Tumors associated with Hereditary Nonpolyposis Colorectal Cancer: Defective Mismatch Repair and Familial Risk of Cancer

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2005

Tumors Associated with Hereditary Nonpolyposis Colorectal Cancer

Defective Mismatch Repair and Familial Risk of Cancer

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Department of Oncology, Lund University, 2005

Doctoral Dissertation

by due permission of
the Faculty of Medicine,
Lund University, Sweden.
To be defended in Segerfalksalen,
Wallenberg Neurocentrum, Lund
at 9.00 am, Friday February 11, 2005.

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Organization
LUND UNIVERSITY

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Document name
DOCTORAL DISSERTATION

Date of issue
February 11th, 2005

Sponsoring organization

Author(s)
Kajsa Ericson

Title and subtitle

Tumors Associated with HNPCC: Defective Mismatch Repair and Familial Risk of Cancer

Abstract

Inactivation of the DNA mismatch repair (MMR) system is a tumorigenic mechanism involved in 15–20% of tumor types such as colorectal and endometrial cancer and is specifically associated with the Hereditary Nonpolyposis Colorectal Cancer (HNPCC) syndrome. These MMR defective tumors are characterized by microsatellite instability (MSI), a phenomenon that reflects alterations in length of repeated sequences, and 90% of MSI tumors show loss of immunohistochemical expression for the MMR protein affected. HNPCC yields an increased risk for several tumor types; cancer of the colorectum (80–90% lifetime risk), endometrium (40–60%), ovary (10–15%), stomach (10–20%), urinary tract, small bowel, skin, and brain. The syndrome is characterized by an early age (mean 45 years) at diagnosis and one third of the patients develop metachronous tumors.

The major aims of this thesis were to assess the contribution of defective MMR to the development of the more rare tumor types associated with HNPCC and to assess cancer risks in children whose parents had developed HNPCC-associated tumors. In study I, patients who developed multiple (at least 4) primary tumors, including two colorectal cancers, were assessed for MSI and immunohistochemical expression of the MMR proteins MLH1 and MSH2. MSI was identified in 63/154 (41%) tumors, 55 of which also showed immunohistochemical loss of MMR protein expression. A concordant finding of MSI and loss of the same MMR protein, which strongly suggest HNPCC, was found in 17/45 (38%) patients, which suggests that a high fraction of such multiple tumors are caused by HNPCC.

In studies II and III, the frequency of defective MMR was studied in adenocarcinomas of the small intestine and in upper urinary tract cancers (UUC). MSI was detected in 16/89 (18%) of cancers of the small intestine
and in 9/194 (5%) UUC. MMR protein expression loss affected 11 cancers of the small intestine and 11 UUC.
Malignant fibrous histiocytoma (MFH) represent one of the largest subsets of soft tissue sarcomas, and occasional MFHs have been described in HNPCC-families. In study IV, we assessed MMR expression in a series of 209 MFH and found loss of MSH2 and MSH6 in 2 MFH.

Study V is based on the national Swedish cancer registry and analyzed familial risk of HNPCC-associated tumors. Cancer risks were calculated in 204 358 offspring whose 102 814 parents had developed HNPCC-associated cancer and the risks were correlated to the age of the parent, metachronous tumors in the parent, and presence of several HNPCC-associated cancers in the family. Significantly increased risks were observed for several tumor types, including colon cancer, rectal cancer, endometrial cancer, gastric cancer, and ovarian cancer. The highest offspring risks were observed in the subgroup with multiple HNPCC-associated cancers in the parent.

In summary, we have demonstrated that MMR defects are common in patients who develop multiple primary tumors, occur at similar frequencies in cancers of the small intestine and the colon, contribute to development of UUC and MFH at low frequencies, and that HNPCC-associated tumor in a parent confer an increased risk of several cancer types in the offspring, especially if the parent developed more than one cancer or cancer at a young age.

Key words: Hereditary Nonpolyposis Colorectal Cancer (HNPCC); Multiple Tumors; Colorectal Cancer; Cancer of the Small Intestine; Cancer of the Upper Urinary Tract; Soft Tissue Sarcomas; Microsatellite Instability; MLH1, MSH2, MSH6; Immunohistochemistry; Familial Risk of Cancer; Population-based; Cohort study.

Classification system and/or index termes (if any):

Supplementary bibliographical information:

Language
English

ISSN and key title:

ISBN
91-628-6379-7

Recipient's notes

Number of pages

Price

Security classification

Tumors Associated with Hereditary Nonpolyposis Colorectal Cancer

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ISBN: 91-628-6379-7
Layout by Ortonova AB, Lund
Printed by Mediatryck
Sweden 2005

Contents

Original studies

This thesis is based on the following papers.

I. Ericson K, Halvarsson B, Nagel J, Rambech E, Planck M, Piotrowska Z, Olsson H, Nilbert M. Defective mismatch-repair in patients with multiple primary tumours including colorectal cancer. European Journal of Cancer 2003; 39(2): 240-8.

II. Planck M, Ericson K, Piotrowska Z, Halvarsson B, Rambech E, Nilbert M. Microsatellite instability and expression of MLH1 and MSH2 in carcinomas of the small intestine. Cancer 2003; 97(6): 1551-7.

III. Ericson K, Isinger A, Isfoss B, Nilbert M. Low frequency of defective mismatch repair in a population-based series of upper urothelial carcinoma. 2005, submitted.

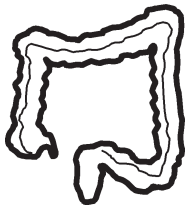
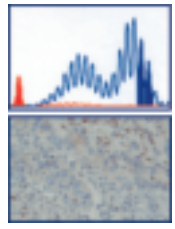
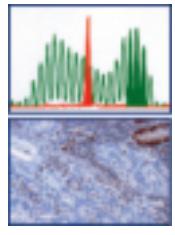
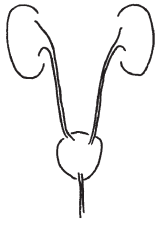
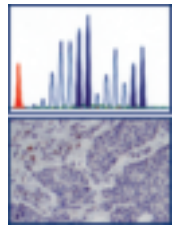
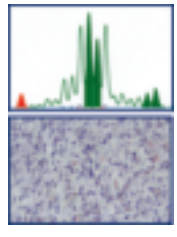
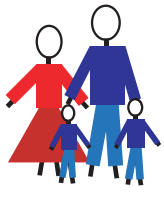
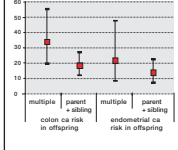
IV. Ericson K, Engellau J, Persson A, Lindblom A, Domanski H, Åkerman M, Nilbert M. Immunohistochemical loss of the DNA mismatch repair proteins MSH2 and MSH6 in malignant fibrous histiocytomas. Sarcoma 2004: 8(4): 123-7.

V. Ericson K, Nilbert M, Bladström A, Anderson H, Olsson H, Planck M. Familial risk of tumors associated with hereditary non-polyposis colorectal cancer: A Swedish population-based study. Scandinavian Journal of Gastroenterology 2004; 12: 1259-65.

Abbreviations

AFAP	attenuated familial adenomatous polyposis
APC	adenomatous polyposis coli
BER	base excision repair
Bp	base pair
BRCA1/2	breast cancer gene 1/2
CI	confidence interval
CRC	colorectal cancer
DSB	double strand break repair
FAP	familial adenomatous polyposis
HNPCC	hereditary nonpolyposis colorectal cancer
H&E	hematoxylin and erythrosine
ICD	international classification of diseases
LOH	loss of heterozygosity
MFH	malignant fibrous histiocytoma
MLH1/3	human MutL homologue 1/3
MMR	mismatch repair
MSH2-6	human MutS homologue 2-6
MSI	microsatellite instability
MSS	microsatellite stability
MutH/L/S	mutator H/L/S
MYH	MutY homologue
NER	nucleotide excision repair
PCR	polymerase chain reaction
PMS1/2	human post meiotic segregation increased 1/2
SIR	standardized incidence ratio
TMA	tissue microarray
UUC	upper urinary cancer

Thesis at a glance

	Question		Method		Results	Conclusion
I	Is defective MMR found among patients with multiple primary tumors?		MSI and MMR protein immunostaining in 156 tumors from 45 patients.		MSI in 41% of the tumors, MMR protein expression loss in 37%. Concordant MMR protein loss in 17/45 patients.	2/5 of the patients with multiple primary tumors, including colorectal cancer, may represent HNPCC-patients.
II	How frequent is defective MMR in cancer of the small intestine?		MSI and MMR protein immunostaining in 89 tumors.		MSI in 18% of the tumors, MMR protein loss in 13%.	Cancer of the small intestine display defective MMR at a frequency similar to colon cancer.
III	How frequent is defective MMR in cancer of the upper urinary tract?		MSI and MMR protein immunostaining in 216 tumors.		MMR deficiency in 5% of the cases.	A low fraction of cancer of the upper urinary tract develop due to defective MMR.
IV	Can defective MMR be found in pleomorphic soft tissue sarcomas?		MMR protein immunostaining in 209 malignant fibrous histiocytomas. MSI assesed in cases with MMR protein loss.		Two tumors showed loss of MSH2/MSH6.	Occasional sarcomas show defective MMR. Sarcoma may be a rare tumor type associated with HNPCC.
V	Cancer risks among relatives of patients with HNPCC-associated cancer?		102 000 parents and 204 000 children identified in a population-based register study. Relative risks in different cohorts calculated.		Children had increased risks for cancer of the colon, rectum, stomach and ovaries. Higher risks were seen if the parent was diagnosed with multiple primary tumors or at a young age.	Increased risks were seen for several types of cancer. Multiple HNPCC-associated tumors in parent, or early age at diagnosis confer the highest risks.

