7 µl of both the BAT26 and D2S123 mixtures and 7.5 µl of the D17S250 mixture. The pooled products were desalted on MicroSpin™ G-25

[1]The sensitivity indicates the percent of true MSI-H samples in which that marker is unstable.
[2]The specificity indicates the percent of true non-MSI-H samples (MSS and MSI-L) in which that marker is stable.

Table 12. Data on microsatellite markers used for MSI analysis

Marker (Genbank accession)	Repeat motif	Primer sequences 5'-3' *=labelled	Gene Chromo-some	Fluorescent label ABI/CEQ
BAT25 (U63834)	$(T)_4GTG(T)_4G(T)_7GA(T)_{\sim 25}$	F:TCGCCTCCAAGAATGTAAGT* R:TCTGCATTTTAACTATGGCTC	*cKIT*, intron 16 4q11-12	TET/ D3
BAT26 (U41210)	$(A)_{\sim 26}$	F:TGACTACTTTTGACTTCAGCC* R:AACCATTCAACATTTTTAACCC	*MSH2*, intron 5 2p22-21	6FAM™/ D4
BAT34C4 (-)	$(T)_3C(T)_6C(T)_{\sim 18}C(T)_5C(T)_3$	F:ACCCTGGAGGATTTCATCTC* R:AACAAAGCGAGACCCAGTCT	*TP53*, 3'-UTR 17p13	HEX/ D2
D2S123/ AFM093xh3 (Z16551)	$(CA)_{\sim 14\text{-}29}$	F:AAACAGGATGCCTGCCTTTA* R:GGACTTTCCACCTATGGGAC	2p16	6FAM™/ D4
D17S250/ Mfd15 (X54562)	$(CA)_{\sim 19\text{-}24}$	F:GGAAGAATCAAATAGACAAT* R:GCTGGCCATATATATATTTAAACC	17q11.2-q12	6FAM™/ D4

columns (GE Healthcare, Piscataway, NJ, USA) and mixed with 40 µl CEQ™ Sample Loading Solution plus 0.5 µl of the CEQ™ 400 size standard mixture and analysed on a CEQ™ 8000 Genetic Analysis System (all supplied by Beckman Coulter Inc., Fullerton, CA, USA).

A tumour was regarded as being MSI-H if at least two of the five markers showed instability compared to a matched normal sample, MSI-L if only one marker was unstable and otherwise MSS. When both MSI and IHC analysis was applied to the same tumours, the researcher scoring MSI was blind to the IHC results and vice versa.

Mutation screening and detection

Screening for sequence variants

The mutation screening in **study II** was performed on genomic DNA from peripheral blood. Primer pairs that amplify all *MLH1* and *MSH2* exons and exon 2-10 of *MSH6* including all intron-exon boundaries were used to amplify genomic DNA prepared from blood. The primer sequences used for PCR amplification of the *MLH1*, *MSH2* and *MSH6* exons were modified after Kolodner *et al.* and Wu *et al.* (Table 13) (219-221). Due to technical difficulties associated with its high content of GC, exon 1 of *MSH6* was not investigated. We cannot therefore ex-clude the possibility that some of the patients may have mutations in this region, or in regulatory elements of any of the genes (which were also not investigated).

The PCR reactions were performed in 50 µl mixtures containing 50 ng DNA, 25 pmol of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1x GeneAmp® PCR-buffert II (Applied Biosystems, Foster City, CA, USA) and 1 U Amplitaq Gold ® DNA polymerase (Applied Biosystems, Foster City, CA, USA) with a touchdown PCR profile: denaturation at 95°C for 12 minutes, then 10 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 60°C with a decrease of 1°C in every cycle and elongation for 30 sec at 72°C, followed by 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 50° and elongation for 30 sec at 72°C, with a final elongation step of 10 min at 72°C. To enhance heteroduplex formation, the PCR products were heat denatured at 95°C for 5 min, then

gradually re-annealed by decreasing the temperature to 20°C over 50 minutes.

The amplified PCR fragments were screened for sequence variants by TMHA using denaturing high-pressure liquid chromatography (DHPLC) in a WAVE DNA fragment analysis system (Transgenomic, Crewe, UK). The running conditions for each amplicon (Table 13) were determined using Wavemaker 3.4.4 software (Transgenomic, Crewe, UK) based on its DNA sequence. Samples that displayed variant elution peaks on basis of visual inspection and comparison with control samples included in each run, were chosen for confirmatory sequence analysis. For some amplicons that displayed many variations of elution profiles also among control samples all patient samples were sequenced.

Screening for large genomic rearrangements

QMPSF, Quantitative Multiplex PCR of Short Fluorescent Fragments

Genomic DNA from nine MSI-positive patients included in **study I**, where no mutations were found by DHPLC of the *MLH1*, *MSH2* or *MSH6* genes in **study II**, were sent to the laboratory of Professor Thierry Frebourg in Rouen, France, for screening large genomic rearrangements of the *MLH1* and *MSH2* genes by QMPSF, Quantitative Multiplex PCR of Short Fluorescent Fragments (222).

MLPA, Multiplex Ligation-dependent Probe Amplification

All of the above samples were later confirmed using the MLPA method in our laboratory. The samples were ligated and amplified using a SALSA MLPA KIT P003 MLH1/MSH2 according to the protocol recommended by the manufacturer (MRC-Holland, Amsterdam, the Netherlands) and analysed with a CEQ™ 8000 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA, USA).

Sequencing

Amplicons with variant DHPLC elution peaks in **study II** were sequenced for detection of sequence variants. Furthermore, sequencing was used in **study IV**, to determine the presence/absence of the SNP rs3136333, *MSH6* c.1345C>T of the disease haplotype in families with the *MSH6* sequence variant c.1346T>C. Independently amplified PCR products of the chosen amplicons were sequenced in both sense and antisense directions, using the same primers as for the original amplification. In some analyses the sequencing was performed with an ABI PRISM® Big Dye™ Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) and the products were analysed using an ABI Prism™ 377 DNA sequencer, while in others a CEQ Dye Terminator Cycle Sequencing with Quick Start kit and a CEQ™ 8000 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA, USA) were used (in both cases following the standard protocols recommended by the manufacturer). Sequences obtained were aligned to and compared with published wild type sequences (GenBank accession numbers U40960-U40978, U41206-U41221 and U73732-73737 for *MLH1*, *MSH2* and *MSH6*, respectively) using Sequencher 3.1.1 analysis software.

Determination of allele frequencies

The allele frequencies of sequence variants detected in **study II** were determined, by PCR amplification of the respective amplicons with the same methods and primers used for mutation screening, in DNA from 92 healthy Swedish donors and compared by DHPLC to sequenced samples with presence/absence of the sequence variant.

Table 13: Primer sequences and DHPLC temperatures for *MLH1*, *MSH2* and *MSH6* amplicons

Gene	Exon	Primers (5'→3')	DHPLC °C
MLH1	1	CAC TGA GGT GAT TGG CTG AA TAG CCC TTA AGT GAG CCC G	61.8, 64
	2	TAC ATT AGA GTA GTT GCA GA CAG AGA AAG GTC CTG ACT C	56.7, 59.7
	3	TTG GAA AAT GAG TAA CAT GAT T TGT CAT CAC AGG AGG ATA T	55.4
	4	CTT TCC CTT TGG TGA GGT GA GAT TAC TCT GAG ACC TAG GC	55, 58.4
	5	TCT CTT TTC CCC TTG GGA TTA G ACA AAG CTT CAA CAA TTT ACT CT	54.7, 58
	6	GTT TTA TTT TCA AGT ACT TCT ATG AAT T CAG CAA CTG TTC AAT GTA TCA GCA CT	57.2
	7	GTG TGT GTT TTT GGC AAC CAT AAC CTT ATC TCC ACC	55.1
	8	AGC CAT GAG ACA ATA AAT CCT TG GGT TCC CAA ATA ATG TGA TGG	53.9
	9	AAG CTT CAG AAT CTC TTT T CTG TGG GTG TTT CCT GTG	56.8
	10	ACT TTG TGT GAA TGT ACA CCT GTG GAG AGC CTG ATA GAA CAT CT	58.7
	11	CTT TTT CTC CCC CTC CCA CTA AAA ATC TGG GCT CTC ACG	56.5, 62
	12A	GGG ACC TGT ATA TCT ATA CT GTT TGC TCA GAG GCT GC	Sequencing
	12B	AGT ACA GAA TAA AGG AGG TAG GC GAT GGT TCG TAC AGA TTC CCG	59.9
	13	AAC CCA CAA AAT TTG GCT AAG CCT TCT CCA TTT CCA AAA CC	58.7, 59.7
	14	TGT CTC TAG TTC TGG TGC TGT TGT AGT AGC TCT GCT TG	57.5, 59.5
	15	ATT TGT CCC AAC TGG TTG TA TCA GTT GAA ATG TCA GAA GTG	55.9
	16	TTG GAT GCT CCG TTA AAG CTT G CCG GCT GGA AAT TTT ATT TG	58.1
	17	AGG CAC TGG AGA AAT GGG ATT TCC AGC ACA CAT GCA TGT ACC GA	59.3
	18	GTC TGT GAT CTC CGT TTA GA AGG TCC TGT CCT AGT CCT	55.5, 58.2
	19	GAC ACC AGT GTA TGT TGG CGG AAT ACA GAG AAA GAA GA	57.8, 58.8
MSH2	1	AGG CGG GAA ACA GCT TAG AAA GGA GCC GCG CCA CAA	66.2, 67.2
	2	GAA GTC CAG CTA ATA CAG TCG CAC ATT TTT ATT TTT CTA CTC TTA A	54.6, 55.3
	3	TAT AAA ATT TTA AAG TAT GTT CAA G TTT CCT AGG CCT GGA ATC TCC TCT	56.7, 58.9
	4	GTA GGT GAA TCT GTT ATC ACT CCT TCT AAA AAG TCA CTA TAG T	52.4, 56
	5	GTG GTA TAG AAA TCT TGC ATT TTT CCA ATC AAC ATT TTT AAC CC	53.4, 57
	6	TTC ACT AAT GAG CTT GCC ATT C	55.5, 58+0.5

		GTA TAA TCA TGT GGG TAA C	
	7	CTT ACG TGC TTA GTT GAT AA CAA CCA CCA CCA ACT TTA TGA	51, 56.1
	8	TTT GTA TTC TGT AAA ATG AGA TCT TT GGC CTT TGC TTT TTA AAA ATA AC	53.6
	9	GTG GGA GGA AAT ATT TGC TTT TTG GGG ACA GGG AAC TTA TA	54.5, 56
	10	TAG TAG GTA TTT ATG GAA TAC TTT T CTT GAC TCT TAC CTG ATG ACT	Sequencing
	11	CAT TGC TTC TAG TAC ACA TTT CAG GTG ACA TTC AGA ACA TTA	51.9, 54.5
	12	ATT CAG TAT TCC TGT GTA CAT TTA CCC CCA CAA AGC CCA A	54.9, 57
	13	CGA TTA ATC ATC AGT GTA C CAG AGA CAT ACA TTT CTA TCT TC	53.8, 57.8
	14	TGG CAT ATC CTT CCC AAT GT GGT AGT AAG TTT CCC ATT AC	55
	15	GCT GTC TCT TCT CAT GCT G CAT CTT AGT GTC CTG TTT AT	58
	16	ATT ACT CAT GGG ACA TTC ACA CCA TGG GCA CTG ACA GTT A	54.4, 56.4
MSH6	2	GCC AGA AGA CTT GGA ATT GTT TAT TTG ACA CAA ACA CAC ACA CAT GGC AGT AG	58
	3	CGT GAG CCT CTG CAC CCG GCC C CCC CAT CAC CCT AAC ATA AA	59
	4A	GTC TTA CAT TAT GGT TTT CC CCA CAT CAG AGC CAC CAA TG	57.1, 58.1
	4B	CGA AGG GTC ATA TCA GAT TC ATA CCA AAC AGT AGG GCG AC	59.3
	4C	CGT TAG TGG AGG TGG TGA TG ATG AAT ACC AGC CCC AGT TC	58.5-0.5
	4D	CTG TAC CAC ATG GAT GCT CTT CTT CCT CTT TTT CTT TGA GG	58.5, 60
	4E	AAA GTA GCA CGA GTG GAA CAG ACT GAG AAC ATC ACC CCA ATG CCA TCA C	57-0.5
	4F	CTG TTC TCT TCA GGA AGG TC AGC CAT TGC TTT AGG AGC CG	57.3-0.5
	4G	ACT TGC CAT ACT CCT TTT GG CCT GCT TTG GGA GTA ATA AG	56.2-0.5
	4H	GAA AAG GCT CGA AAG ACT GG CCA AAG GGC TAC TAA GAT AAA AGC GAG	56.6, 57.6, 58.6
	5	GGG GAG ATC GTT GGA CTG TAA TTG TTG CTT CCT ATT AAG TCA CTG GCT G	Sequencing
	6	GTT TAT GAA ACT GTT ACT ACC GCA AAT ATC TTT TAT CAC AT	59, 62
	7	GCC CAG CCA ATA ATT GCA TA TCT TCA AAT GAG AAG TTT AAT GTC TT	53, 56
	8	CCT TTT TTG TTT TAA TTC CT CAA CAG AAG TGC CCT CTC AA	57
	9	TCT GTT GCT AGC ACA TGT ATC G TGT TTC TTT GAA ACT TAA GGT CAG T	57, 59
	10	GGA CAT AGA AAA GCA AGA GAA AGT GTA TGC ATG TAC CAT AAC A	Sequencing

Restriction fragment length polymorphism, RFLP

Segregation of three of the sequence variants found in **study II** in the respective families were analysed by restriction fragment length polymorphism (RFLP) analysis in **studies III and IV**. Presence/absence of the *MLH1* c.62C>T and *MSH6* c.2931C>G changes was detected by restriction analysis of PCR fragments with *Fnu*4HI endonuclease. Presence/absence of the c.1346T>C change was detected by restriction analysis of PCR fragments with *Hpa*II endonuclease, whose restriction site is created by the C introduction. The C/T polymorphism in position 1345 (SNP rs3136333) would, if present, interfere with the restriction digestion method described, since there would be no *Hpa*II restriction site whether there was a c.1346T>C change or not. Several alleles from members of different parts of the family were sequenced, none of which contained this polymorphism, indicating that that it does not reside in the mutation haplotype (Figure 2). The PCR reactions were performed in 50 µl mixtures containing 200 ng genomic DNA, 10 pmol of each primer, 1x Accu-Prime™ PCR-buffer II and 1 µl Accu-prime™ Taq DNA High Fidelity Poly-merase (Invitrogen Life Technologies, Carlsbad, CA, USA). The primer sequences are listed in Table 14. The PCR programs were as follows. For *MLH1* c.62C>T: initial denaturation at 94°C for 2 min, 10 cycles of 94°C for 15 sec, 60°C for 15 sec, -1°/cycle and 68°C for 30 sec, then 30 cycles of 94°C for 15 sec, 50°C for 15 sec and 68°C for 30 sec, followed by a final extension step of 10 min at 68°C. For both *MSH6* changes: initial denaturation at 94°C for 2 min, 15 cycles of 94°C for 15 sec and 55°C for 15 sec, then 25 cycles of 89°C for 15 sec and 55°C for 15 sec, followed by a final extension step of 10 min at 68°C. The PCR products were digested overnight according to the instructions of the manufacturer (New England Biolabs, Beverly, MA, USA) and analysed on an 8% PAGE gel.