Populärvetenskaplig sammanfattning

Defekt mismatch-reparation och familjär cancerrisk

Cancer är på cellnivå en genetisk sjukdom. Förändringar i cellens arvsmassa (DNA) drabbar tre huvudtyper av gener; onkgener, tumörsuppressorgener och DNA-reparationsgener. Onkgener är en grupp gener som befrämjar cellens tillväxt. Vid cancerutveckling aktiveras onkgener via mutation eller genom ökat uttryck, vilket leder till ökad tillväxt. Tumörsuppressorgener hämmar normalt celldelning, bromsar cellcykeln, styr defekta celler till programmerad celldöd och verkar för stabilitet i genomet. När båda kopiorna av en tumörsuppressorgen inaktiveras förloras dess funktion, vilket befrämjar tumörbildning. Normalt finns alla gener i dubbel uppsättning, en kopia från vardera föräldern. Vid ärftlig cancer finns den första erforderliga DNA-förändringen (mutationen) i kroppens alla celler (konstitutionell mutation), medan icke-ärftlig (sporadisk) cancer uppkommer genom förvärvade (somatiska) mutationer av båda kopiorna. DNA-reparationsgener motverkar att förändringar i arvsmassan uppstår genom att ta hand om spontana mutationer vid celldelning via cellens olika DNA-reparationssystem.

Defekt DNA-reparation är en tumörbiologisk mekanism som styr utvecklingen i flera vanliga tumörtyper, bl. a. i en andel av tjocktarmscancer och livmodercancer. Dessutom karakteriserar defekt DNA-reparation av typen mismatch repair (MMR) specifikt de tumörer som uppkommer genom det ärftliga syndromet hereditär nonpolyposis colorektal cancer (HNPCC). I MMR-systemet samverkar sex olika proteiner och vid HNPCC är någon av MMR-generna *MLH1, MSH2, MSH6* eller *PMS2* defekta. Personer som bär en av mutation i någon av dessa gener löper ca 90% risk att drabbas av cancer, vanligast i tjock-/ändtarm och livmoder, men även i njurbäcken/urinledare, tunntarm eller äggstockar. Tumörer som uppkommit via defekt MMR karakteriseras av så kallad mikrosatellitinstabilitet (MSI) och immunhistokemisk förlust av det defekta MMR-proteinets uttryck.

Denna avhandlings fyra första delarbeten innefattar tumörbiologiska studier i vilka vi har undersökt förekomst av defekt MMR i olika tumörtyper associerade med HNPCC.

I arbete I studerades individer med fyra primära (separat uppkomna) tumörer varav minst två i tjocktarm eller ändtarm. Defekt MMR förekom i 41% av tumörerna. Hos 17/45 patienter (38%) var samma MMR protein förlorat i flera tumörer. Studien visade en ökad frekvens MMR-defekter vid multipla tumörer och HNPCC är en diagnos som bör övervägas hos patienter som drabbas av flera tumörer.

I arbete II undersöktes defekt MMR i tunntarmscancer, vilket förekom i 18% av fallen. MMR defekter var något vanligare bland de unga patienterna. Fynden visar att MMR-defekter i tunntarmscancer är ungefär lika vanliga som i tjocktarmscancer.

I arbete III studerades cancer i de övre urinvägarna, dvs. i njurbäcken och urinledare. MSI och/eller immunhistokemisk MMR-proteinförlust påvisades i cirka 5% av fallen. Trots en ökad risk för cancer i urinvägarna hos individer med HNPCC indikerar studien att endast en liten andel av tumörer i de övre urinvägarna uppkommer via defekt MMR.

I arbete IV påvisades att defekt MMR också kan förekomma i sarkom, en ovanlig tumörform som uppkommer i kroppens stödjevävnader. Denna tumörtyp är normalt inte associerad med ärftlighet, men en koppling mellan sarkom och HNPCC finns sannolikt.

I Sverige sker en rapportering av alla cancerdiagnoser till ett nationellt cancerregister. Det finns även ett familjeregister där individers barn kan identifieras. Genom att länka cancerregister och familjeregister till varandra kan cancerrisker

hos barn till föräldrar med cancer räknas ut. Delarbete V är en epidemiologisk studie (en studie av samband och riskfaktorer i en befolkning) där vi beräknat cancerrisker hos individer vars föräldrar drabbats av de cancertyper som är vanligast vid HNPCC. Analyserna visade att barn till personer med någon HNPCC-associerad diagnos löper en förhöjd risk att själva drabbas av cancer, framför allt i samma organ som föräldern. Störst cancerrisk har de barn vars föräldrar fått sin tumör före 50 års ålder, barn som har både en förälder och ett syskon med cancer och barn vars förälder utvecklat flera primära tumörer. I studien påvisades ett starkt samband mellan canceruppkomst och HNPCC-associerad tumör hos en förälder och resultaten bekräftar vikten av att ta hänsyn till förekomst av flera olika tumörtyper när HNPCC övervägs som diagnos. Studien visade även att flera primära tumörer hos en individ är en stark riskfaktor för ärftlighet, jämförbar med tumörförekomst hos flera individer i familjen.

Sammanfattningsvis har vi i arbetena I–V

- påvisat defekt MMR i hög frekvens hos individer som utvecklat flera primära cancrar,
- visat att defekt MMR förekommer i samma frekvens i tunntarmscancer som i tjocktarmscancer,
- funnit låg andel defekt MMR i cancer i de övre urinvägarna,
- visat att defekt MMR förekommer i mjukdelssarkom samt
- beräknat cancerrisker hos individer vars föräldrar utvecklat HNPCC-relaterad cancer.

Background and Aims

In 1993, microsatellite instability (MSI) was observed in tumors from patients with a family history of colorectal cancer (Aaltonen *et al.* 1993; Aaltonen *et al.* 1994; Parsons *et al.* 1993; Perucho 1996). In parallel, studies of mutated DNA mismatch repair (MMR) genes in yeast revealed increased genetic instability (Strand *et al.* 1993). These combined findings lead to the identification of several human MMR genes and the discovery of their role in cancer development (Modrich 1994). MMR defects represent a tumorigenic mechanism in a subset of e.g. colon cancer, gastric cancer, and endometrial cancer and germline MMR gene mutations cause the familial cancer syndrome Hereditary Nonpolyposis Colorectal Cancer (HNPCC) (Lynch *et al.* 1993). This thesis studies the contribution of defective MMR in different tumor types associated with HNPCC and analyzes familial risks of HNPCC-associated cancer. The aims of the different studies were to:

- evaluate the role of defective MMR in patients who develop multiple primary tumors,
- characterize the contribution of defective MMR in rare HNPCC-associated tumor types, i.e. cancer of the small intestine and the upper urinary tract,
- investigate whether soft tissue sarcoma can develop due to defective MMR, and
- calculate cancer risks among children to patients who have developed tumor types associated with HNPCC.

Tumor biology

Cancer affects 1/3 individuals in the western world and is the second most common cause of death in Sweden (25% of all deaths in Sweden and 12% worldwide) (www.socialstyrelsen.se, www.who.org). Cancer cells acquire capabilities that make the cells independent and self-sufficient in several ways; production of growth signals, insensitivity to anti-growth signals, escape from apoptosis, increased growth potential, sustained angiogenesis, invasion of surrounding tissue, and seeding of metastases (Hanahan and Weinberg 2000). At the cellular level, cancer has been characterized as a genetic trait, with dozens of genes altered and thousands of genes showing an aberrant expression profile. The majority of altered genes are somatically mutated, but some are mutated in the germline and confer an increased risk of cancer in carriers. Genes involved in tumorigenesis are divided into three main categories, which are presented below.

Oncogenes

Oncogenes are altered or activated versions of protooncogenes, genes that normally promote cell proliferation (Strachan and Read 2004). These changes are usually dominant and can be quantitative or qualitative, involving increased production or normal production of an altered gene product. The existence of oncogenes was suggested in the 1960s through studies of viral factors in animal leukemias and lymphomas (Bishop 1983; Stehelin *et al.* 1976). Currently, there are over 100 oncogenes identified, and these can be divided into different classes, e.g., secreted growth factors (e.g. *EGF*), cell surface receptors (e.g. the *ERBB2* family), components of intracellular signal transduction systems (e.g. *RAS*), DNA-binding nuclear proteins (e.g. *MYC*), and genes in the network cascades that govern progress through the cell cycle (e.g. cyclins and cyclin-dependant kinases) (Balmain *et al.* 2003; Vogelstein and Kinzler 1998).

Tumor suppressor genes

In contrast to oncogenes, tumor suppressor genes function as negative regulators of cell proliferation and growth, e.g. through prevention of cell cycle

progression (e.g. cyclin-dependant kinase inhibitors), initiation of apoptosis (e.g. *BAX*), guarding of genomic stability, suppression of angiogenesis, and stimulation of cell differentiation. Mutations in tumor suppressor genes typically act in a recessive manner and inactivation of both copies is usually required for tumor development (Sherr 2004; Stanbridge 1990). Mutant tumor suppressor genes often cause inherited cancer syndromes with one mutant copy being inherited from either parent and the second being somatically inactivated, according to the "Knudson-two-hit-hypothesis" (Knudson 1971, 2001). Knudson characterized the first tumor suppressor gene, the Retinoblastoma gene *(RB1),* through studies of familial clustering of retinoblastoma and the gene was later characterized to encode a key cell cycle protein (Hansen and Cavenee 1988).

DNA repair

Defects in DNA repair have gradually been found to be important in tumor development and the four major DNA repair systems, which through different mechanisms guard genetic stability, have been associated with cancer development (Ljungman and Lane 2004; Tuteja and Tuteja 2001).

Base excision repair (figure 1a)

The base excision repair (BER) system repairs the most common type of DNA damage in the cell; single nucleotide changes that are mainly caused by endogenous oxidative and hydrolytic decay of DNA. BER uses glycosidase enzymes to remove abnormal bases, whereafter an endonuclease and a phosphodiesterase cut the sugar-phosphate backbone and remove the sugar-phosphate residue. The gap is filled with new nucleotides by a DNA polymerase and is sealed by DNA ligase (Fortini *et al.* 2003; Tuteja and Tuteja 2001). Biallelic inactivation of the *MYH* gene provides an example of the role of BER in cancer development and has been demonstrated in patients with multiple colorectal adenomas (Jones *et al.* 2002; Sieber *et al.* 2003). *MYH* maps to 1p32-34 and functions in the scanning of the daughter strand after replication, and thereby removes adenosine residues mispaired with either guanosine or oxidatively damaged nucleotides (Chow *et al.* 2004; Fortini *et al.* 2003; Lu *et al.* 2001).

Nucleotide excision repair (figure 1b)

The nucleotide excision repair (NER) system removes bulky lesions that are induced by UV-light or chemicals. In *E. Coli* the three polypeptides UvrA, UvrB and UvrC can locate and remove DNA damage. In humans, the NER system involves six proteins (Rpa, Xpa, Xpc, TFIIH, Xph and Xpf-ERCCI) that remove a large patch around the damage, even if the defect comprises only a single nucleotide (Batty and Wood 2000; Sancar *et al.* 2004). Defective NER is associated with Xeroderma pigmentosum, a recessively inherited syndrome in which the cells fail to properly eliminate UV-induced DNA lesions. This leads to ultraviolet induced skin neoplasms. NER capacity has also been linked to the development of lung cancer (Park *et al.* 2002; Shen *et al.* 2003) and breast cancer (Ramos *et al.* 2004; Shi *et al.* 2004).

Double strand break repair (figure 1c)

DNA double strand breaks (DSBs) result from two opposite nicks in the DNA double helix. Such DSBs can be induced by exogenous agents such as ionizing radiation and topoisomerase inhibitors, or by endogenous factors such as free radicals (Karagiannis and El-Osta 2004). Defective DSB repair may lead to carcinogenesis by causing deletion of genomic segments or aberrant joining of broken chromosomal ends. The DSB repair functions through a signaling pathway that involves detection of the DSB by the sensor proteins ATM and ATR and two complexes formed by Rad9, Rad1, Hus1, and Rad17-RFC (Sancar *et al.* 2004). These sensor proteins activate transduction cascades in response to DSBs. The Mre11/Rad50/Nbs1-complex processes the DNA termini of the DSB, before initation of strand invasion by Rad51. ATM functions both as a sensor and a transducer and in response to DSBs, it binds directly to DNA termini and phosphorylates several proteins including CHK2, p53, and BRCA1. ATM sensed DSBs are

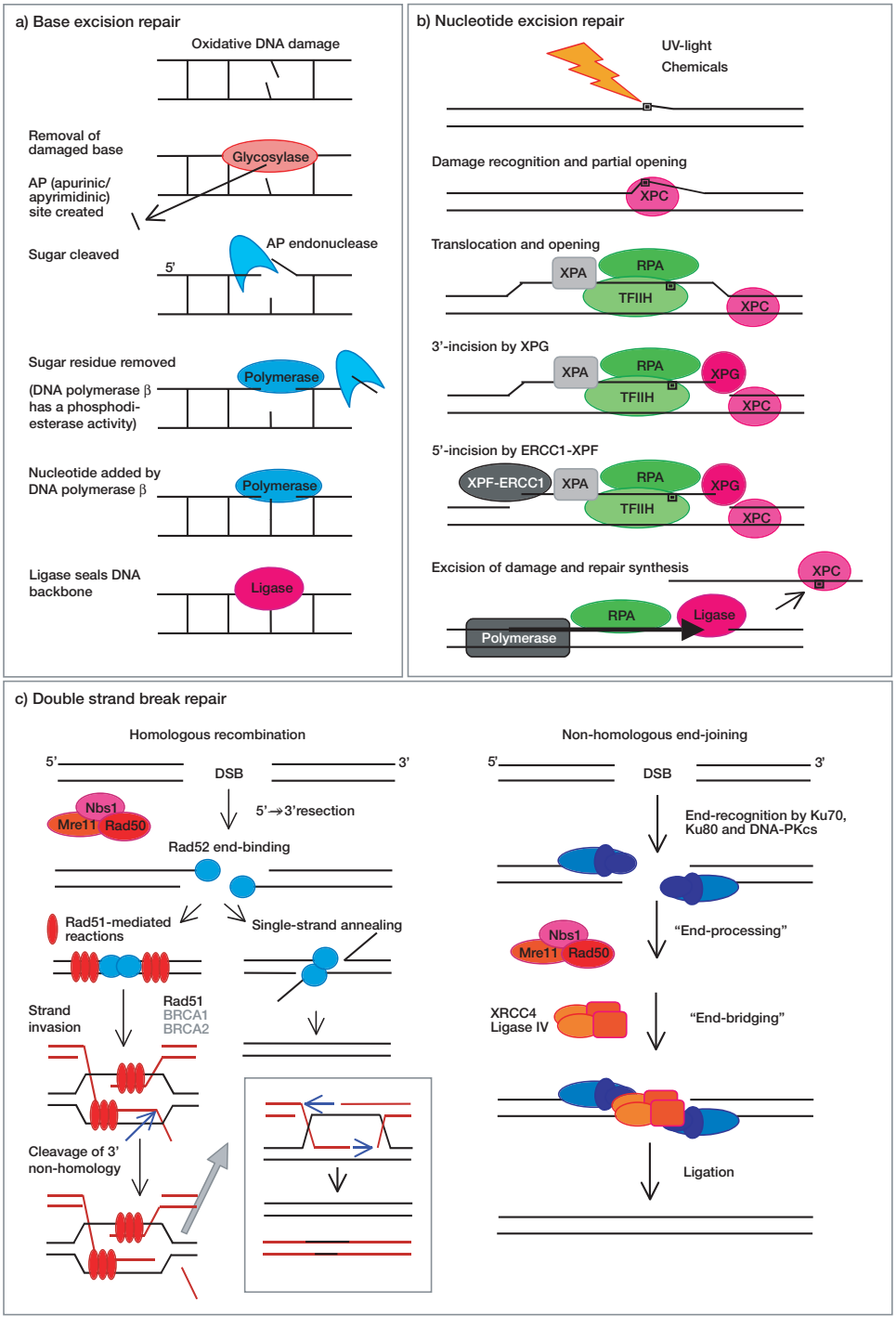


Figure 1. Schematic view over mechanisms in base excision repair (a), nucleotide excision repair (b), and double strand break repair (c)

transduced by CHK2, which phosphorylates either p53 or BRCA1 leading to apoptosis or to DNA repair (Bartek and Lukas 2003; Karagiannis and El-Osta 2004; Sancar *et al.* 2004). DBS in mammalian cells involves two complementary pathways, homologous recombination and non-homologous end-joining. Homologous recombination relies on sequence homology and copies the missing information from the undamaged homologous chromosome (Karagiannis and El-Osta 2004; Sancar *et al.* 2004). Non-homologous end-joining does not require undamaged DNA molecules, although the damaged DNA ends are processed to become more compatible for ligation, which usually results in loss of a few nucleotides at each end. This is therefore an error-prone DNA-repair system, and it is the dominating DSB repair in higher eukaryotes (Karagiannis and El-Osta 2004). The two main genes causing hereditary breast and ovarian cancer, *BRCA1* and *BRCA2,* are involved in DSB signaling pathways via homologous recombination (Nkondjock and Ghadirian 2004) as is also *CHK2,* which has been found to be mutated in a subset of families with hereditary breast and colon cancer (Dong *et al.* 2003; Meijers-Heijboer *et al.* 2003).