Immunohistochemistry, IHC

IHC analysis was used in **studies III and IV**. Sections from formalin fixed and paraffin embedded tissue were dried, deparaffinized and rehydrated before microwave treatment in EDTA, pH 8.0. A semi-automatic staining machine (Ventana ES, Ventana Inc., Tucson, AZ, USA) was used for the following immunohistochemical procedures. Monoclonal MLH1, MSH2 and MSH6 antibodies were all applied at a concentration of 1:50. Bound antibody was visualised according to the Ventana program, using DAB as the chromogen. The slides were subsequently counterstained with Mayer's hematoxylin. The MLH1 antibody, clone G168-15 (BD Biosciences Pharmingen, Belgium), is a mouse monoclonal antibody raised against full-length human MLH1 protein. The epitope of the MSH2 antibody, clone FE11 (Oncogene Research Products, San Diego, CA), which is also a mouse monoclonal

Table 14. RFLP primers

Genetic change	Primers(5'→3')
MLH1 c.62C>T	CCA AAA TGT CGT TCG TGG CA
	TCG ACT CCC TCC GTA CCA GTT
MSH6 c.1346T>C	ACT CCT GGG ATG AGG AAG TG
	TTT CAG GAA AGC CAG AAT GG
MSH6 c.2931C>G	AGA GAA ACA GCG CAA CA
	TAC AGC CCT TCT TGG TAG ATT

antibody, is the carboxyl terminal region. The mouse MSH6 antibody clone 44 (BD Biosciences Pharmingen, Belgium) has the most specific epitope; amino acids 225 to 333 of the human MSH6 protein. When evaluating the stained sections a case was considered positive for expression of a given protein if any tumour cells displayed positive nuclear staining. Cases that did not yield positive internal control staining (lymphocytes, benign colonocytes) were considered non-informative. The expression was classified without any grading of the staining intensity, as present, absent or non-informative.

Genome-wide scan

A genome-wide scan was performed in **study V**. Genomic DNA was prepared from whole blood using standard salting-out methods. All available samples were analysed using the ABI linkage panel set of 400 microsatellite markers with an average spacing of 10 cM (ABI Linkage Mapping Set 10 cM version 2.5 from Applied Biosystems, Foster City, CA, USA). The primers were amplified according to the manufacturer's instructions utilising multiplex PCR reactions. PCR products were resolved through 36 cm capillary arrays using POP-4 polymer and an ABI PRISM 3100 DNA sequencer, and genotypes were analysed using GeneMapper 3.0 (Applied Biosystems, Foster City, CA). Genotyping data were checked for Mendelian inheritance incompatibilities by the PEDCHECK program (223).

Statistical analyses

Standard incidence ratio, SIR

The standard incidence ratio (SIR) is the observed number of cancer cases divided by the expected number of cases. SIRs were calculated for a number of different cancer diagnoses for the FDRs of probands who had double primary colorectal cancers, or both primary endometrial and colorectal cancers (**Study I**). Using their unique personal identification numbers, the cohort was linked to the Swedish National Cancer Register between the years 1958 and 1996 and the numbers of cancer cases observed among the 649 FDRs were obtained. The number of person-years involved (17088 in total) was calculated from January 1 1958 to the date of death or December 31 1996 using the program PYRS (224). Age, gender and calendar year were used as stratification variables in the calculations of person-years. The reference population was defined as all individuals in the northern health care region of Sweden. The expected number of cases was calculated by multiplying the stratum-specific incidence rates in the reference population by the corresponding numbers of person-years in the cohort and then summed over calendar strata. The population rates for the period 1958-1996 were obtained from the Swedish Cancer Register. The SIR was defined as the ratio between the observed and expected numbers of cases. Using the formula suggested by Byar (225), 95% confidence limits of the SIR estimates were calculated. Differences between the observed and expected number of cases were considered significant if the confidence interval did not include the point one. The total cohort was divided into subgroups according to the type of tumours present (colon/colon or colon/uterus), MSI status (MSI-positive or MSI-negative) and age at first cancer diagnosis (over or under 50 years of age) in the proband.

Cumulative risk analysis

Cumulative cancer risk by attained ages was estimated by the Kaplan-Meyer method using SPSS software in **study IV**. Only cancer diagnoses verified in the Swedish National Cancer Register in proven and obligate carriers were included. Tumours where MSH6-involvement was ruled out in the molecular analyses were excluded. In the analysis of cumulative risk for all Lynch syndrome-related tumours, only the first

diagnosis of a person was included. In analyses of cumulative risks for specific organs, only the first malignancy was included if more than one tumour developed in the same organ. Follow-up for malignancies started on the date of birth or January 1, 1958, whichever was later, and ended at death, emigration or December 31, 2004, whichever came first.

Linkage analysis

Linkage analysis was performed in **study V**. using the robust multipoint linkage statistic proposed by Göring and Terwilliger (226), herein referred to as the TLOD (theta LOD). This statistic method is analogous to a two-point LOD score, but utilizes full multi-point haplotype information. Analysis was performed using MCLINK, a program developed at Myriad Genetics Inc. (227), which allows the analysis of very large pedigrees with any number of genotyped markers. MCLINK uses a Markov chain Monte Carlo approach to reconstruct haplotypes across the markers analysed; an analytical tool that has been successfully employed to map multiple susceptibility genes (228, 229). An autosomal dominant disease inheritance model with a disease allele frequency of 0.003 was applied. Affected individuals were assumed to be carriers of the disease allele with a 15% phenocopy rate, whereas all unaffected individuals were assumed to be of an unknown phenotype. Marker allele frequencies were estimated using genotype data for all 19 typed individuals within the family and an additional set of 31 men affected with prostate cancer ascertained from the same population. Three different diagnostic criteria were used to classify subjects as affected or not in the linkage analysis. For the most stringent classification (I), individuals with a confirmed diagnosis of colorectal cancer were considered affected. For the less stringent classification (II), individuals with villous adenomas and/or adenomas larger than 10 mm and/or adenomas with severe dysplasia were also considered affected. Finally, for the least stringent classification (III), also individuals with any tubulovillous or tubular adenomas were classified as affected.

Results and Comments

Study I

The main goals of this study were to identify persons at a high risk for hereditary colorectal cancer, in particular Lynch syndrome, to estimate the risk of colorectal and other Lynch syndrome-associated tumours in their FDRs, and to test the hypothesis that young age at diagnosis and/or MSI-positive tumours are associated with higher risk for heredity and thus higher cancer risk. The advantage of the study design, a population-based study, is that the chance of overestimating the cancer risks was lower than if a cohort of selected cases had been examined. Availability of the highly accurate information in the Swedish Cancer Registry for confirmation of diagnoses was also advantageous.

Multiple primary tumours are a hallmark of hereditary predisposition to cancer; hence we identified a cohort of patients with double primary tumours of the two main tumour sites of Lynch syndrome; the colorectum and the endometrium. We analysed their tumours for MSI, another hallmark of Lynch syndrome. Representative MSI and MSS tumour analyses are shown in Figure 1. The cancer risk in their FDRs was analysed in the whole cohort and in two pairs of subgroups (FDRs to probands diagnosed before or after 50 years of age, and probands with MSI or MSS tumours). The cut-off age of 50 years was used in accordance with the Amsterdam criteria. In all the analyses we combined data from probands with double primary colorectal cancers and probands with colorectal/ endometrial cancers, since no significant differences were found between them, either for the overall risk or for specific tumours (data not shown). We expected the MSI subgroup to be enriched in hereditary cancers (Lynch syndrome), but also to contain some sporadic cancers MSI due to *MLH1* hypermethylation. The MSS sub-group was expected to contain sporadic cases and, possibly, non-Lynch syndrome hereditary cases. In both subgroups, the number of hereditary cases was expected to increase when only probands <50 years old was included.

As expected, the cancer risk in the <50 years group was increased compared to the whole cohort, as were the risks of both colon and endometrial cancer. We found the same increased risks of ovarian cancer as Pal *et al.* (4.2 vs. 4.3), but in contrast to their findings we also found an increased risk of gastric cancer and no risk of pancreas cancer. In the >50 years cohort, the only significantly increased risks were for rectal cancer in men (SIR 4.36; 95% CI 1.17-11.16) and endometrial cancer in women (Table 15).

The cancer risk was also, as anticipated, higher in the MSI subgroup than in the whole cohort, or in the MSS subgroup. In the MSS subgroup the increased risks were restricted to colon and endometrial cancers. The MSS and >50 years subgroup had no increased cancer risks at all, which is important for counselling. None of the increased risks in the MSS group were due to the MSI-L families, but are associated with other hereditary colon and/or endometrial cancer predispositions other than Lynch syndrome. The MSI subgroup had increased risks of colon cancer (men and women), rectal cancer (men: SIR 8.14; 95% CI 2.19-20.85) and endometrial cancer. The rectal and endometrial cancer risks were confined to the >50 MSI years subgroup. The colon cancer risk was confined to and further increased in the <50 years MSI subgroup, which had the highest overall cancer risks of all patients. This <50 years MSI group is expected to contain Lynch syndrome patients, and can thus be com-

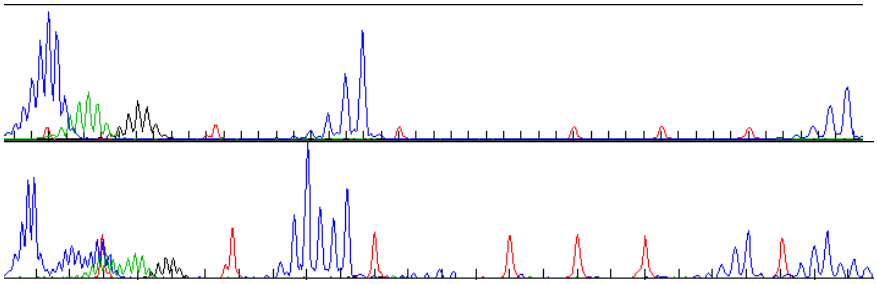


Figure 1. Representative MSS and MSI tumours. The markers are, from left to right, BAT26, BAT25, BAT34C4, D17S250 and D2S123.

pared to the mutation-positive cases in the study by Millar *et al.* of women with double primary colorectal/endometrial cancer and *MLH1/MSH2* mutations: they found an overall cancer risk of 5.4 compared to our 3.2 and a colorectal cancer risk of 24.5 compared to our 21.1 (201).

Reclassification of the re-analysed MSS family to MSI-H would move five colon cancer cases from the MSS<50 group to the MSI<50 group. This would increase the already very high colon cancer risks in the MSI group, but not remove all of the risk from the MSS group. Reclassification of the MSI-L family and the family with unknown MSI to MSI-H would only add one gastric cancer case to the MSI<50 group.

To conclude **study I**, first degree relatives of probands with double primary colorectal or colorectal/endometrial tumours have a significantly increased risk for developing colorectal, endometrial cancer. This risk is further increased if the proband is diagnosed with at least one of the tumours before the age of 50 years. If the proband's tumour is diagnosed after the age of 50, the overall cancer risk is not increased. If the probands tumour is MSI, the risk is even further increased and extended to other cancers of the Lynch syndrome spectra and to some extent to probands over 50 years of age. These families are candidates for genetic counselling and surveillance programmes.

Study II

The aim of **study II** was to investigate the *MLH1, MSH2* and *MSH6* mutation spectra in patients with MSI-positive double primary colorectal cancer or colorectal/

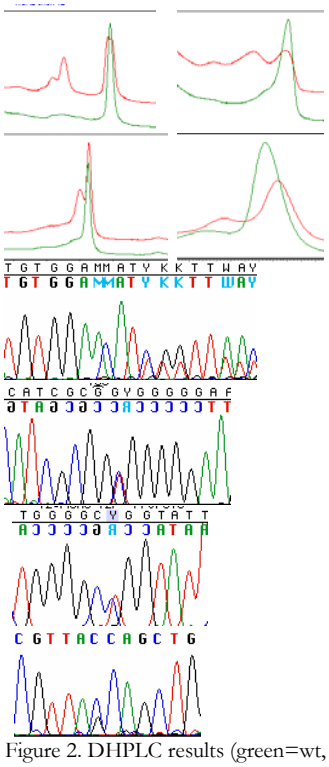


Figure 2. DHPLC results (green=wt, red= variant) and sequences of *MSH2* c.1986delG, *MLH1* p.62C>T, *MSH6* c.1346T>C and *MSH6* c.2931C>G.

endometrial cancer tumours. We hypothesized that the MSI-H probands included in **study I** were candidates for Lynch syndrome, and thus screened their *MLH1*, *MSH2* and *MSH6* genes for sequence variants. Since *MSH6* mutations sometimes predispose to MSI-L and even MSS tumours (207, 230), and the apparent MSI status could be dependent on the choice of markers (95), one MSI-L proband, the family in which the MSI status could not be determined but clinical Lynch syndrome was present, and two MSS probands with clinical Lynch syndrome diagnoses were all also included. As described above, the MSI staus of some of these families was later reclassified.