Mismatch repair (figure 2)

The MMR system recognizes and repairs errors of DNA replication, i.e. single mismatched base pairs and nucleotide insertions/deletions, due to DNA polymerase slippage during recombination (Sancar *et al.* 2004). The MMR proteins MutH, MutL, and MutS were initially identified in *E. Coli* (Kolodner 1995; Modrich 1994). The MutS protein binds to the mispaired base and functions as a regional lesion sensor. It has an ATPase activity that is activated by exposure to DNA ends, and the mismatch-provoked ADP-ATP exchange is a determining factor in MutS recognition specificity (Acharya *et al.* 2003). Thus, interaction of MutS with mismatched DNA provokes releases of bound ADP, consequent binding of ATP, dissociation of the mismatch and initiation of the MutS ATPase cycle. MutS does not significantly interact with MutL although they form a sliding clamp together (Acharya *et al.* 2003) (figure 2 a). The MutL protein can associate with the ATP-bound MutS sliding clamp, can remove unused sliding clamps, and is required for the activation of MutH endonuclease activity (Acharya *et al.* 2003). ATP-bound MutS is hydrolysis-independent and can diffuse 1 kb along the DNA adjacent to the mismatch. The diffusion of the sliding MutS/MutL clamp exposes the mismatch again and enables multiple clamps to form. Since MutS is activated by exposure to DNA ends, this could theoretically initiate MMR at different sites and MMR is therefore initiated by multiple localized ATP-bound MutS/MutL sliding clamps (Acharya *et al.* 1996). Binding of the MutS/MutL sliding clamp to MutH triggers ATP binding to MutL, which subsequently enhances the endonuclease activity of MutH. The process that follows involves strand excision, resynthesis, and ligation and requires helicases, exonucleases, polymerases, and ligases (Buermeyer *et al.* 1999).

In eukaryotes, the MMR system resembles the bacterial MutHLS system and several human homologues to the bacterial MutL and MutS gene products have been identified. There are three MutS homologues; MSH2, MSH3, and MSH6, and the recognition of mispaired bases is made by two MutS-related protein complexes; the MutSα complex (MSH2-MSH6) responsible for base: base mismatches and single base insertion/deletion mispairs and the MutSβ (MSH2-MSH3) responsible for the repair of larger (2-4 bp) insertion/deletion mispairs (figure 2 b) (Buermeyer *et al.* 1999; Edelmann and Edelmann 2004). There are two additional MutS homologues known in eukaryotes, MSH4 and MSH5, which have been demonstrated to be involved in meiotic recombination (Snowden *et al.* 2004). The MutSα and MutSβ complexes require interaction with MutL homologues for activation of repair events. Several human MutL homologues have been described; MLH1, PMS1, PMS2, and MLH3. These also interact in complexes; MutLα (MLH1-PMS2), MutLβ (MLH1-PMS1), and MutLγ (MLH1-MLH3) (Buermeyer *et al.* 1999; Edelmann and Edelmann 2004). The MutLα complex interacts with the other two MutL complexes, but no specific function has so far been described for MutLβ and MutLγ (Edelmann and

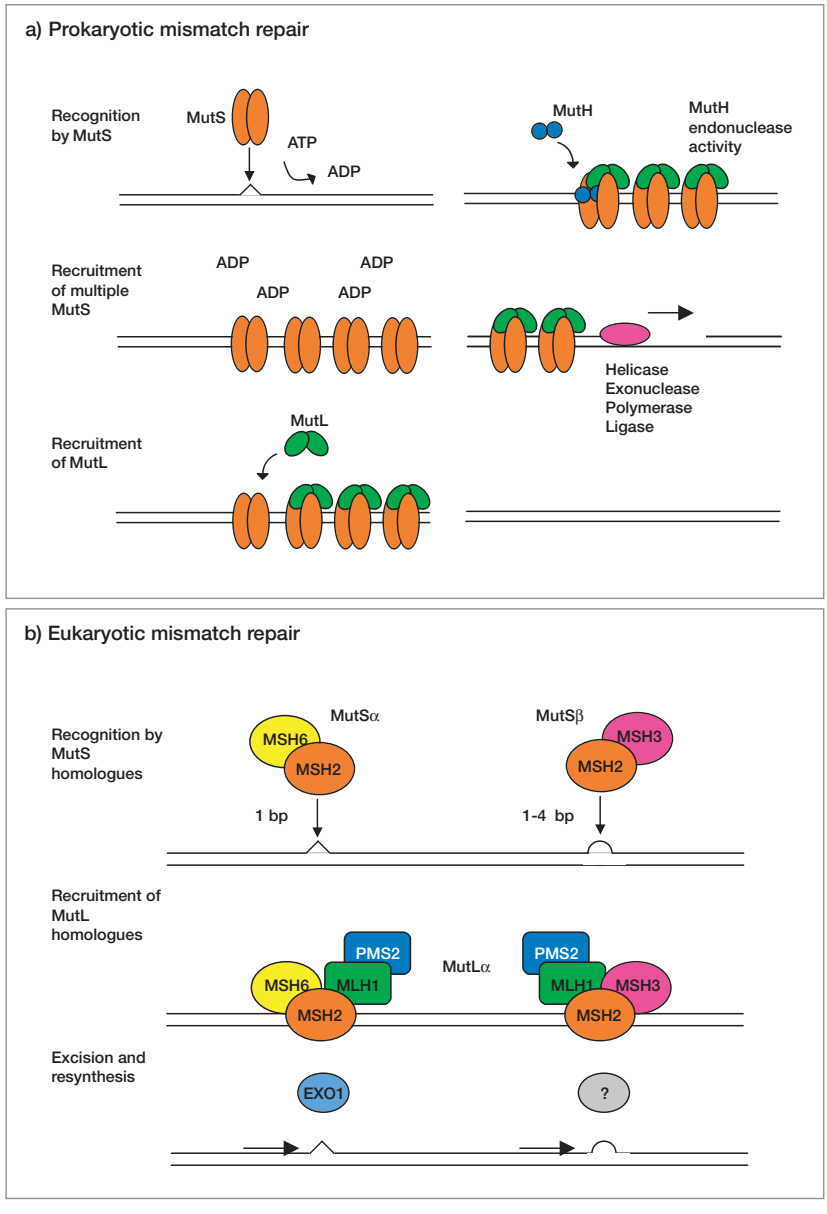


Figure 2. The mismatch repair system in prokaryotes and eukaryotes

Edelmann 2004). The mechanisms underlying the further MMR steps include excision and resynthesis of the DNA strand carrying the mismatch, but are less well understood. However, interaction with two different proteins have been demonstrated; EXO1 is a 5'-3' exonuclease that binds to MSH2, MSH3, and MLH1 and the proliferating cellular nuclear antigen (PCNA) binds to MLH1, MSH3, and MSH6 (Surtees and Alani 2004). Other functions of these proteins such as response to DNA damage, signaling pathway regulation, cell proliferation, and apoptosis are poorly understood.

In cells with defective MMR, DNA replication errors, such as single mismatched base pairs and insertions/deletions of nucleotides accumulate, which results in MSI. Microsatellites consist of 1–4 nucleotides long tandem repeats, and are mostly located in non-coding regions throughout the genome, but also appear within coding regions (Ionov *et al.* 1993; Lengauer *et al.* 1998; Thibodeau *et al.* 1993). There are as many as 50 000 to 100 000 microsatellites, with (A/T)n and (CA/GT)n being the most common repetitive elements. Microsatellites are frequently used for linkage analysis since they normally exhibit length polymorphisms. MSI is characterized by altered length of the repetitive sequences and is classified as either high or low, depending on the extent of instability. Nearly all HNPCC-associated tumors display a MSI-high phenotype, although MSI-low tumors have been described in patients with germline *MSH6* mutations (Wu *et al.* 1999). However, the existence of MSI-low tumors is debated (Pawlik *et al.* 2004), and, according to Tomlinson *et al.* (Tomlinson *et al.* 2002), a majority of MSI-low cancers are biologically similar to MSS tumors. This is opposed in studies of gene expression using cDNA microarray technique, where distinct global molecular pheonotypes are found to distinguish MSI-low tumors from MSI-high and MSS tumors (Mori *et al.* 2003).

Defective MMR results in a 100 to 1000-fold increased mutation rate, which leads to a "mutator" or "replication-error" phenotype (Parsons *et al.* 1993; Strand *et al.* 1993) (Loeb 2001; Markowitz *et al.* 1995; Perucho 1996) with an accumulation of mutations in genes with tandem nucleotide repeats. This pathway may explain how loss of tumor suppressor function or gain of oncogenic potential occurs in the MSI tumors.

Hereditary colorectal cancer

Since Knudson's pioneer work on hereditary retinoblastoma (Knudson 1971), our knowledge about mechanisms of cancer susceptibility has increased dramatically and several common cancer types (e.g. of the colorectum, endometrium, breast, and ovary) have now been linked to genetic defects that yield increased risks, most often through autosomal dominant inheritance, for example *BRCA1* and *BRCA2* mutations in breast and ovarian cancer (Miki *et al.* 1994; Wooster *et al.* 1995). However, in the majority of families with suspected hereditary cancer, the genetic cause has not been understood. Some of these cases may be explained by polygenic or recessive mechanisms or by low-penetrant traits (Antoniou *et al.* 2001; Nwosu *et al.* 2001).