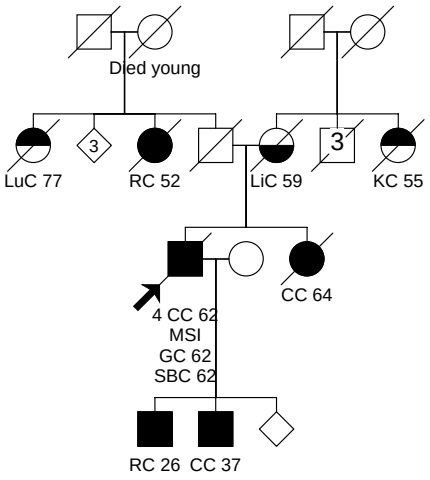


Figure 3. Pedigree of family Gen266, with the c.1846_1848delAAG, p.Lys616del mutation in *MLH1*. Abbreviations: CC: colon cancer; GC: gastric cancer; KC: kidney cancer; LiC: liver cancer; LuC: lung cancer; RC: rectal cancer; SBC: small bowel cancer. Numbers denote age in years at diagnosis.

Of the 25 patients screened for mutations in *MLH1*, *MSH2* and *MSH6*, mutations were found in 16 (Figure 5). Mutations in these genes were detected in 17% of the 88 probands originally included in **study I**. In comparison, 7% of 40 women with colorectal/endometrial cancers were found to have *MLH1* and *MSH2* mutations in a Canadian registry-based study (201) and 38% of patients with at least four primary malignancies, including two colorectal cancers, were MSI-positive with concordant loss of MLH1 or MSH2 according to immunohistochemical analysis, indicative of Lynch syndrome (197). In a study of patients with double primary colorectal/ endometrial cancer, MSI and concordant loss of MLH1 or MSH2 by IHC was found in 44% (200).

Twelve of the 16 mutations were found in probands diagnosed before the age of 50 years. In three mutation-positive families with probands diagnosed after they were 50 years old (Gen266 [Figure 3], CU013 [Figure 22] and Gen082), other family members were diagnosed before they were 50 years old. In the fourth (CU003, Figure 18), no family member was diagnosed before the age of 50 years. This family had an *MSH6* mutation, which generally predisposes to later onset cancer (180, 209, 211, 230).

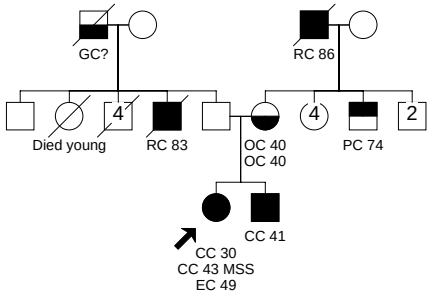


Figure 4. Pedigree of family CU004, in which no mutation was found. Abbreviations: EC: endometrial cancer; GC: gastric cancer; OC: ovarian cancer; PC: prostate cancer; RC: rectal cancer. Numbers denote age in years at diagnosis.

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Table 15: Cancer risks in first degree relatives of probands with double primary colorectal/endometrial tumours

		No	SIR	95% CI	No	SIR	95% CI	No	SIR	95% CI	No	SIR	95% CI	No	SIR	95% CI
Cancer	**Sex**	**Whole cohort**			**Proband<50**			**Proband>50**			**Proband MSI**			**Proband MSS**		
All	Both	111	**1.69**	(1.39-2.03)	70	**2.67**	(2.08-3.37)	41	1.04	(0.74-1.40)	51	**2.16**	(1.61-2.84)	59	**1.41**	(1.08-1.82)
Colon	Both	28	**6.13**	(4.04-8.86)	23	**12.57**	(7.96-18.86)	5	1.83	(0.59-4.27)	18	**11.04**	(6.54-17.45)	10	**3.42**	(1.64-6.30)
	F	12	**4.91**	(2.54-8.58)	9	**8.48**	(3.87-16.09)	3	2.17	(0.44-6.35)	7	**7.80**	(3.13-16.07)	5	**3.25**	(1.05-7.59)
	M	16	**7.53**	(4.30-12.23)	14	**18.06**	(9.87-30.31)	2	1.48	(0.17-5.35)	11	**15.16**	(7.56-27.13)	5	**3.62**	(1.17-8.44)
Rectal	Both	8	**2.96**	(1.27-5.83)	3	1.08	(0.56-8.12)	5	1.63	(0.99-7.16)	4	**4.17**	(1.12-10.67)	4	2.31	(0.62-5.92)
Endom.	F	10	**5.45**	(2.61-10.02)	4	**4.93**	(1.33-12.62)	6	**5.86**	(2.14-12.75)	5	**7.19**	(2.32-16.79)	5	**4.42**	(1.42-10.31)
Ovarian	F	4	1.87	(0.50-4.79)	4	**4.29**	(1.15-10.99)	0	0.00	(0.00-3.04)	2	2.45	(0.28-8.85)	2	1.53	(0.17-5.51)
Gastric	Both	6	1.22	(0.45-2.66)	6	**3.33**	(1.22-7.26)	0	0.00	(0.00-1.18)	1	0.58	(0.01-3.25)	4	1.27	(0.34-3.25)
					Proband<50, MSI			**Proband<50, MSS**			**Proband>50, MSI**			**Proband>50, MSS**		
All	Both				33	**3.17**	(2.18-4.45)	36	**2.34**	(1.64-3.24)	18	1.36	(0.81-2.15)	23	0.87	(0.55-1.31)
Colon	Both				15	**21.13**	(11.82-34.85)	8	**7.27**	(3.13-14.33)	3	3.30	(0.66-9.63)	2	1.10	(0.12-3.97)
	F				5	**12.17**	(3.92-28.41)	4	**6.23**	(1.68-15.95)	2	4.11	(0.46-14.84)	1	1.12	(0.01-6.22)
	M				10	**33.17**	(15.88-61.01)	4	**8.74**	(2.35-22.38)	1	2.36	(0.03-13.12)	1	1.08	(0.01-6.01)
Rectal	Both				1	2.33	(0.03-12.94)	2	3.13	(0.35-11.28)	3	**5.66**	(1.14-16.54)	2	1.83	(0.21-6.62)
Endom.	F				1	3.00	(0.04-16.68)	3	**6.41**	(1.29-18.72)	4	**11.07**	(2.98-28.34)	2	3.02	(0.34-10.89)
Ovarian	F				2	5.24	(0.59-18.93)	2	3.72	(0.42-13.42)	0	0.00	(0.00-8.44)	0	0.00	(0.00-4.75)
Gastric	Both				1	1.45	(0.02-8.06)	4	**3.74**	(1.01-9.57)	0	0.00	(0.00-3.56)	0	0.00	(0.00-1.76)

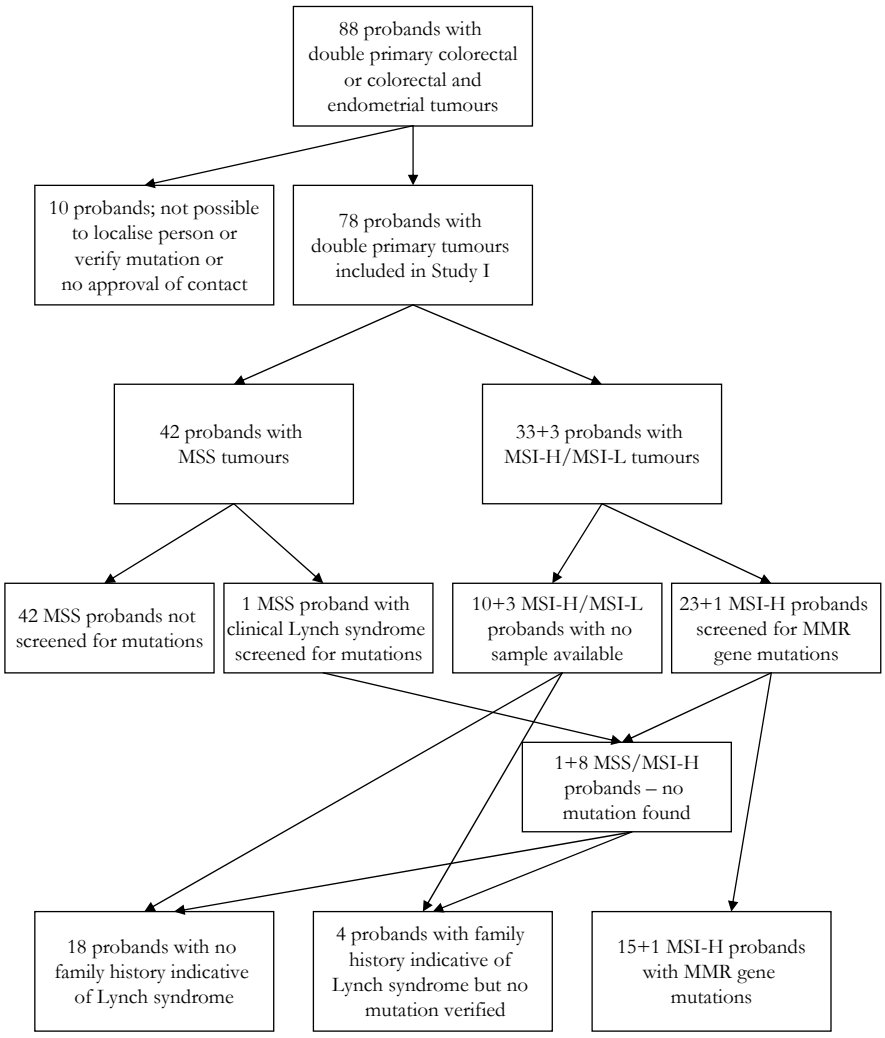

Figure 5. Flow chart of studies I and II.

Six of the seven MSI-positive probands in which no mutation was found did not fulfil the Amsterdam I or II criteria and did not have the typical Lynch syndrome tumour spectra in their families. The seventh family (CU006, Figure 6), which includes many endometrial and ovarian cancer cases, may harbour a mutation in a region of *MSH6* that was not examined. An IHC analysis of MSH6 expression could possibly aid further investigation of this family.

No mutation was found in any of the two MSS probands screened for mutations. Family CU004 is clinically diagnosed with Lynch syndrome (Figure 4). Further investigation of this family should be undertaken, including MSI testing of more tumours and IHC analysis. In family Gen013, fulfilling the Amsterdam I criteria, another tumour (the probands colon cancer at age 43) have since study II was completed been tested and found MSI-posi-

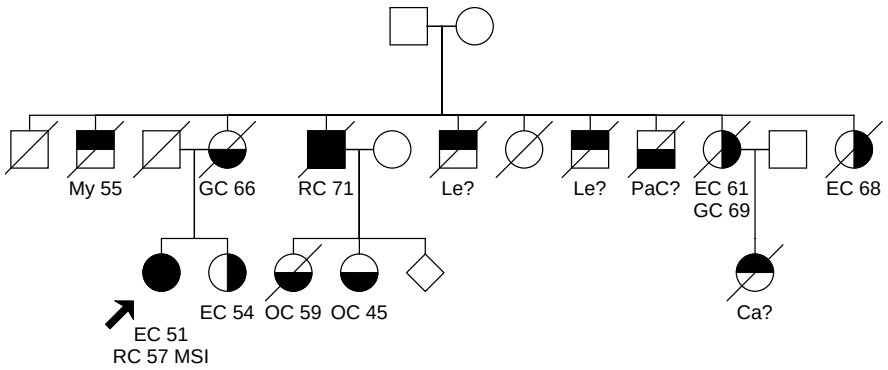

Figure 6. Pedigree of family CU006, in which no mutation was found. Abbreviations: EC: endometrial cancer; GC: gastric cancer; Le: leukaemia; My: myeloma; OC: ovarian cancer; PaC: pancreas cancer; RC: rectal cancer. Numbers denote age in years at diagnosis.

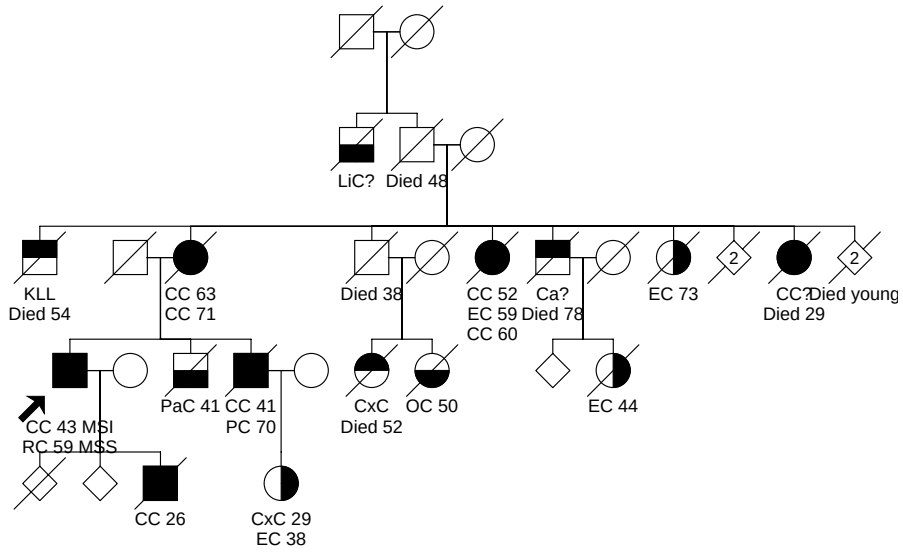

Figure 7. Pedigree of family Gen013, in which no mutation was found. Abbreviations: CC: colon cancer; KLL: chronic lymphatic leukaemia; CxC: cervix cancer; EC; endometrial cancer; LiC: liver cancer; OC: ovarian cancer; PC: prostate cancer; PaC: pancreas cancer; RC: rectal cancer. Numbers denote age in years at diagnosis or death.

tive (Figure 7). Thus, this family is a candidate for IHC analysis and further screening for MMR gene mutations, possibly of *PMS2*.

Thirteen MSI probands included in **study I** were not screened for mutations, due to a lack of blood samples from the affected individuals. In nine of these families, the proband had no first-degree relative with cancer. In two of the families, the proband had just one first-degree relative with cancer, which was not Lynch syndrome-related in either case. In one of the families, the proband had three first-degree relatives with cancer, but none of them were Lynch syndrome-related. These twelve probands most likely have sporadic MSI cancers due to hypermethylation of *MLH1*. The thirteenth proband (family Enk001, Figure 8),

who was diagnosed with endometrial and colon cancers at ages of 57 and 58 years, respectively, had a father with rectal cancer and a son with brain cancer diagnosed at the age of 31 years. Of her seven siblings, five died young with tuberculosis. On one that immigrated to Canada at age 22 years we have no further information. This family is a candidate for MMR gene screening if any new case shows up.

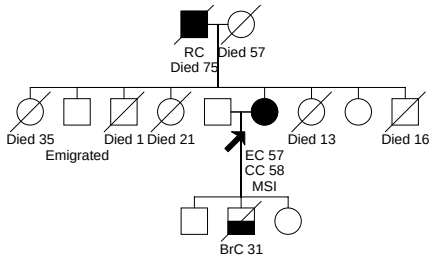

Figure 8. Pedigree of family Enk001, in which no sample was available for mutation screening. Abbreviations: BrC: brain cancer; CC: colon cancer; EC: endometrial cancer; RC: rectal cancer. Numbers denote age at diagnosis or death.

Five of the mutations were known at the Cancer-genetic counselling service at the University Hospital of Umeå's Departments of Clinical Genetics and Oncology, or other cancer-genetic counselling services in Sweden in families already attending screening programmes (Table 16: Sthlm69, Gen028, Gen082, Gen058/Sthlm167, and Gen086/Sthlm80).

The mutation found in family Gen266 (Figure 3), *MLH1* c.1846_1848delAAG, p.Lys616del, was not known in this family, but has been reported several times in the literature and the InSiGHT database in patients from various parts of the world. This mutation has been suggested to make the resultant protein unstable. If so, the pathogenicity is not linked to loss of function but to shortage of the functional protein (155). The family was offered genetic counselling, surveillance and the possibility of presymtomatic testing.

The two novel large deletions in *MSH2* (Table 2) and the novel frameshift mutation in *MSH2* (Figure 2) were all regarded as being pathogenic and the families (CC011 [Figure 9], Gen024 [Figure 10], and Gen051 [Figure 11]) were offered genetic counselling, surveillance and the possibility of presymtomatic testing.

The novel missense mutation in *MLH1* (Figure 2) was consi-dered to need further evaluation (**study III**) before use in clinical practice. Therefore this family (CU001, Figure 12) was offered counselling and surveillance but not genetic testing.

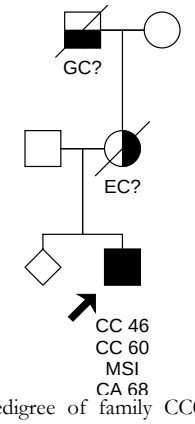

Figure 9. Pedigree of family CC011 with the *MSH2* c.1986delG, p.Gln662fs23X mutation. Abbreviations: CA: colonic adenoma; CC: colon cancer; EC: endometrial cancer; GC: gastric cancer. Numbers denote age in years at diagnosis or death.

The novel nonsense mutation in *MSH6* (Figure 2) was found in two probands from the same geographical area. This mutation was deduced to be pathogenic and the two families (CU009 and CU013; Figures 21 and 22 respectively) were offered genetic counselling, surveillance and the possibility of presymtomatic testing. Furthermore, these two families were included in the additional studies of *MSH6* in **study IV**. The novel missense mutation in *MSH6* (Figure 2) was found in four probands from the same geographical area. This missense mutation was also considered for further evaluation

Table 16. MMR gene mutations in MSI patients with double primary colorectal or colorectal and endometrial tumours.

Family no	Gene	Exon	Mutation	Consequence	Reference
CU001	*MLH1*	1	c.62C>T	p.Ala21Val	This study
Sthlm69	*MLH1*	2	c.199G>A	p.Gly67Arg	(231)
Gen028	*MLH1*	2	c.203T>A	p.Ile68Asn	(231)
Gen082	*MLH1*	16	del 578-632+intron	In-frame del of exon 16	(232)
Gen266	*MLH1*	16	c.1846_1848delAAG	p.Leu616del	This study
Gen024	*MSH2*	1-6	del exon 1-6	Del exon 1-6	This study
Gen051	*MSH2*	1-11	del exon 1-11	Del exon 1-11	This study
Gen058/ Sthlm167	*MSH2*	7-10	del exon 7-10	Out-of-frame del exon 7-10	Liu *et al.* InSiGHT database
Gen086/ Sthlm80	*MSH2*	10	c.1550_1551delCA	p.Gln518fs10X	(233)
CC011	*MSH2*	12	c.1986delG	p.Gln662fs23X	This study
CU002	*MSH6*	4	c.1346T>C	p.Leu449Pro	This study
CU003	*MSH6*	4	c.1346T>C	p.Leu449Pro	This study
CU008	*MSH6*	4	c.1346T>C	p.Leu449Pro	This study
CU012	*MSH6*	4	c.1346T>C	p.Leu449Pro	This study
CU009	*MSH6*	4	c.2931C>G	p.Tyr977X	This study
CU013	*MSH6*	4	c.2931C>G	p.Tyr977X	This study

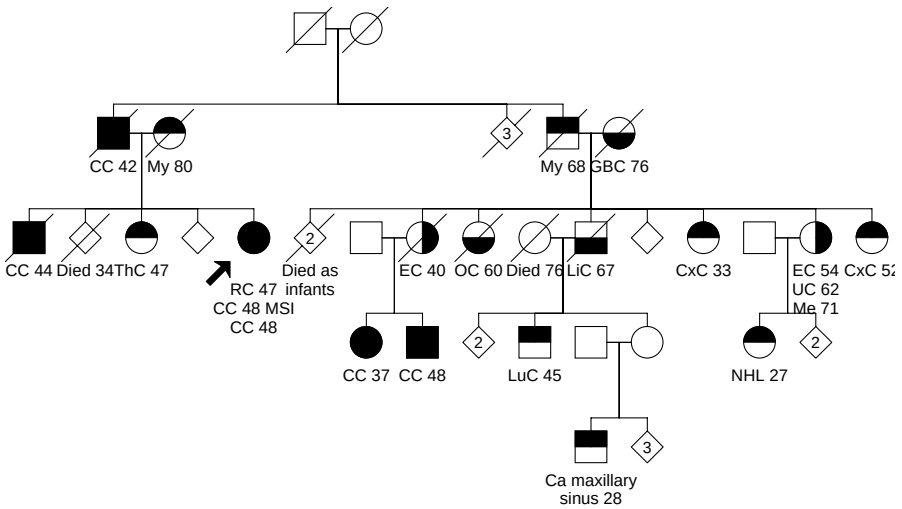

Figure 10. Pedigree of family Gen024 with a deletion of *MSH2* exons 1-6. Abbreviations: CC: colon cancer; CxC: cervix cancer; EC: endometrial cancer; GBC: gall bladder cancer; LiC: liver cancer; LuC: lung cancer; Me: meningioma; My: myeloma; NHL: Non-Hodgkins lymphoma; OC: ovarian cancer; RC: rectal cancer; ThC: thyroid cancer; UC: urinary tract cancer. Numbers denote age in years at diagnosis or death.

(**study IV**) before use in clinical practice. The four families (CU008, Enk103, CU012, CU002 and CU003; Figures 15-19 respectively) were offered counselling and surveillance but not testing.

Reconsidering the results from **study I**, in the light of knowledge acquired from **study II**, some details can be noted. In the cohort with probands diagnosed after the age of 50, the only significantly increased risks were for rectal cancer in men, and endometrial cancer in women. These risks, which are confined to the MSI subgroup, are to a large extent due to *MSH6* cases, which in the >50 years, MSI subgroup include one of

the four rectal cancer cases and three of the four endometrial cancer cases. This further supports the notion that if the proband is diagnosed after the age of 50 and Lynch syndrome is excluded, there relatives have no increased risk of cancer.

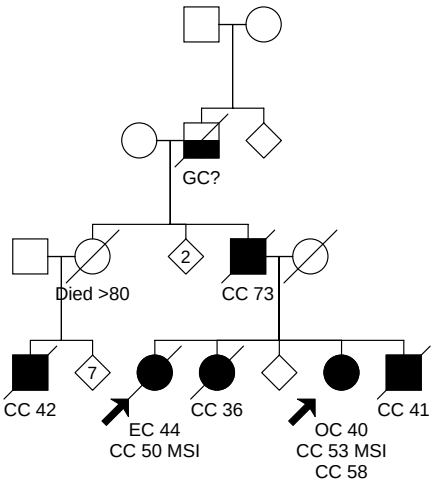

Figure 11. Pedigree of family Gen051 with a deletion of *MSH2* exons 1-11. Abbreviations: CC: colon cancer; EC: endometrial cancer; GC: gastric cancer; OC: ovarian cancer. Numbers denote age in years at diagnosis or death.

To conclude **study II**, patients with microsatellite-unstable double primary cancers of the colorectum and the endometrium have a very high risk of carrying a mutation not only in *MLH1* or *MSH2*, but also in *MSH6*.

Study III

The aim of study III was to assess the pathogenicity and clinical significance of the *MLH1* c.62C>T, p.Ala21Val sequence variant found in a large Lynch syndrome family fulfilling Amsterdam I criteria in **study II** (family CU001, Figure 12). MSI, protein expression by IHC and segregation of the mutation were analysed in ten tumours from each proband and seven relatives with Lynch syndrome-related tumours.

The amino acids alanine and valine are both aliphatic non-polar amino acids with small hydrocarbon side chains, but they strongly differ in alphahelix propensity. Thus, an alanine-valine substitution represents a major change that might disturb the structure and, thus, function of the protein. PolyPhen (a tool which predicts the possible impact of an amino acid substitution on the structure and function of a human protein) (144) predicts this change to be possibly damaging and the SIFT program (143) predicts it to affect protein function. Regarding evolutionary conservation, interspecies sequence comparison using ClustalW (145) shows that the alanine is conserved from bacteria (*E. coli*) through yeast (*Saccharomyces cerevisiae* and *S. pombe*), flatworm (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*), and even plant (*Arabidopsis thaliana*), to chicken (*Gallus gallus*), rat (*Rattus norvegicus*) and mouse (*Mus musculus*). Furthermore, it is positioned in a block of nine amino acids that are conserved from yeast to human.

The c.62C>T sequence variant was not found in 184 alleles from healthy Swedish blood donors, nor in 50 alleles from other colon cancer patients (**study II**). It has also not been reported as a polymorphic variant in the literature, or in any of the common mutation- or SNP databases. In exon 1 of *MLH1*, where the p.A21V mutation resides, six missense mutations (p.E23D, p.I25F, p.I25T, p.P28L, p.A29S and p.M35R) have been reported to be pathogenic to date, but none as polymorphic or non-pathogenic (231, 234-236).

The proband had been examined for mutations in the remainder of the *MLH1* gene and also in the *MSH2* and *MSH6* genes, but no other suspected variations had been found (**study II**). None of the ten analysed tumours showed any reduction in IHC staining for MSH2 or MSH6 protein. With 16/19 confirmed cancers in the family being intestinal and the remaining three endometrial, the family shows a phenotype

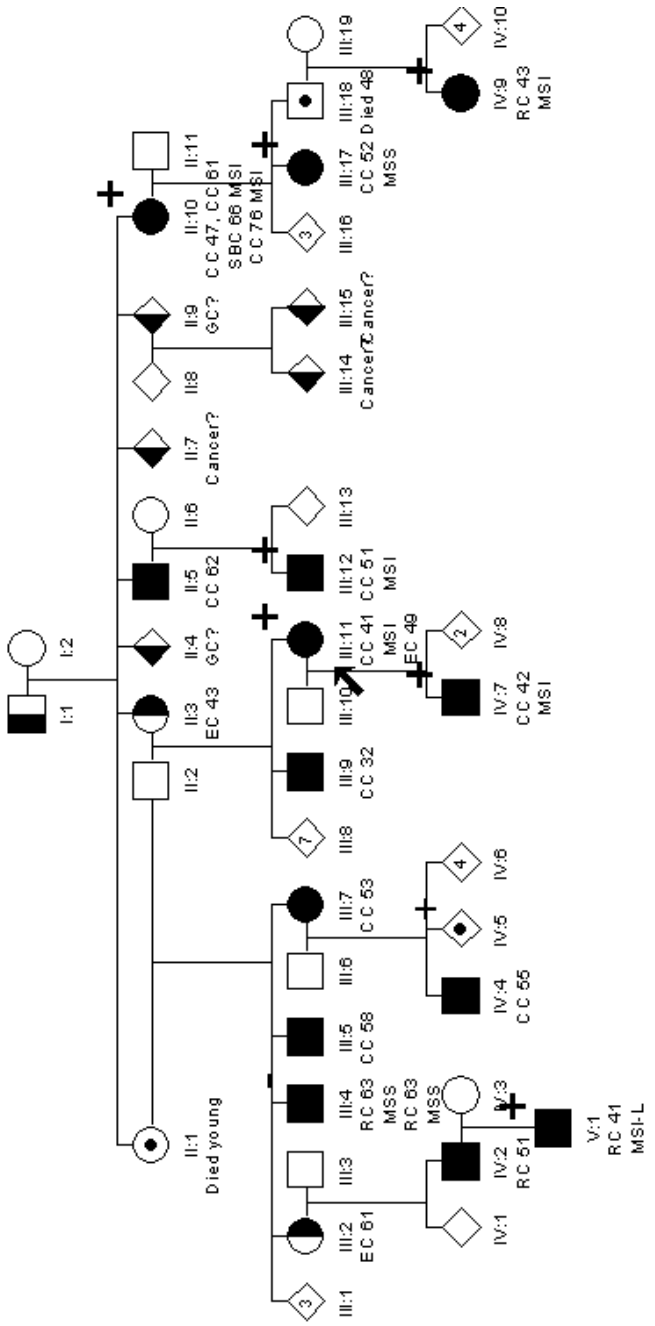

Figure 12. Pedigree of family CU001, with the MLH1 c.62C>T, p.Ala21Val mutation. A plus sign denotes a verified carrier, a minus sign a verified non-carrier. Abbreviations: CC: colon cancer; EC: endometrial cancer; GC: gastric cancer; RC: rectal cancer; SBC: small bowel cancer. Numbers denote age in years at diagnosis or death.