Large twin studies have observed significant effects of heritable factors in about 35% of all colon cancers (Lichtenstein *et al.* 2000). Colon cancer in a first-degree relative has been estimated to confer a 2–3 fold increased risk for the disease, whereas the presence of two first-degree relatives with colon cancer or, alternatively, colon cancer at younger age (<50) in one first-degree relative, has been estimated to confer a 3–4 fold increased risk for colon cancer (Burt 2000). Most data on familial colorectal cancer risks derive from case-control studies, although population-based cohort studies provide more unselected and accurate information (Johns and Houlston 2001).

Development of multiple tumors is a hallmark of hereditary cancer, and can be exemplified by the multiple colorectal adenomas and the increased risks of periampullary carcinomas, thyroid cancer, and brain tumors in FAP, and by the development of leukemias, sarcomas, brain tumors, and breast cancer in the Li-Fraumeni syndrome (Cruz-Correa and Giardiello 2003; Li *et al.* 1991). About 1–3% of the patients with sporadic colorectal cancer develop other primary tumors (Chiang *et al.* 2004; Wang *et al.* 2004). Others studies estimate that the cumulative incidence of a second primary colorectal cancer is 1.5% at 5 years, with a higher risk for patients who developed their first colorectal cancer at young age (Cali *et al.* 1993; Hemminki *et al.* 2001). Furthermore, an increased risk of colorectal cancer has been detected also among individuals whose mother has developed other HNPCC-associated tumor types such as endometrial cancer and cancer of the small intestine (Hemminki and Vaittinen 1999). Double primary tumors of the endo-

metrium and the colon have been implicated as an indicator of susceptibility of HNPCC (Cederquist *et al.* 2004; Planck *et al.* 2002).

Hereditary nonpolyposis colorectal cancer

History

The history of HNPCC, or Lynch Syndrome, goes back to 1895, and to the pathologist Aldred Warthin at the University of Michigan School of Medicine, whose seamstress was afraid of dying from cancer since many of her relatives had. She did indeed die from endometrial cancer at a young age, and Warthin published the pedigree in 1913 (Warthin 1913). This original family, known as Family G (figure 3), has served as a prototype of HNPCC, and was updated in 1971 by Lynch and Krush (Lynch and Krush 1971) who characterized the following features to be associated with the syndrome; autosomal dominant inheritance, 85–90% penetrance, colorectal carcinoma at an early age, synchronous and metachronous colorectal cancer, a better prognosis, tumors in the proximal colon, and an increased risk of extracolonic cancer such as cancer of the endometrium, ovary, stomach, small bowel, hepatobiliary tract, pancreas, ureter, and renal pelvis (Lynch *et al.* 1998). The syndrome was denoted Lynch syndrome; type I when colorectal cancer only was presented in the family and type II when other tumor types also occurred in the family. Linkage to the gene loci on chromosomes 2p and 3p was found in 1993 (Lindblom *et al.* 1993; Peltomäki *et al.* 1993) and studies on tumor and normal material from HNPCC-patients revealed insertions and deletions in various repetitive noncoding microsatellite sequences in the tumor tissue (Aaltonen *et al.* 1993; Ionov *et al.* 1993; Thibodeau *et al.* 1993). In parallel, investigations in yeast cells with mutations in the MutHLS mismatch repair pathway displayed MSI (Strand *et al.* 1993). The human homologues *MSH2* and *MLH1*, were cloned in 1993, and mutations of both genes were identified in HNPCC families (Bronner et al. 1994; Fishel *et al.* 1993; Leach *et al.* 1993; Papadopoulos *et al.* 1994).

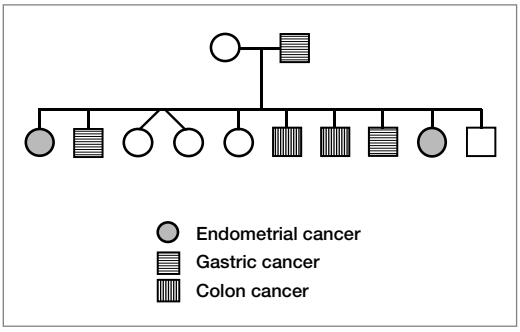


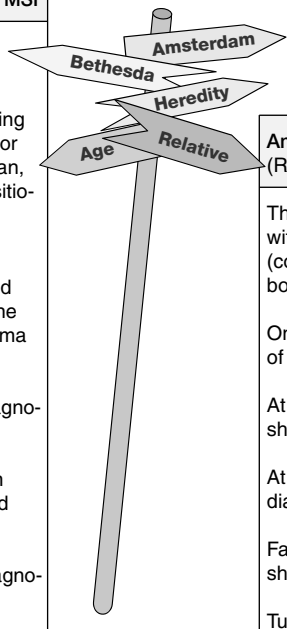
Figure 3. Pedigree over "Family G", the first described HNPCC-family

Prevalence and clinical criteria

HNPCC has been estimated to account for 20–50% of familial colorectal cancer (Fuchs *et al.* 1994; Hemminki and Li 2001; Hemminki and Vaittinen 1999) and 1–8% of colorectal cancer (Aaltonen *et al.* 1998; Lynch and de la Chapelle 1999; Mecklin 1987; Peel *et al.* 2000; Ponz de Leon *et al.* 1993; Samowitz *et al.* 2001). In order to provide uniformity in the classification of HNPCC, diagnostic criteria, the so-called Amsterdam criteria, were established in 1991 (table 1) (Vasen *et al.* 1991). A revised version of the criteria, Amsterdam II, was established in 1999 and included, in addition to colorectal cancer, also cancers of the endometrium, small bowel, urinary pelvis, and ureter (Vasen *et al.* 1999). Ovarian cancer and gastric cancer occurs at increased frequencies in HNPCC-families, but are not included in the Amsterdam II criteria. Gastric cancer is often seen in the earlier generations in HNPCC pedigrees, but is less frequently observed nowadays, perhaps as a result of the decreasing frequency of this tumor type in the western world. However, gastric cancer is the second most common HNPCC-associated malignancy in Asian HNPCC-families (Liu *et al.* 2004). Ovarian cancer was not included in the Amsterdam II criteria because of a risk of confusing HNPCC families with families with hereditary breast-ovarian cancer, which causes the majority of the hereditary ovarian cancer cases. The Amsterdam criteria can be criticized for only identifying families with a highly penetrant genetic defect and families that are large enough to contain the required number

Table 1. Clinical criteria for the classification of HNPCC

Bethesda guidelines for testing colorectal tumors for MSI
Individuals with cancer in the family that meet the Amsterdam II criteria or Individuals with two HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated extracolonic cancers (endometrial, ovarian, gastric, hepatobiliary, or small bowel cancer or transitional cell carcinoma of the renal pelvis or ureter) or 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 <45 years, and the adenoma <40 years or Individuals with colorectal or endometrial cancer diagnosed <45 years or Individuals with right-sided colorectal cancer with an undifferentiated pattern on histopathology diagnosed <45 years or Individuals with signet-ring cell colorectal cancer diagnosed <45 years or Individuals with adenomas diagnosed <40 years



Amsterdam II Criteria (Revised ICG-HNPCC Criteria)
There should be at least 3 relatives with HNPCC-associated cancer (colorectum, endometrium, small bowel, ureter or renal pelvis) and One should be a first-degree relative of the other two and At least two successive generations should be effected and At least one individual should be diagnosed < age 50 and Familial adenomatous polyposis should be excluded and Tumors should be verified by pathological examination

of cancers. Moreover, the accuracy of reporting a family history has been estimated to be >90% in first-degree relatives, but decreases to 70–85% among second-degree relatives (Theis *et al.* 1994). Some families with HNPCC do not fulfill the Amsterdam II criteria because of a predominance of extracolonic cancers, reduced penetrance, *de novo* mutations, small families or late onset. Also, the sensitivity of the MSI analysis is 95%, and mutation analyses do not always include *MSH6* and methods that allow detection of large intragenic alterations, which may account for 20–30% of the mutations in *MSH2* (Charbonnier *et al.* 2000; Di Fiore *et al.* 2004). Also, the geographic variation of HNPCC may vary (Nyström-Lahti *et al.* 1995). Therefore, these criteria should not be used as exclusion criteria for performing MSI testing and immunohistochemical staining. In order to identify individuals who may benefit from MMR gene analysis the Bethesda guidelines have been developed (Rodriguez-Bigas *et al.* 1997) (table 1), and about 1/3 of the tumors identified using these are estimated to display defective MMR (Umar *et al.* 2004; Vasen *et al.* 2004).