Table 17. Results of *MLH1* mutation, MSI and IHC analysis of family CU001

Patient no.	C.62 C>T	Tumour Age	MSI marker					MSI	IHC		
			BAT 25	BAT 26	BAT 34C4	D2S 123	D17 S250		MLH1	MSH2	MSH6
II:10	Yes	SBC 66	**U**	**U**	**U**	NA	**U**	MSI	P	P	P
		CC 76	NA	**U**	NA	**U**	**U**	MSI	**A**	P	P
III:4	No	RC 63	S	S	S	NA	S	MSS	P	F	P
		RC 63	S	S	S	NA	NA	MSS	P	P	P
III:11	Yes	CC 41	**U**	**U**	**U**	S	S	MSI	P	P	P
III:12	Yes	CC 51	**U**	**U**	S	**U**	S	MSI	P	P	P
III:17	Yes	CC 52	NA	S	S	S	S	MSS	P	P	P
IV:7	Yes	CC 42	**U**	**U**	NA	**U**	**U**	MSI	P	P	P
IV:9	Yes	RC 43	**U**	**U**	S	**U**	S	MSI	P	P	P
V:1	Yes	RC 41	S?	**U**	S	S	S	MSI-L	P	P	P

CC=colon cancer, RC=rectal cancer, SBC=small bowel cancer, NA=not amplified/not conclusive, S=stable, U=unstable, A=absent, P=present, F=failed

that correlates well with a *MLH1* mutation, since carriers of *MLH1* mutations have a lower risk for extracolonic cancers compared to *MSH2* and *MSH6* mutation carriers (174, 179).

Of the eight examined tumours from verified mutation carriers all but one was MSI positive (Table 17). The MSS tumour, a colon cancer from a woman aged 52, is considered sporadic.

According to the IHC results, six of the seven MSI-positive tumours retained MLH1 expression (Figure 13, Table 17). Only one tumour, an MSI-H colon tumour from a 76 year-old woman, showed loss of MLH1 expression. About 80% of all MSI-H colorectal cancers (including sporadic cancers) show loss of MLH1 expression (237), particularly in patients more than 65 years old (238), due to hypermethylation of the *MLH1* promotor, so this tumour is thus regarded as sporadic.

In hereditary cases, IHC has a sensitivity of only 74% in detecting germline *MLH1* mutations (129). This is to a large extent due to the over 30% missense mutations (66), which often result in proteins that are inactive in MMR but antigenically intact (129). Furthermore, even truncating mutations in *MLH1* may result in proteins that react with the commonly used antibodies (129). We cannot exclude the possibility that the retained staining is a sign of the proposed mutation not being disease causing *per se,* but a polymorphism segregating with the actual mutation. However, all other evidence indicates that the sequence change is pathogenic.

To conclude **study III**, the *MLH1* c.62C>T, p.Ala21Val sequence variant is regarded as being pathogenic based on its segregation with MSI-positive Lynch syndrome-associated tumours in a large Lynch syndrome family fulfilling Amsterdam I criteria, evolutionary conservation of the amino acid, non-conservative amino acid change and absence in the general population. The family has been offered genetic counselling, surveillance and presymtomatic testing.

Study IV

The aims of study IV were to assess the pathogenicity and clinical significance of the *MSH6* c.1346T>C, p.Leu449Pro sequence variant, to investigate if it is a founder mutation, and to describe the clinical and genetic characteristics of *MSH6*-related Lynch syndrome families. MSI, protein expression by IHC and segregation of the

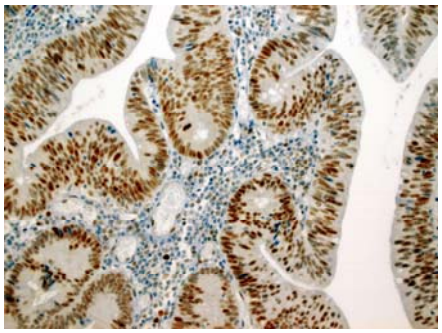

Figure 13. Immunostaining for MLH1 in a colon cancer from family CU001. The MLH1 expression is retained in the tumour cells.

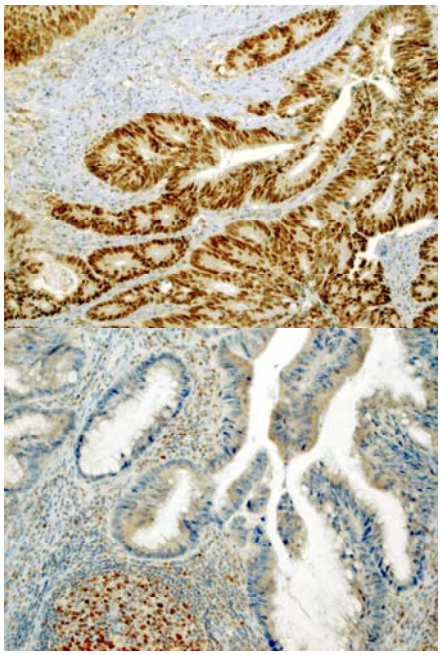

Figure 14. Immunohistochemical staining of MSH6 in colorectal cancers. A. MSH6 staining present in tumour cells. B. Absence of MSH6 staining in tumour cells from a patient with *MSH6* mutation.

mutations were analysed in tumours from seven probands carrying the *MSH6* mutations and 23 of their relatives with Lynch syndrome-related tumours.

The *MSH6* c.1346T>C, p.L449P sequence change was found in five different probands from the same geographical area (families CU008, Enk103, CU012, CU003 and CU002; Figures 15-19 respectively). Through studies of parish registers they were all traced to common ancestors in the late 17th century (Figure 20). The sequence change segregates with disease in 15 persons with Lynch syndrome-associated cancers, the majority of which are MSI-H and lack MSH6 staining (Table 18, Figure 15)

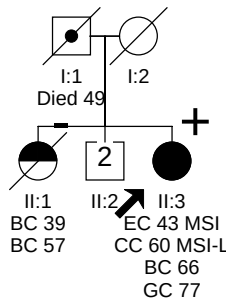

Figure 15. Pedigree of family CU008 with the *MSH6* c.1346T>C, p.Leu449Pro mutation. A plus sign denotes a verified carrier, and a minus sign a verified non-carrier. Abbreviations: BC: breast cancer; CC: colon cancer; EC: endometrial cancer; GC: gastric cancer. Numbers denote age in years at diagnosis or death.

Four of the probands had been examined for mutations in *MLH1* and *MSH2*, and no other suspected variations were found (**study II**); findings that were also supported by the IHC analyses of MLH1 and MSH2 (Table 18). Nine mutation-carriers were unaffected by Lynch syndrome at ages of 38-89 years. The sequence change was not found in 184 chromosomes from healthy Swedish blood-donors or 50 alleles from other colon cancer patients (**study II**), and has not been reported as a sequence variant in the literature or any public SNP database.

Interspecies sequence comparisons using ClustalW (145) showed that the leucine is conserved in chimpanzee (*Pan troglodytes*), mouse (*M. musculus*), rat (*R. norvegicus*), dog

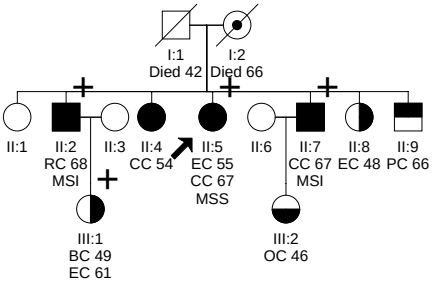

Figure 16. Pedigree of family Enk103 with the *MSH6* c.1346T>C, p.Leu449Pro mutation. A plus sign denotes a verified carrier, and a minus sign a verified non-carrier. Abbreviations: BC: breast cancer; CC: colon cancer; EC: endometrial cancer; OC: ovarian cancer; PC: prostate cancer. Numbers denote age in years at diagnosis or death.

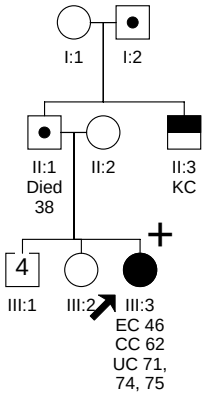

Figure 17. Pedigree of family CU012 with the *MSH6* c.1346T>C, p.Leu449Pro mutation. A plus sign denotes a verified carrier, and a minus sign a verified non-carrier. Abbreviations: CC: colon cancer; EC: endometrial cancer; KC: kidney cancer; UC: urinary tract cancer. Numbers denote age in years at diagnosis or death.

(*Canis familiaris*), chicken (*G. gallus*) and yeast (both *S. cerevisiae* and *S. pombe*), but not in fruit fly (*D. melanogaster*), flatworm (*C. elegans*), the plant *A. thaliana* or the bacterium (*E. coli*). The corresponding bacterial residue lies in a linker in domain I of MutS (239, 240), close to the highly conserved mismatch binding motif (241) where mutations have devastating effects on MMR in both yeast (242) and human cells (241). Introduction of a helix-breaker such as proline certainly has implications for the structure, and thus function, of this very important domain, consequently Poly- Phen (243) predicts this change to be possibly damaging and the SIFT program (244) predicts it to affect protein function.

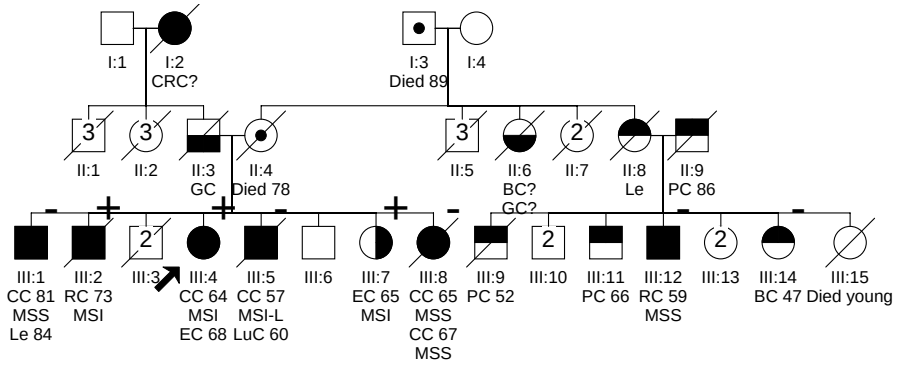

Figure 18. Pedigree of family CU003 with the *MSH6* c.1346T>C, p.Leu449Pro mutation. A plus sign denotes a verified carrier, and a minus sign a verified non-carrier. Abbreviations: BC: breast cancer; CC: colon cancer; CRC: colorectal cancer; EC: endometrial cancer; GC: gastric cancer; Le: leukaemia; LuC: lung cancer; PC: prostate cancer; RC: rectal cancer. Numbers denote age in years at diagnosis or death.

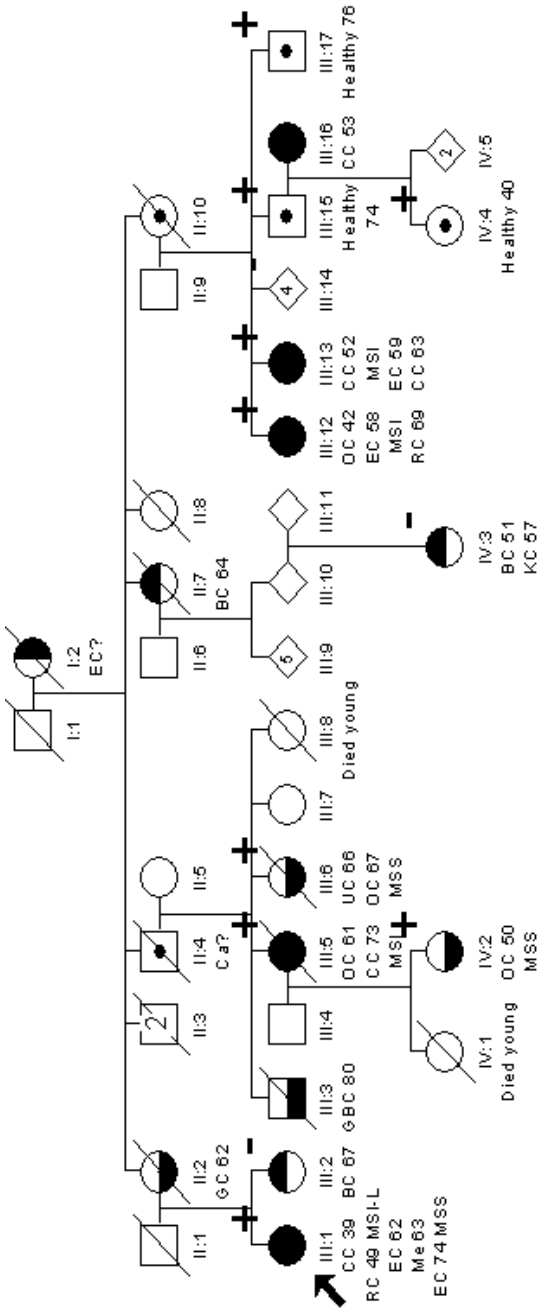

Figure 19. Pedigree of family CU002 with the *MSH6* c.1346T>C, p.Leu449Pro mutation. A plus sign denotes a verified carrier, a minus sign a verified non-carrier. Abbreviations: BC: breast cancer; CC: colon cancer; EC: endometrial cancer; GBC: gall bladder cancer; GC: gastric cancer; KC: kidney cancer; Me: meningioma; OC: ovarian cancer; RC: rectal cancer; UC: urinary tract cancer. Numbers denote age in years at diagnosis or death.

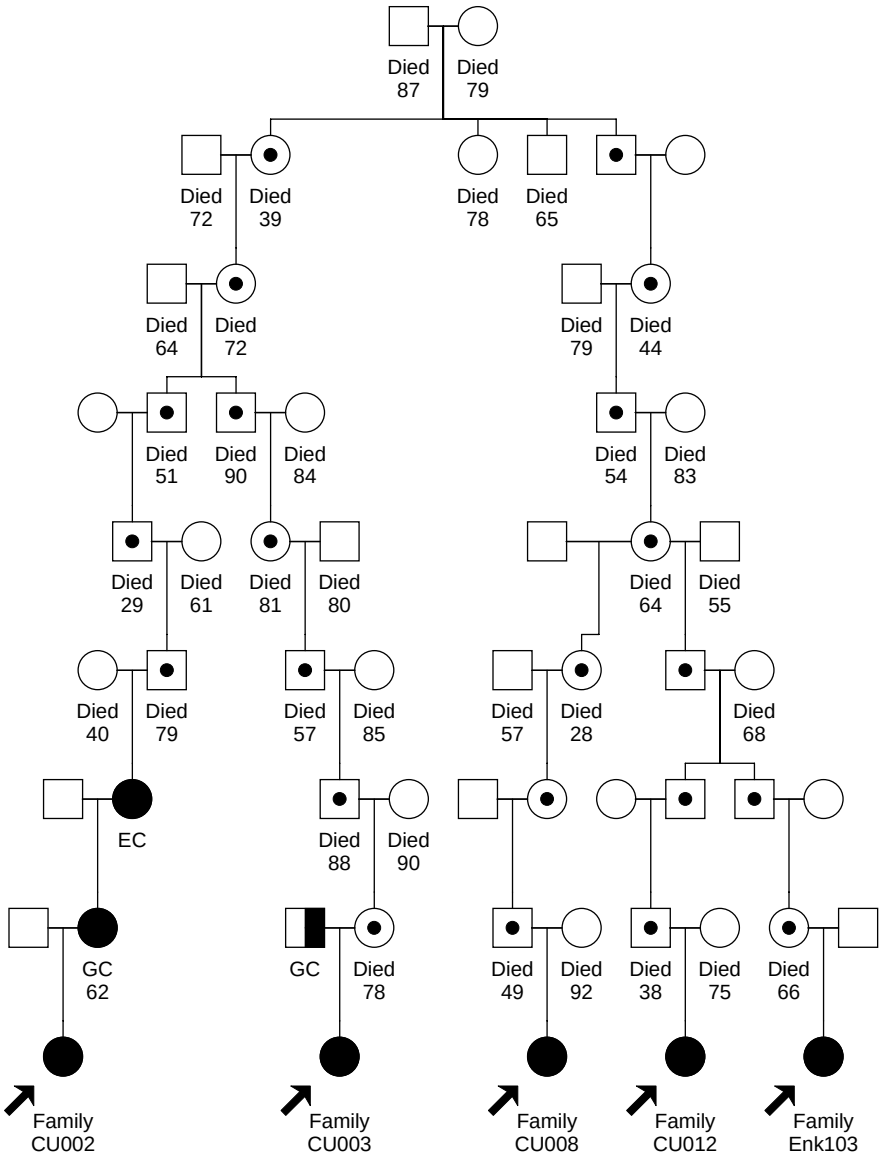

Figure 20. Pedigree of the large, merged family with the *MSH6* c.1346T>C, p.Leu449Pro mutation. Abbreviations: EC: endometrial cancer; GC: gastric cancer. Numbers denote age in years at diagnosis or death.

A structural change in the protein could also modify the epitope recognized by the antibody used (amino acids 225-233), and thus explain the loss of IHC staining. On the other hand, the lack of IHC staining could be due to the amount of the mutant protein being reduced, due for instance to instability caused by the amino acid change, as has been shown for other MMR gene missense mutations (155, 157). However, as

Table 18. Results of mutation, MSI and IHC analysis of families with the *MSH6* c.1346T>C, p.L449P mutation

Fam No.	Patient	Tumour Age	Mut.	MSI marker					MSI	IHC		
				BAT 25	BAT 26	BAT 34C4	D2S 123	D17S 250		MLH1	MSH2	MSH6
CU 002	III:1	RC 49	Yes	**U**	**U**	S	NA	NA	MSI-H	P	P	F
		EC 74		S	S	NA	S	S	MSS	F	F	F
	III:5	CC 73	Yes	**U**	**U**	**U**	S	S	MSI-H	P	F	P
	III:6	OC 67	Yes	S	S	S	S	S	MSS	P	**A**	**A**
	III:12	EC 58	Yes	**U**	**U**	S	S	S	MSI-H	P	P	**A**
	III:13	CC 52	Yes	**U**	**U**	S	S	S	MSI-H	**A**	P	P
	IV:2	OC 50	Yes	S	S	S	S	S	MSS	P	P	P
	IV:3	BC 51	No	-	-	-	-	-	ND	P	P	P
CU 003	III:1	CC 81	No	S	S	S	S	S	MSS	P	P	P
	III:2	RC 73	Yes	**U**	**U**	S	S	S	MSI-H	P	F	**A**
	III:4	CC 64	Yes	**U**	**U**	**U**	S	S	MSI-H	P	P	**A**
	III:5	CC 57	No	S	S	S	NA	**U**	MSI-L	F	F	F
	III:7	EC 65	Yes	**U**	**U**	S	**U**	**U**	MSI-H	P	P	**A**
	III:8	CC 65	No	S	S	S	S	S	MSS	P	P	P
		CC 67		S	S	S	S	S	MSS	P	P	P
	III:12	RC 59	No	S	S	S	S	NA	MSS	P	P	P
	III:15	BC 47	No	-	-	-	-	-	ND	F	P	P
CU 008	II:1	BC 39	No	-	-	-	-	-	ND	F	P	F
		BC 57		-	-	-	-	-	ND	P	P	P
	II:3	CC 60	Yes	**U**	**U**	S	S	S	MSI-H	P	P	**A**
		EC 43		NA	NA	**U**	**U**	NA	MSI-H	P	P	P
		BC 66		-	-	-	-	-	ND	F	P	P
CU 012	III:3	CC 62	Yes	**U**	**U**	**U**	S	**U**	MSI-H	P	P	**A**
Enk 103	II:2	RC 68	Yes	**U**	**U**	S	S	**U**	MSI-H	P	P	**A**
	II:5	CC 67	Yes	S	S	S	S	S	MSS	F	P	P
	II:7	CC 67	Yes	**U**	**U**	**U**	**U**	**U**	MSI-H	P	P	**A**
	III:1	BC 49	Yes	-	-	-	-	-	ND	P	P	F

BC: breast cancer; CC: colon cancer; EC: endometrial cancer; OC: ovarian cancer; RC: rectal cancer; U: unstable; S: stable; NA: not conclusive/not amplified; P: present; A: absent; F: failed

we have no functional data on the mutated protein, we can only speculate on possible pathogenicity mechanisms. We cannot exclude the possibility that the L449P genetic change is an innocent sequence variant in linkage disequilibrium with another, undiscovered *MSH6* mutation, e.g. a large deletion involving exons up- or downstream of the mutation site.

In the L449P-families, ten carriers and seven non-carriers were found among the 17 persons with Lynch syndrome-associated tumours and breast cancer analysed. Three of the eight non-carriers had breast cancer (Table 18) and four (ages 57-81 years) had colorectal cancer. Three of those colorectal cancer patients are siblings whose father (not in line with the sequence variant) had gastric cancer, indicating that there may be an additional hereditary cancer predisposition in this family (family CU003, Figure 18). None of the nine tumours from non-carriers analysed had a molecular phenotype suggestive of Lynch syndrome (Table 18).

Of the 18 tumours from carriers, three were considered to be possibly sporadic: an MSS endometrial tumour in which MSH6 staining failed, an MSS colon cancer with retained MSH6 staining, and an MSI-H colon cancer lacking MLH1 staining (Table

18). Breast cancer is not commonly regarded as part of the Lynch syndrome spectrum, but a few *MSH6* mutation-carriers with breast cancer have been found, and at least one breast tumour has been reported to be MSI-H with loss of MSH6 staining (179, 180) (179, 180, 211, 218). A recent study of 38 Finnish breast cancer families with colorectal and/or endometrial cancer ruled out *MSH6* mutations as the cause of breast cancer in such families (218). In our study, four breast cancers were from non-carriers (Table 18). The two breast cancers from mutation-carriers were MSS with no proof of MSH6 loss, implying that the *MSH6* mutations do not cause breast cancer. Unfortunately, only two ovarian tumours were available for molecular analyses and the results were inconclusive: both were MSS, one lacked both MSH2 and MSH6 staining and the IHC analysis of the other one failed. There is little molecular information on ovarian cancers in *MSH6* mutation-carriers, but one or two previously reported ovarian tumour were MSI-H (179) (179, 211). Of the remaining 11 MSI-H colorectal and endometrial tumours, MSH6 staining was absent in nine and inconclusive in the other two.

To conclude, the *MSH6* c.1346T>C, p.Leu449Pro sequence variant is regarded as being pathogenic based on its segregation with MSI-positive Lynch syndrome-related tumours lacking MSH6 expression in a large Lynch syndrome family fulfilling Amsterdam I criteria, evolutionary conservation, non-conservative amino acid change and absence in the general population. Based on genealogical studies it is considered a founder mutation in the Skellefteå area in Northern Sweden.

The two initial families with the *MSH6* c.2931C>G, p.Tyr977X mutation (CU009 and CU013; Figures 21 and 22 respectively), who also originated from the Skellefteå river area, were merged into one large family with two different possible common

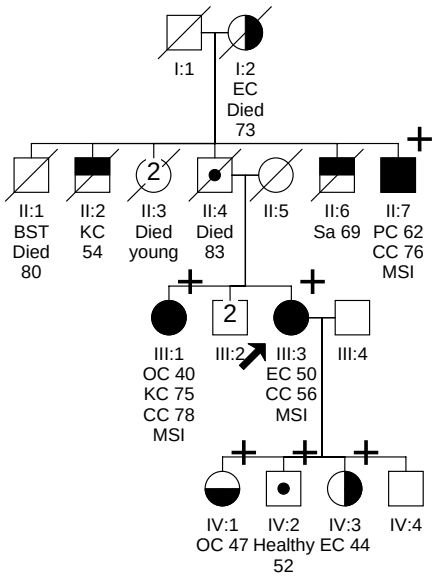

Figure 21. Pedigree of family CU009 with the *MSH6* c.2931C>G, p.Tyr977X mutation. A plus sign denotes a verified carrier, and a minus sign a verified non-carrier Abbreviations: BST: benign skin tumour; CC: colon cancer; EC: endometrial cancer; KC: kidney cancer; OC: ovarian cancer; PC: prostate cancer; Sa: sarcoma. Numbers denote age in years at diagnosis or death.

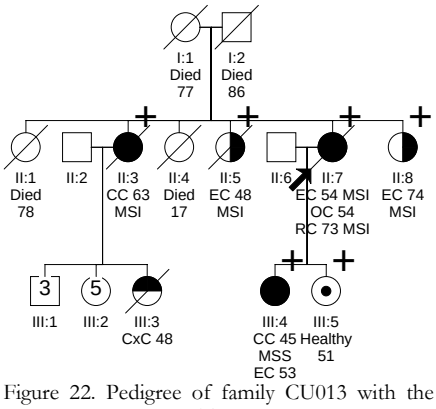

Figure 22. Pedigree of family CU013 with the *MSH6* c.2931C>G, p.Tyr977X mutation. A plus sign denotes a verified carrier, and a minus sign a verified non-carrier Abbreviations: CC: colon cancer; CxC: cervix cancer; EC: endometrial cancer; OC: ovarian cancer; RC: rectal cancer. Numbers denote age in years at diagnosis or death.