Cancer risk

The lifetime risk of cancer in HNPCC mutation carriers is estimated to be 80–90% (Aarnio *et al.* 1995; Aarnio *et al.* 1999; Vasen *et al.* 1996; Watson *et al.* 1998; Watson and Lynch 2001). Compared to sporadic cases, HNPCC patients develop tumors 15–20 years earlier, with a mean age of diagnosis of the first malignancy of 45 years in patients not undergoing preventive control programs, and the 10-year incidence of a second primary cancer is 40% (Aarnio *et al.* 1995; Lynch *et al.* 1998; Lynch *et al.* 1993; Watson and Lynch 2001). The tumor spectrum in HNPCC has been evaluated in several studies and the risks for different HNPCC-associated tumors have been calculated by observing the frequency, compared to the general population, of different tumor types either in clinically

defined HNPCC families fulfilling the Amsterdam II criteria or in mutation carriers (Aarnio *et al.* 1999; Dunlop and Campbell 1997; Vasen *et al.* 1996). The highest cancer risks, 70–80%, apply to colorectal cancer, which corresponds to an increased risk of about 15–18 times and endometrial cancer, 40–60%, which translates to a 13–20 times increased risk (Aarnio *et al.* 1999; Dunlop and Campbell 1997; Vasen *et al.* 1996). Sex differences have been observed with an increased risk of colorectal cancer in male mutation carriers (Aarnio *et al.* 1999; Dunlop and Campbell 1997; Vasen *et al.* 1996). Increased risks are observed also for gastric cancer (10–20%) and ovarian cancer (10–15%) (Aarnio *et al.* 1995; Aarnio *et al.* 1999). Rare tumor types such as transitional cell carcinoma of the upper urothelial tract, carcinomas of the small intestine, hepatobiliary cancer, skin tumors, and brain tumors also occur at increased frequencies, although the lifetime risk does not exceed 2–5% (Aarnio *et al.* 1999; Watson and Lynch 2001). Occasional sarcomas associated with HNPCC have also been reported (den Bakker *et al.* 2003; Lynch *et al.* 2003; Sijmons *et al.* 2000).

Pathology

There are no macroscopic or microscopic features exclusively associated with HNPCC, but several diagnostically useful features exist (Hamilton and Aaltonen 2000). These include a predilection for tumors in the proximal colon and well-circumscribed tumors that grow with a pushing margin. Subsets of HNPCC tumors are mucinous and show poor differentiation. In addition, tumor-infiltrating lymphocytes and a Crohn-like lymphocytic reaction are often found (Jass 2000). Adenomas are not numerous, but occur at increased frequency in HNPCC (Jass *et al.* 1994). Adenomas associated with HNPCC develop earlier in life, become larger, are more often villous, contain more areas of high-grade dysplasia, and are more prone to malignant conversion than sporadic adenomas (Beck *et al.* 1997; Jass and Stewart 1992). Moreover, HNPCC-adenomas, in contrast to sporadic adenomas, display MMR-defects, although at a lower rate than the carcinomas (De Jong *et al.* 2004; Konishi *et al.* 2004).

Genetic analysis of HNPCC

MSI analysis is estimated to have a sensitivity of 95% and a panel of five MSI markers, including BAT25, BAT26, D5S346, D2S123, and D17S250, has been established as an international standard (Boland *et al.* 1998; Dietmaier *et al.* 1997). Although, a recent publication reviewing a second Bethesda consensus meeting, suggest the use of mononucleotide repeats only, instead of dinucleotide repeats (Buhard *et al.* 2004). BAT26 alone has in several studies been shown to have the highest sensitivity and detects 97% of the MSI tumors (Loukola *et al.* 2001). It has been suggested that BAT25 and BAT26 predict MSI-high properly (Brennetot *et al.* 2005). The use of immunohistochemical staining with monoclonal antibodies against MLH1 and MSH2 was first reported in 1996 (Moslein *et al.* 1996). MMR protein immunostaining has a specificity of 95–100% and a sensitivity of about 90% (Halvarsson *et al.* 2004; Lindor *et al.* 2002; Marcus *et al.* 1999; Ruszkiewicz *et al.* 2002) and also has the advantage of pinpointing the inactivated gene to which mutation analysis can be directed.

Currently more than 400 different germline *MMR* gene mutations from over 700 families have been registered in the HNPCC-database (http://www.insight-group.org/) (Peltomäki and Vasen 2004). Of these, mutations in *MLH1* account for 50%, *MSH2* account for 39% of the mutations and *MSH6* in 7% of the cases (Miyaki *et al.* 1997; Peltomäki and Vasen 2004; Peltomäki and Vasen 1997; Wijnen *et al.* 1999). In the remaining cases several different causes exist, including rare mutations in *PMS2*. Lifetime risk of extracolonic cancer is somewhat greater in *MSH2* mutation carriers than in *MLH1* carriers (Lin *et al.* 1998; Vasen *et al.* 1996). Moreover, mutations in *MSH6* are associated with incomplete penetrance of HNPCC, later onset of cancer; and a high risk of gynecological cancer in female mutation carriers (Cederquist *et al.* 2004; Wagner *et al.* 2001; Wijnen *et al.* 1999). The type and distributions of mutations so far identified

in *MLH1* and *MSH2* are diverse, although founder mutations have been identified e.g. in Finland, in Newfoundland, and in the Ashkenazi Jewish population (Aaltonen *et al.* 1998; Froggatt *et al.* 1995; Nyström-Lahti *et al.* 1995; Peltomäki and Vasen 1997). The mutation spectra differ between the two major HNPCC-associated genes; in *MSH2*, frameshift mutations and nonsense mutations account for the majority of the mutations identified, whereas the proportion of missense mutations is greater in *MLH1* (Peltomäki and Vasen 2004; Peltomäki and Vasen 1997). Large genomic alterations have been demonstrated to constitute 20-30% of the *MSH2* mutations and 5–10% of the mutations within *MLH1* (Charbonnier *et al.* 2002; Gille *et al.* 2002; Wang *et al.* 2003; Wijnen *et al.* 1998). In a small subset of the families, mutations in the genes *MLH3, EXO1,* and *TGFßRII* are found (Hayward *et al.* 2004; Markowitz *et al.* 1995; Nicolaides *et al.* 1994; Thompson *et al.* 2004; Wu *et al.* 2001; Wu *et al.* 2001).

The exonuclease gene *EXO1* is involved in MMR and therefore mutations in this gene have been suspected to increase the cancer risk, and germline mutations have been found in occasional families suspected of having HNPCC (Wu *et al.* 2001). However, this has been opposed by investigations that has concluded that tumors with loss of both copies of *EXO1* do not have MSI (Alam *et al.* 2003). Germline mutation in the MMR gene *MLH3* has been identified in a few families with atypical or classical HNPCC, but the contribution of mutations in these genes to HNPCC has not yet been clarified, but likely account for only a small fraction of the cases (Wu *et al.* 2001; Wu *et al.* 2001). *Cyclin D1* is the major cyclin involved in transition from G1 to S phase and had been investigated as being a modifier gene in HNPCC patients (Bala and Peltomäki 2001). It exhibits alternate splicing and expression of the two different transcripts correlated to colorectal cancer risk in HNPCC patients.

The Muir-Torre syndrome is an HNPCC-subtype associated with colorectal carcinoma and sebaceous skin lesions such as sebaceous adenomas and carcinomas and these tumors display MSI and MMR gene mutations, most often in *MSH2* (Kruse *et al.* 1998; Schwartz and Torre 1995). Turcot syndrome is characterized by development of colon tumors and primary brain tumors, typically glioblastomas or medulloblastomas, and has been associated with germline mutations either in the *APC* gene or in the MMR genes (Hamilton *et al.* 1995). The histological type of brain tumor may be indicative of the underlying mutation; in families with HNPCC-associated mutations glioblastomas multiforme predominate, whereas cerebellar medulloblastomas occur in the *APC* kindreds (Hamilton *et al.* 1995). Germline mutations in *PMS2* have been found in families with Turcot syndrome, but this variant seems to be heterogenous with recessive modes of inactivation found in some families and yet unidentified genes may underly some of these families (De Rosa *et al.* 2000; Hamilton *et al.* 1995).

Clinical management

Identification of HNPCC-families enables inclusion of high-risk individuals into control programs in order to detect early-stage tumors and to prevent cancer development, decrease morbidity, and prolong survival. Colonoscopic screening every third year in HNPCC-families has been shown to reduce the risk of colorectal cancer by more than 50%, prevents death from colorectal cancer, and decreases overall mortality by about 65% (Järvinen *et al.* 2000). The surveillance programs recommended for colorectal cancer, which include biannual colonoscopy from age 20–25 and gynecologic ultrasound and endometrial biopsy starting from age 30–35 (www.insight-group.org), have been shown to be cost-effective (Lynch *et al.* 1993; Vasen *et al.* 1998). However, neither are there any data on the benefit of the screening programs recommended for endometrial cancer, nor evidence for control programs for hepatobiliary cancer, cancer of the small intestine, gastric cancer, and urothelial cancer, and therefore screening for the latter tumor types is reserved for families in which these tumor types have developed (Thorson *et al.* 1999).