Table 19. Results of mutation, MSI and IHC analysis of families with the *MSH6* c.2931C>G, p.Y977X mutation

Fam No	Patient	Tumour Age	Mut	MSI marker					MSI	IHC		
				BAT 25	BAT 26	BAT 34C4	D2S 123	D17S 250		MLH1	MSH2	MSH6
CU 009	III:1	CC 78	Yes	**U**	**U**	S	S	S	MSI-H	P	F	**A**
	III:3	CC 56	Yes	**U**	**U**	S	NA	**U**	MSI-H	P	P	P
	II:7	CC 76	Yes	**U**	**U**	S	S	S	MSI-H	P	P	**A**
CU 013	II:3	CC 63	Yes	**U**	**U**	**U**	S	U	MSI-H	P	P	**A**
	II:5	EC 48	Yes	**U**	**U**	NA	NA	NA	MSI-H	P	P	**A**
	II:7	RC 73	Yes	**U**	**U**	S	**U**	S	MSI-H	P	F	**A**
	II:8	EC 74	Yes	**U**	**U**	**U**	NA	S	MSI-H	P	P	**A**
	III:4	CC 45	Yes	S	S	S	S	S	MSS	P	P	**A**

CC: colon cancer: EC: endometrial cancer: RC: rectal cancer: U: unstable: S: stable: NA: not conclusive/not amplified: P: present: A: absent: F: failed

ancestors in the late 18th century (Figure 23). All of the eight analysed persons with Lynch syndrome-related tumours in the Y977X-families carried the mutation, as did one healthy 51-year-old woman and one healthy 52-year-old man.

Seven tumours from Y977X-carriers showed no MSH6 staining, including one MSS colon tumour (Table 19). MSS tumours in which MSH6 expression has been lost have been occasionally reported in *MSH6* truncating mutation-carriers (180, 207, 245).

There were no significant differences between the two large families in either tumour spectra or mean age at diagnosis in proven or obligate carriers (56.2 years for all Lynch syndrome-related cancers, 64.3 years for colorectal, 56.0 years for endometrial and 51.2 years for ovarian cancers, with only one, colon cancer, case diagnosed before the age of 40 years).

The cumulative risk of all Lynch syndrome-related tumours, of colorectal, endometrial and ovarian cancer were estimated for each family separately; however, as there was no significant differences between the two families (p values >0.05) all estimates presented are based on both families together.

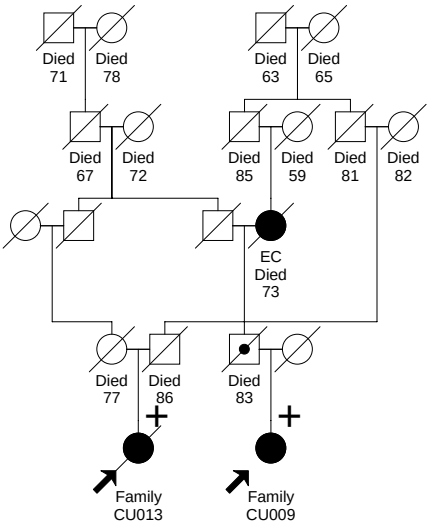

Figure 23. Pedigree of the merged family with the *MSH6* c.2931C>G, p.Tyr977X mutation. A plus sign denotes a verified carrier, and a minus sign a verified non-carrier Abbreviations: EC: endometrial cancer. Numbers denote age at diagnosis or death.

In the Kaplan-Meyer analysis 39 proven or obligate carriers were included, 27 of whom were affected with at least one Lynch syndrome-related tumour. Cumulative risks of different tumours are shown in Table 20. There were no statistically significant differences in cumulative risk for colorectal

Table 20. Cumulative cancer risks (%) ± standard errors for *MSH6* mutation-carriers.

Cancer type	Age (years)		
	50	65	80
All HNPCC-related tumours	24.3 ± 7.0	58.8 ± 8.6	82.2 ± 7.8
HNPCC, men	-	-	68.8 ± 23.7
HNPCC, women	33.3 ± 9.1	79.2 ± 8.2	88.9 ± 6.8
Colorectal cancer	5.4 ±	22.5 ± 7.6	60.5 ± 11.0
Colorectal cancer, men	- 3.7	-	68.8 ± 23.7
Colorectal cancer, women	7.6 ± 5.1	31.1 ± 9.9	59.1 ± 12.5
Endometrial cancer	14.8 ± 6.8	57.4 ± 10.1	70.2 ± 10.5
Ovarian cancer	11.3 ± 6.1	25.3 ± 9.1	32.8 ± 10.8

cancer between men and women (p=0.66), but for Lynch syndrome-related tumours in total the cumulative risk differed significantly (p=0.003) between men (69%) and women (89%), illustrated also by the findings that far larger numbers of women are affected than men. Admittedly, the male risk is based on a limited number of cases (eleven), but is identical to the risk of 69% at this age reported by Hendriks *et al.* (179) The cited authors report 30% and 60% risks of colorectal cancer in women by the ages of 70 and 80 years respectively. At the same ages, we found cumulative risks of 37% (+/-11) and 59%, and no significant difference from the male risk. The overall Lynch syndrome-risk differences between male and female *MSH6*-carriers can be attributed to the high risks of endometrial cancer (70% in our study and 71% in the study by Hendriks *et al.* by 80 years of age) and the unusually high risk (33%, again by 80 years of age) of ovarian cancer in women in addition to the high risk of colorectal cancer.

Several previous studies of *MSH6*-patients reported a late age of onset, which is supported by our data. However, this did not result in a lower lifetime risk in our families. Early studies of *MSH6* indicated that it had low penetrance (230) compared to *MLH1* or *MSH2*: 32% for colorectal and 52% for endometrial cancer at the age of 80 years in a large Dutch family with a truncating mutation (211). However, Berends *et al.* pointed out that the Dutch founder mutation had high penetrance, since several families with this mutation meet Amsterdam criteria (207). Later studies also indicated that it had high penetrance; Buttin *et al.* reported an overall penetrance of 58% among carriers of six truncating mutations (210) and Hendriks *et al.* a 73% cumulative risk at the age of 70 years among carriers of 17 truncating mutations (179). The cumulative risk at the age of 80 years among carriers of both non-sense and missense mutations in our study was 82%. However, the calculations are based on a limited number of cases and may represent an overestimate as not all healthy eligible relatives were tested.

To conclude **study IV**, we show a putative pathogenic *MSH6* missense mutation segregating with MSI-H, Lynch syndrome-spectrum tumours lacking MSH6 expression in a large 17th century pedigree and an *MSH6* non-sense mutation segregating in the same manner in an 18th century pedigree. We conclude that despite the late age of onset, these families have a high lifetime risk of Lynch syndrome-related tumours, and a significantly higher risk in women than in men due to the high risk of endometrial and ovarian cancer in addition to the high risk of colorectal cancer. The several occurrences of breast cancer are not due to the *MSH6* mutations. These findings are of great importance for counselling, management and surveillance of families with *MSH6* mutations.

Study V

The aim of study V was identify a novel locus for predisposition to hereditary colorectal cancer and adenomas. To accomplish this, a genome-wide scan was performed in a single large Swedish family (Family 19, Figure 24) with an apparently autosomal dominant predisposition to late onset colorectal cancer and adenomas.

At least ten members of Family 19 have been affected with colorectal cancer, with a mean age at diagnosis of 66 years. Many members of the family have been regularly examined by colonoscopy, and colorectal adenomas have been detected in at least 23 family members in three generations. This enabled phenotyping of many family members who would otherwise have been classified as "unknown" in the linkage analysis. It is not known whether individuals in the older generations with colorectal cancer also had adenomas. Of the family members under surveillance, none have developed colorectal cancer, possibly because of regular colonoscopies with polypectomi. The adenomas occured throughout the colon and include lesions of different histological types; tubular, tubulovillous and villous as well as hyperplastic polyps. The youngest person presenting with adenomas (detected at screening) was 35 years. The number of adenomas varied from one to over 15. The cancers and adenomas were equally distributed between males and females. In addition to the cases of colorectal cancers and adenomas, two family members presented with gastric cancer at ages 78 and 43.

As both colorectal cancer and adenomas are common in the general population, a number of phenocopies were expected in the family. This was accounted for in the model used, by assuming a phenocopy rate of 15%. The linkage analysis was performed using three different affected status criteria: colorectal cancer, advanced adenomas, and any adenomas. The least stringent criteria was included since >50% of individuals in high-risk families develop adenomas and these adenomas rarely exceed 5 mm if the patients undergoes biannual colonoscopies (246). Genetic information from 374 markers, spanning all autosomes and the X chromosome, were analysed using multipoint linkage analysis. Figure 25 graphically presents the genome-wide results for each of the three different diagnostic criteria.

In general the linkage evidence was moderate and only three chromosomes (2, 7 and 13) with TLODs over 1.0 were observed (Table 21). When using the most stringent classification (I), no TLODs reached the required thresholds for significant (3.3) or suggestive (1.9) linkage (247). Suggestive evidence of linkage (TLOD=1.9 at marker D7S515) was observed on chromosome 7q21 when using the second most stringent classification (II). Furthermore, this locus was the only region providing linkage evidence irrespective of which classification criteria used. This finding supports the hypothesis of the existence of a susceptibility gene predisposing to both colorectal cancer and adenoma.

In addition, we found moderate evidence of linkage on chromosome 2p (TLOD=1.5 at marker D2S2333), 2q (TLOD=1.6 at marker D2S126), and 13q (TLOD=1.7 at marker D13S173) when considering the two most stringent status classification criteria. Neither of these regions has been reported as susceptibility locus for colorectal cancer in previous linkage analyses. However, an increased frequency of allelic imbalances in colorectal tumours from patients with familial colorectal cancer, compared to tumours from sporadic colorectal cancer patients, has been reported to chromosome 7q21 (248), providing further support for the 7q locus.

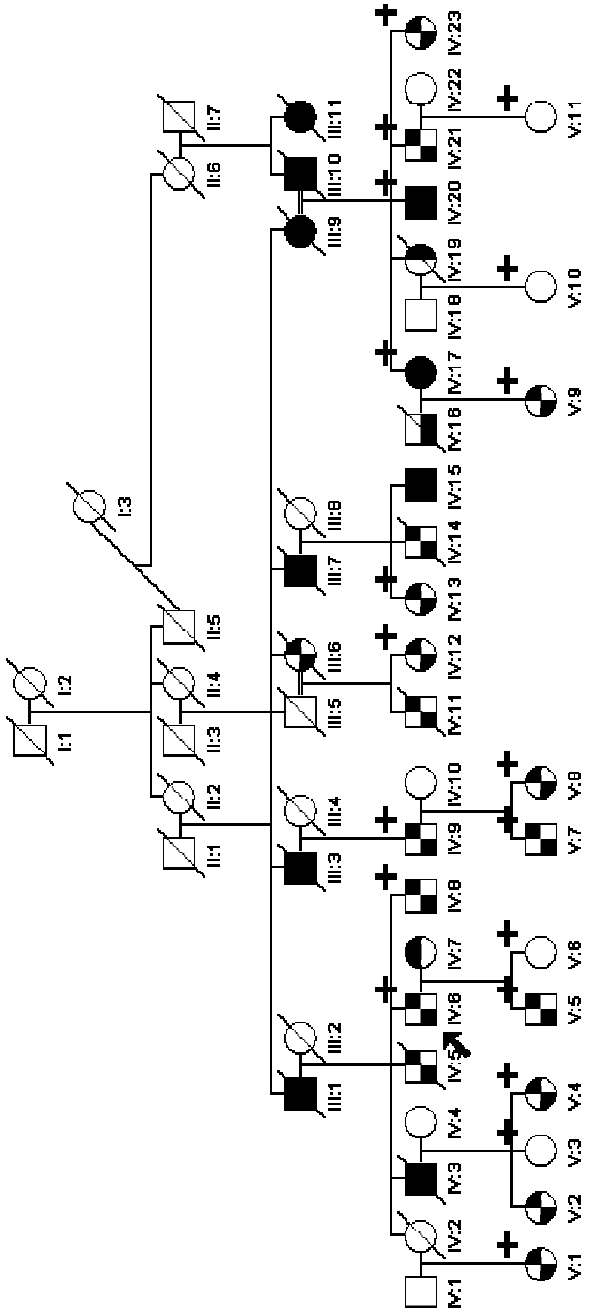

Figure 24. Pedigree of Family 19, a large Swedish family with predisposition to hereditary late onset colorectal cancer and adenomas. Filled symbol: colorectal cancer. Checked symbol: colorectal adenoma(s). Right part of symbol filled: gastric cancer. Lower part of symbol filled: prostate cancer. Upper part of symbol filled: breast cancer. Plus sign: sample available for genome-wide scan.

Table 21. Chromosomal regions with LOD scores over 1.0

Chromosome	Phenotype	Marker	Position (cM)[1]	LOD score
2	I	D2S286	92.5	1.04
		D2S2333	101.8	1.16
		D2S2216	109.4	1.15
	II	D2S2382	214.8	1.39
		D2S126	222.9	1.63
7	I	D7S657	100.5	1.10
		D7S515	108.2	1.06
	II	D7S657	100.5	1.84
		D7S515	108.2	1.90
		D7S486	120.6	1.35
		D7S530	131.7	1.19
	III	D7S486	120.6	1.31
		D7S530	131.7	1.20
		D7S640	135.0	1.86
13	I	D13S285	105.5	1.48
	II	D13S159	74.2	1.06
		D13S158	79.6	1.44
		D13S173	88.6	1.74
		D13S1265	94.4	1.59
		D13S285	105.5	1.39

[1]The marker positions are based on the Généthon Human genetic map

Surprisingly few new colorectal cancer candidate loci have been reported in the literature. The putative locus for Hereditary Mixed Polyposis Syndrome (MIM 601228) in families of Ashkenazi descent has been mapped to chromosome 15q13-q14 (36, 37). Another colorectal cancer susceptibility locus on chromosome 9q22.32-31.1 was proposed based on a linkage analysis of sib pairs concordant or discordant for colorectal cancer or advanced adenomas (38) and very recently confirmed by linkage in a large Swedish colorectal cancer family (39). Another recent genome-wide linkage analysis in 18 Swedish families with familial colorectal cancer showed suggested linkage to chromosomes 11q, 14q and 22q in different subsets of families (40). In our analysis, we found no evidence for linkage to any of the above regions.