Table 2. Hereditary syndromes predisposing for colorectal cancer

Syndrome	Gene	Function/pathway	Associated features
Familial adenomatous polyposis (FAP)	*APC*	Cell-cell adhesion, transcription regulation, chromosomal segregation	Cancer of the small intestine and thyroid cancer
Gardner syndrome	*APC* (codons 1403–1578)		Osteomas, skin fibromas, epidermoid cysts
Attenuated FAP	*APC* (5' uppstream of codon 157 or 3' end of the gene)		Fewer adenomas and higher age at onset compared to FAP
Hereditary nonpolyposis colorectal cancer (HNPCC)	*MLH1* *MSH2* *MSH6* *PMS2*	Mismatch repair	Cancer development in the colon, rectum, endometrium, small intestine, upper urinary tract, ovaries, stomach, skin, brain and sarcomas
Hamartomatous syndromes			
Peuts-Jeghers syndrome	*LKB1*	Serine/theonine kinase	Pigmentation of the perioral region, fingers and toes
Juvenile polyposis	*SMAD4* (50%) *BMPR1A* (50%)	TGF-β signaling pathway	–
Cowden disease	*PTEN*	Cell growth and apoptosis	Oral and facial papules, and cancer of the breast and thyroid
Bannayan-Ruvalcaba-Riley syndrome	*PTEN*	Cell growth and apoptosis	Skeletal malformations, macrocephaly, mental retardation, hemangiomas, lipomas, intestinal polyps, lymphomas, thyroid cancer
Hereditary colorectal cancer	*AXIN2* *TGFβR2* *POLD* *MYH*	Wnt-signaling pathway Cell growth DNA polymerase Base excision repair	Tooth agenesis – – Multiple adenomas, although fewer than in FAP

Other forms of hereditary colorectal cancer

Polyposis syndromes

Up to 20% of colorectal cancer is estimated to develop due to heredity and various genetic predispositions probably underly these cases (Chapelle 2004; Lynch and de la Chapelle 2003) (table 2).

Familial adenomatous polyposis (FAP) is an autosomal dominant syndrome characterized by development of hundreds to thousands of polyps in the colorectum and virtually 100% risk of colorectal cancer at age 40. The disease is estimated to affect approximately 1/8–12 000 individuals in Scandinavia (Björk *et al.* 1999) and accounts for less than 1% of colorectal cancer (Järvinen 1992). FAP is caused by mutations in the *APC* gene located at 5q21 (Groden *et al.* 1991; Kinzler *et al.* 1991). FAP-patients are at increased risk of also developing cancer of the small intestine, in particular periampullary adenocarcinomas, tumors of the hepatobiliary tract, and thyroid cancer (Björk *et al.* 2001; Soravia *et al.* 1997). Gardner syndrome is a variant of FAP caused by mutations between codon 1403 and 1578 of the *APC* gene and is in addition to colonic polyposis associated with development of osteomas, skin fibromas, and epidermoid cycts (Beroud and Soussi 1996; Davies *et al.* 1995; Gardner 1962). Attenuated FAP (AFAP) is associated with development of fewer polyps (tens or hundreds rather than thousands) and mutations upstream of codon 157 or in the 3´ end of the gene (Heppner Goss *et al.* 2002; Nilbert *et al.* 2000).

Mutations in the *MYH* gene have been found in families with multiple adenomas (Chow *et al.* 2004). These patients may be clinically difficult to differentiate from patients with FAP or AFAP. Although the number of polyps is slightly lower, the age at onset is somewhat higher, median 47–55 years. A recessive mode of inheritance is suggested since the patients present with biallelic missense mutations, most commonly the variants Tyr165Cys and Gly382Asp (Croitoru *et al.* 2004; Sieber *et al.* 2003; Wang *et al.* 2004).

Mutations in other genes, such as *AXIN2, TGFβR2* and *POLD,* have been described only in a small subset of families with hereditary colorectal cancer (table 2) (Lu et al. 1998, da Costa et al. 1995). The *AXIN2* gene is a regulator of the wnt-signaling pathway, involved in formation and morphogenesis of most organs, and *AXIN2* gene mutation have been reported in a family with tooth agenesis and colorectal cancer (Lammi *et al.* 2004).

Hamartomatous syndromes

Development of intestinal hamartomatous polyps is associated with four rare syndromes (table 2). The Peutz-Jeghers syndrome is an autosomal dominant trait associated with hamartomatous polyposis of the gastrointestinal tract, melanin pigmentation of the lips, perioral region, buccal mucosa, toes and fingers (Jeghers 1944; McGarrity *et al.* 2000; Peutz 1921) and an increased risk of cancer e.g. of the breast, pancreas, and ovaries (Giardiello *et al.* 2000). The Peutz-Jeghers syndrome is caused by germline mutations in the *LKB1/STK11* gene, a tumor suppressor gene involved in control of cell growth (Lim *et al.* 2003). Cowden disease is a rare syndrome estimated to affect 1 in a million individuals, and is associated with development of benign adenomas and malignant neoplasms of the thyroid, breast, endometrium, and skin (Eng and Peacocke 1998; Lloyd and Dennis 1963; Marsh *et al.* 1998; Weary *et al.* 1972). Cowden disease is caused by mutations in *PTEN*, a tumor suppressor gene located at 10q23. The Bannayan-Ruvalcaba-Riley syndrome is associated with mutations in the *PTEN* gene and is characterized by microcephaly, mental retardation, skeletal malformations, hemangiomas, lipomas, intestinal polyps, lymphomas, and thyroid cancer (Cohen 1990; Merg and Howe 2004). Juvenile polyposis present with a family history and intestinal polyposis in childhood with a 50% risk of developing gastrointestinal cancer (Howe *et al.* 1998; Merg and Howe 2004). Of patients with juvenile polyposis 20% carry *SMAD4* gene mutations and an additional 20% carry mutations in *BMPR1A*, both of which are involved in the TGFβ-signaling pathway (Howe *et al.* 2004).

HNPCC-associated tumor types studied in this thesis

Colorectal cancer

Prevalence and risk factors

Colorectal cancer accounts for one tenth of the cancer cases in the Western world and affects about 1 million individuals annually, approximately 0.5 million of whom die from the disease (Boyle and Leon 2002; Parkin 2001).

The cumulative probability of developing colorectal cancer is 5–7% for colon cancer and 2–3% for rectal cancer (figure 4) (National Board of Health and Welfare). Life-style factors have been associated with development of colorectal cancer, and people emigrating from low-risk areas (Japan) to high-risk areas (the United States) adopt the higher risk (Potter 1999). Life-style factors associated with increased risk of the disease are high intake of animal fat and alcoholic beverages (Berlau *et al.* 2004), a high body-mass-index (Giovannucci 2003), insulin resistance, and hyperinsulinemia (Giovannucci 1995). A possible protective effect from dietary fiber has been debated, and prospective cohort studies have failed to demonstrate any strong associations between fruit and vegetable intake and colorectal cancer (Hung *et al.* 2004). Proposed benefits include dilution and binding of potential carcinogens, decreased transit time, primary bile acid binding, and fermentation of fibers to short fatty acids (Hung *et al.* 2004; Kim 2000; Peters *et al.* 2003). Animal fat enhances secretion of primary bile acids that are converted to more cytotoxic forms, although this has not yet been confirmed by epidemiologic studies (Kushi and Giovannucci 2002). Physical activity has a preventive effect through stimulation of intestinal transit and reduction of growth factors and bile acid levels, which may promote growth and proliferation (Potter 1999). A family history of colorectal cancer is one of the strongest risk factors for the disease (Houlston *et al.* 1990). Individuals with a first-degree relative diagnosed with colorectal cancer have a 2-fold increased risk of developing the disease, and the risk is further increased if the relative is young at diagnosis or if more than one

Figure 4. The 10 most common cancer types in Swedish men and women 2002, according to the National Board of Health and Welfare

relative is affected by colorectal cancer (Carstensen *et al.* 1996; Fuchs *et al.* 1994; Hemminki and Vaittinen 1999). Individuals from families who do not fulfill the Amsterdam criteria for HNPCC may be at increased risk of colorectal cancer as well as of extraintestinal tumors (Fuchs *et al.* 1994).

Pathology

The earliest precursor lesion identified in the colonic mucosa is the aberrant crypt foci, with enlarged crypts and thickened epithelium. There are two major forms of aberrant crypt foci, one with features of a hyperplastic polyp, and one with a dysplastic epithelium. Aberrant crypt foci of the dysplastic subtype may develop to an adenoma, which is considered to represent a precursor lesion of cancer. Adenomas are by definition intraepithelial neoplasias with hypercellularity, varying degrees of nuclear stratification, and loss of polarity. Non-neoplastic polyps include hyperplastic polyps and juvenile polyps. Hyperplastic polyps have previously been regarded as harmless lesions, but have been suggested to be associated with MSI tumors with methylation of the *MLH1* promoter (Higuchi and Jass 2004; Jass 2004), although contradictory results exist (Rijcken *et al.* 2003). Adenocarcinoma is the major histological subtype of colorectal cancer, and accounts for

90–95% of the colorectal malignancies. Adenocarcinomas can be of the mucinous type, where >50% of the lesion is composed of mucin, often in extracellular pools. Signet-ring cell carcinoma consists of >50% cells with mucin vacuoles, which displace the nucleus. Adenosquamous carcinomas have components of both adencarcinoma and squamous carcinoma and are rare within the large bowel. Medullary carcinomas show sheets of cells with prominent nucleoli, vesicular nuclei, and infiltration of intraepithelial lymphocytes and have a more favorable prognosis compared to other carcinoma subtypes. The rare undifferentiated adenocarcinomas have variable histopathological appearances. Subtypes associated with MSI include mucinous adenocarcinoma, signet-ring cell cancer, medullary adenocarcinoma, and undifferentiated carcinoma (Hamilton and Aaltonen 2000). The risk of metastasis is associated with invasion through the muscularis mucosae into the submucosa and the TNM classification is based on tumor infiltration, presence of regional lymph node metastasis, and distant metastasis (Sobin and Wittekind 2002).