The number of phenocopies accounted for in our disease model was possibly too high. Furthermore, using the "affected only" method, we lost a lot of information and thus power, particularly in the more stringent classifications. We recognize that our finding on chromosome 7q21 does not reach the threshold for significant linkage in a genome-wide scan. Therefore, further analysis of families with predisposition to late onset colorectal cancer and adenomas are warranted. In an attempt to verify and possibly strengthen our finding, more markers will be genotyped in the 7q21 region in order to increase the resolution and thereby the linkage information content. The regions on chromosomes 2 and 13, with moderate evidence of linkage, will also be further analysed.

To conclude **study V**, we report a suggested linkage, with a maximum TLOD score of 1.9 at marker D7S515, to chromosome 7q21.3 in a large Swedish family with hereditary late onset colorectal cancer and adenomas. This chromosomal region has previously been implicated in familial colorectal cancer. More markers will be analysed in the areas of interest to increase the resolution and possibly strengthen our findings.

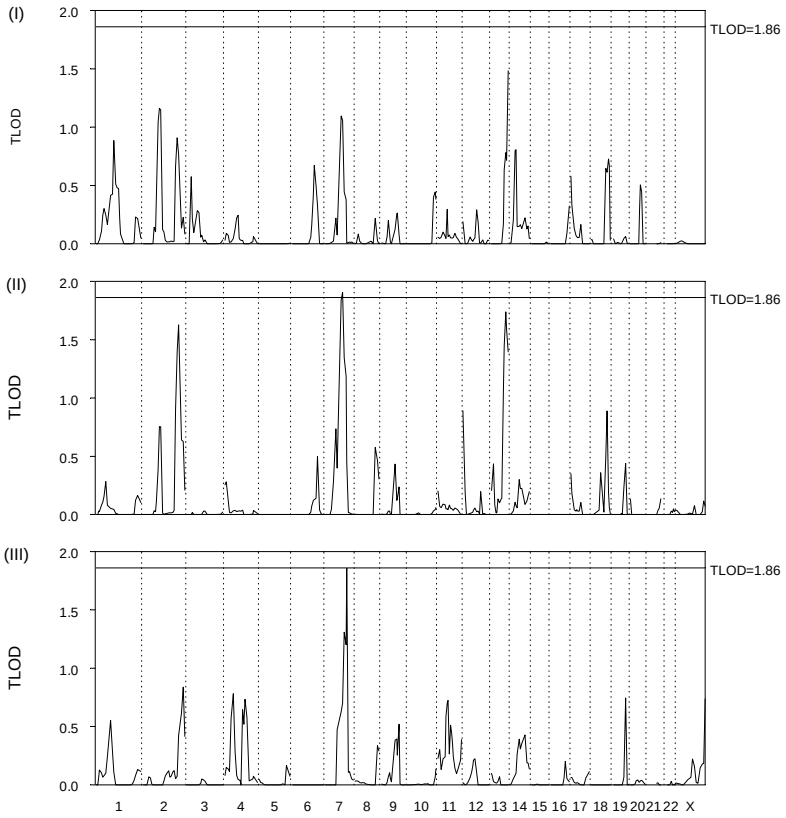

Figure 25. TLOD scores for all autosomes and chromosome X. Three different sets of diagnostic criteria (I-III) were used in the linkage analysis. The required threshold for suggestive linkage (1.86) is marked.

Discussion and perspectives

The foci of the work underlying this thesis lay at the interface between clinical practice and research. The main aims were to identify persons at risk of hereditary colorectal cancer, especially Lynch syndrome, to estimate the cancer risks associated with these predispositions, and to identify the underlying genetic causes.

The identification of persons at risk for hereditary cancer allows them and their families to attend counselling and surveillance programmes, thereby increasing the likelihood that any cancers they may develop will be detected early enough to allow them to be effectively treated. In **study I** we identified a number of high-risk families, which we have offered the possibility of genetic counselling and surveillance. The cancer risk estimates among first-degree relatives of probands with double primary colorectal or colorectal/endometrial tumours, obtained in **study I**, improve the counselling of these and other families with similar appearances. Of special importance are the findings that most of the cancer risk found among young probands with MSI tumours is due to Lynch syndrome, which requires special clinical handling with more intense surveillance programmes and that old onset in probands confer no overall cancer risk to relatives.

The identification of the disease-causing mutations in sixteen families in **study II** makes their Lynch syndrome diagnoses definite. Moreover, it enables presymtomatic testing of their relatives at risk, halving the number of persons who need to attend surveillance.

When we started investigating the *MSH6* families (**study IV**), the risks associated with these mutations were not well known. The findings that the *MSH6* mutations are associated with high life-time risks (especially of endometrial and ovarian cancers), despite the late age of onset, are important not only for counselling in these families, but also for identification and classification of Lynch syndrome families.

The unexpectedly large proportion of *MSH6* mutations found, in **study IV** shown to be due to founder effects, has implications for future mutation screening strategies for families from this geographical region. Studies of the population-based frequencies of the two *MSH6* mutations in unselected colorectal and endometrial cancer cases in the Skellefteå area would be useful, and screening for those two mutations in all newly diagnosed cases in the area may be justified.

In retrospect, given unlimited time and resources, I would have done some things differently. I would have analysed both tumours from all the probands not only for their MSI status, but also for their MMR gene expression (using IHC), for the *BRAF* V600M mutation and (where implicated by the previous analyses) for MMR gene mutations. If both tumours had been analysed, the possible misclassification of patients with one sporadic tumour among the hereditary tumours could have been avoided. For the MSI analysis, it would have been better to have used a panel of more mononucleotides and one or a few dinucleotides for quality control. Since mononucleotides are more sensitive for MSI, perhaps more MSI tumours would have

been detected using this approach. The use of IHC to detect *MLH1*, *MSH2*, *MSH6* and *PMS2* expression would have pinpointed the genes to screen, minimising the effort required to find mutations. Screening all MSI-positive tumours or all tumours lacking MLH1 expression, for the *BRAF* V600M mutation before further mutation screening would have excluded a large proportion of the sporadic tumours, thus further minimising the number of mutation screenings needed. To complement the mutation screening with *PMS2*, exon 1 of *MSH6* and regulatory regions of all four genes could have identified more mutations. Thus, the patients could have been classified more accurately for the risk analysis, the risk estimates in different subgroups would have been even more useful, and the mutation spectra perhaps widened.

When assessing the pathogenicity of the novel missense mutations, the use of functional assays would have been useful. When calculating cumulative risks on *MSH6* families, the possibility of including and mutation test all eligible relatives to the mutation-carrying probands would have given more accurate risk estimates.

The use of both MSI and IHC as prescreening tools is perhaps excessive, but at least the missense mutation in *MLH1* would have been missed if only IHC had been used. On the other hand, the two tumours that were initially misclassified would perhaps have been correctly identified from the start if IHC had been used initially instead of MSI analysis. IHC not only has the undisputable advantage of pinpointing the MMR gene of interest, but is also less labour intense than MSI. The debate about the relative merits of the possible methods for predicting MMR gene deficiencies will probably become more intense since recent studies have shown that chemotherapy responses and survival outcomes differ for tumours associated with various MMR gene anomalies compared to MSS tumours (128). Generally, tumours exhibiting MSI have a better overall prognosis than MSS tumours (55). MSI status is also a strong predictor of the benefit of adjuvant chemotherapy with fluorouracil in stage II and III colorectal cancer. Patients with sporadic MSI tumours do not benefit from chemotherapy and may even have worse prognosis than they would otherwise. It is currently not possible to conclude whether germline MMR gene mutation carriers might also show disadvantageous chemotherapy responses, but cell line work suggests that this might be the case (55).

Finally, the identification of a new chromosomal area linked to colorectal cancer in the large family 19 is very exciting, but warrants further studies. The large chromosomal region with suggested linkage on chromosome 7 needs to be fine-mapped. Candidate genes in the hopefully decreased area must be examined for possible disease-causing mutations and a plausible biological model for the contribution to tumorigenesis must be constructed. My hope is that eventually we will be able to offer this family more than surveillance, starting with the possibility of presymtomatic testing to restrict the surveillance solely to people at risk. My belief is that in the not-too-distant future, not only surveillance but also prevention and treatment will be tailored on basis of the genetic cause of the cancer.

Populärvetenskaplig sammanfattning

Colorektalcancer (tjock- och ändtarmscancer) är en av de allra vanligaste cancerformerna, med över en miljon nya fall i världen och 5000 i Sverige varje år. Det är också en cancerform som till en stor del beror på ärftliga faktorer. Det finns flera olika typer av **ärftlig colorektalcancer**, som ärvs på olika sätt och med olika stark genomslagskraft. Personer med ärftlig cancer får i allmänhet sin cancer tidigare och löper större risk att drabbas av mer än en tumör än personer med en sporadisk (icke-ärftlig) cancer.

Den vanligaste typen av ärftlig colorektalcancer är **Lynch syndrom** [också kallat Hereditär nonpolyposis colorektalcancer (HNPCC)], som står för upp till 3% av all colorektalcancer. Personer med Lynch syndrom löper risk inte bara för colorektalcancer utan också flera andra cancerformer, framförallt **livmodercancer** men också cancer i t.ex. äggstockar, magsäck, tunntarm, hjärna och hud. Det är viktigt att identifiera personer med Lynch syndrom, eftersom de då kan ges möjlighet att regelbundet kontrollera framförallt tarmen och livmodern för att en eventuell cancer ska upptäckas och opereras bort så tidigt som möjligt.

Lynch syndrom orsakas av en mutation i någon av de gener (*MLH1*, *MSH2*, *MSH6* och *PMS2*) som kodar för proteiner i ett DNA-reparationssystem. Normalt finns alla gener i två kopior, en från vardera föräldern. Lynch syndrom ärvs dominant, vilket innebär att det räcker med att ärva en skadad gen från den ena av sina föräldrar för att löpa risk att få sjukdomen. I cellerna är det däremot en recessiv sjukdom, vilket innebär att den normala genkopian som ärvts av den andra föräldern måste slås ut i en cell innan sjukdomen kan uppstå. När båda kopiorna är utslagna blir DNA-reparationssystemet ur funktion och mutationer ansamlas i cellerna, med en ökad cancerrisk som följd. Tumörer som uppkommit på grund av ett defekt DNA-reparationssystem visar en instabilitet i repetitiva DNA-sekvenser, en s.k. **mikrosatellitinstabilitet**.

Målen med detta avhandlingsarbete var att identifiera personer med hög risk för ärftlig colorektalcancer, framförallt Lynch syndrom, att bedöma risken för cancer hos deras släktingar, samt att försöka identifiera de bakomliggande genetiska orsakerna.

Med hjälp av det regionala cancerregistet vid Universitetssjukhuset i Umeå identifierades 78 personer (probander) i norra sjukvårdsregionen (Norrbotten, Västerbotten, Västernorrland och Jämtland) med dubbla tumörer; antingen två colorektalcancrar eller en colorektalcancer och en livmodercancer. Probanderna delades upp i undergrupper beroende på om den första tumören diagnosticerades före eller efter 50 års ålder samt om tumören var mikrosatellitinstabil eller inte. Därefter beräknades cancerriskerna hos probandernas förstagradssläktingar (föräldrar, syskon och barn) jämfört med en likadan grupp människor ur den allmänna befolkningen. Risken för cancer (framförallt colorektal- och livmodercancer) hos släktingarna visade sig vara störst om probanden fått sin diagnos före 50 års ålder och tumören var mikrosatellitinstabil, båda tecken på en ärftlighet. Om probanden fått sin diagnos efter 50 års ålder och med mikrosatellitstabil tumör sågs inga överrisker alls hos

släktingarna, vilket är viktigt att veta vid genetisk vägledning.

Nästa steg var att leta efter förändringar i DNA-reparationsgenerna hos probanderna med mikrosatellitinstabila tumörer, dvs. hos de personer som löper störst risk att ha Lynch syndrom. Hos tio personer hittades förändringar som förutsågs förstöra proteinet (mutationer). Hos fem personer hittades genetiska förändringar av en typ som inte alltid förstör proteinerna. För att försöka avgöra om dessa förändringar ändå kunde vara orsaken till cancerförekomsten undersöktes tumörer från flera familjemedlemmar i dessa familjer. Då det visade sig att tumörerna var av Lynch syndromtyp, samt att de familjemedlemmar som fått cancer alla hade den genetiska förändringen bedömdes även dessa genetiska förändringar vara sjukdomsframkallande mutationer.

Alla familjer där mutationer hittats erbjöds genetisk vägledning vid Cancergenetiska mottagningen vid Klinisk Genetik och Onkologiska kliniken vid Umeå Universitetssjukhus, samt möjligheten till regelbundna undersökningar. Friska familjemedlemmar erbjöds att testa om de bar på mutationen, för att veta om de behöver delta i kontrollprogrammen eller inte.

Fem familjer hade samma mutation, i den gen som heter *MSH6*. Släktforskning visade att alla fem familjerna härstammar från samma par i Skellefteåtrakten på tidigt 1700-tal. Den typ av Lynch syndrom som orsakas av *MSH6*-genen undersöktes och visade sig vara annorlunda jämfört med den vanligare typ som orsakas av *MLH1*- och *MSH2*-generna. I familjerna med *MSH6*-mutationer fick man i allmänhet cancer vid högre ålder samt hade en större risk för livmoder- och äggstockscancer.

Den sista studien gjordes på en annan stor, norrländsk familj med ärftlig colorektalcancer av en annan, okänd, typ. För att ta reda på den genetiska bakgrunden till cancern i denna familj gjordes en studie där genetiska markörer över hela genomet (den totala arvsmassan) undersöktes hos många familjemedlemmar, för att se om någon kromosomdel följer sjukdomen, dvs. finns bara hos de drabbade och inte hos de friska individerna. Ett område på kromosom 7 bedömdes vara kopplat till sjukdomen och kommer att undersökas närmare för att om möjligt hitta den gen som orsakar den ärftliga colorektalcancern i denna familj.

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