Genetic alterations in colorectal cancer

Colorectal cancer serves as a model for how genetic alterations accumulate during tumor progression and several distinct genetic alterations associated with the adenoma-carcinoma sequence have been identified (Fearon and Vogelstein 1990). In the original model described by Vogelstein, four to 7 mutations are thought to accumulate (figure 5) (Fearon and Vogelstein 1990; Fodde *et al.* 2001; Leslie *et al.* 2002). The major alterations identified include *KRAS, APC, SMAD4,* and *p53* (Fodde *et al.* 2001). Loss of heterozygosity (LOH) studies have revealed several genes involved in colorectal cancer development; LOH has been found at 5q (*APC*), 8p, 17p (*p53)* and 18q (*SMAD4)*. Loss of chromosome 17p is present in 75% of CRC, but rarely in earlier lesions, which suggests inactivation late in tumorigenesis. Similarly LOH at 18q has been identified in 50% of large adenomas and 75% of carcinomas (Fodde *et al.* 2001). Inactivation of the *APC* gene is the earliest event and can be found already in the aberrant crypt foci (Smith *et al.* 1994) and *KRAS* and *APC* seem to act synergistically in the development of a carcinoma (Jen *et al.* 1994; Leslie *et al.* 2002). *SMAD2* and *SMAD4* have key functions in the TGF-β signaling pathway. Loss of SMAD4 has been found in pancreatic and colorectal carcinomas (Hahn *et al.* 1996; Thiagalingam *et al.* 1996) and SMAD2 have been specifically lost in colorectal carcinomas (Eppert *et al.* 1996). Recently, the tyrosine kinase gene *PIK3CA*, involved in regulation of cellular growth and proliferation, has been found to be altered in about 30% of colorectal cancers, which suggests an importance in colorectal cancer development (Samuels *et al.* 2004).

Chromosomal instability

In CRC development, there are two major forms of genetic instability involved, chromosomal instability (CIN) and microsatellite instability (MSI). The majority of the tumors are of the CIN type, which refers to structural and numerical genomic alterations (Lengauer *et al.* 1998). Karyotypic order is maintained by checkpoints that operate at mitosis and guard genomic stability; i.e. genes encoding proteins that function in mitotic spindle assembly and dynamics, chromosome metabolism, cell-cycle regulation, and check point control. In the spindle checkpoint, the sister chromatids are not separated until the chromosomes are appropriately aligned along the mitotic spindle. Genes involved in these mechanisms; e.g. *APC, MAD1, MAD2, BUB1,* and *BUB2,* have been demonstrated to be altered in e.g. breast cancer and colon cancer (Lengauer *et al.* 1998; Rajagopalan and Lengauer 2004). *BUB1,* being a mitotic checkpoint gene, has been reported to be involved in CIN, although only a few *BUB1* mutations have been found in colorectal cancers (Cahill *et al.* 1998). The functions of *APC* involve promotion of cell-cell adhesion through binding to β-catenin, which is essential in cell-cell adhesion through complex formation with the cell adhesion protein E-cadherin. In under-stimulated cells, β-catenin can be found unbound in the cytoplasm, and be destabilized by GSK3β and APC (Christofori and Semb 1999).

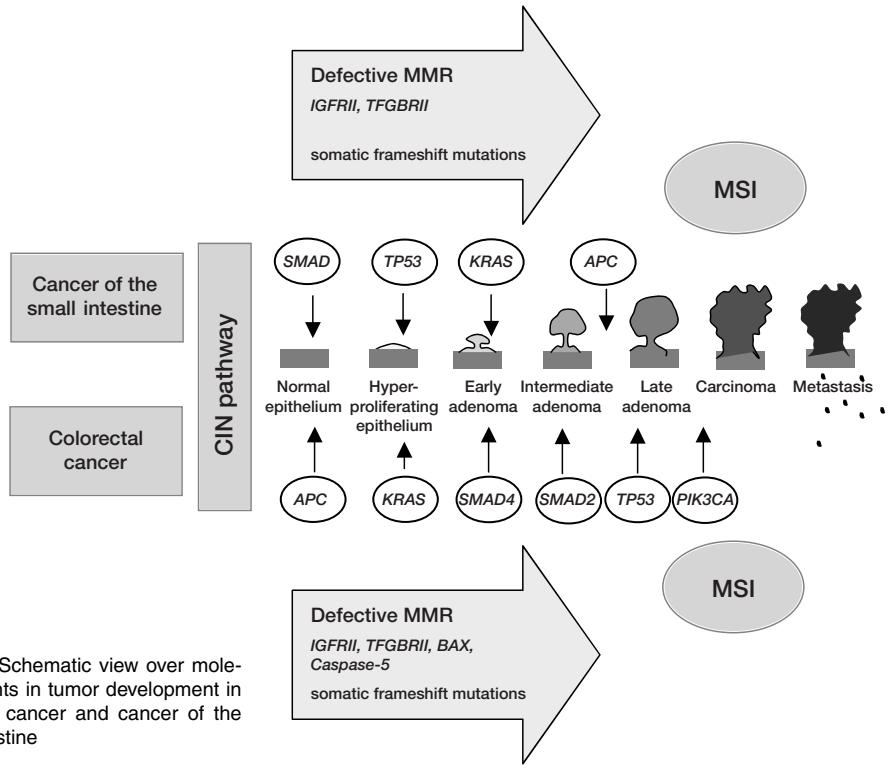


Figure 5. Schematic view over molecular events in tumor development in colorectal cancer and cancer of the small intestine

The wnt-pathway is initiated through binding of low-density lipoprotein receptor-related protein (LRP6) to Frizzled-receptors, that in turn bind wnt factors inactivating GSK3β and the deregulation complex. This leads to an increased amount of β-catenin, which can be shuttled to the nucleus where it binds DNA-binding proteins in the T-cell factor family and acts as an essential co-factor in transcriptional activation (Christofori and Semb 1999). Thus, APC is important in cellular adhesion and in the wnt-signaling pathway. Downstream targets of the APC-β-catenin pathway include MYC and cyclin D1 that are important in proliferation, apoptosis, and cell-cycle progression (Fodde *et al.* 2001). Other targets in the wnt-pathway, such as CD44, MYC, and urokinase-type plasminogen activator receptor seems to be involved in the promotion of tumors rather than the initiation (Fodde *et al.* 2001). Lately, involvement in maintaining the chromosomal stability during mitosis has been identified as a function of the *APC* gene. The C-terminus of APC contains a region interacting with the microtubule-associated protein EB1 and thus facilitates the binding of spindle microtubules to the kinetochore (Fodde *et al.* 2001). Cells with truncating *APC* mutations (that may be intact in the β-catenin-binding domains) thus fail in the EB1 or microtubule interaction, which leads to chromosomal instability (Rajagopalan and Lengauer 2004).

Microsatellite instability

The approximately 15% of CRC that show MSI (HNPCC-associated as well as sporadic cases) have several clinical and pathological features in common, such as a preferred proximal localization within the colon and poor histopathologic differentiation (Jass 2000; Lynch *et al.* 1993). The frequency of MSI within the colon varies from 20% in the proximal to <5% in the rectum (Fernebro *et al.* 2002; Hoogerbrugge *et al.* 2003). Generally, MSI tumors are diploid or near-diploid, and

carry only few chromosomal aberrations. HNPCC tumors develop because of a germline MMR gene mutation and somatic inactivation of the wild-type allele in the tumor tissue, whereas most sporadic tumors associated with the MSI tumorigenic pathway develop because of somatic hypermethylation of the *MLH1* promotor (Kane *et al.* 1997; Wheeler *et al.* 2000). *BRAF* mutations have been found in 40–74% of sporadic MSI-high colorectal cancers, whereas such mutations are not found in MSI-high tumors associated with HNPCC (Domingo et al. 2004; McGivern *et al.* 2004). This suggests that screening for *BRAF* mutations might be useful in classification of MSI-high tumors. The defective correction of DNA replication errors primarily affects repetitive DNA sequences and tumors with defective MMR display somatic mutations in coding repetitive tracts of genes involved in growth control, apoptosis or DNA-repair (Markowitz *et al.* 1995). A large number of such repeat-containing genes have been proposed as potential targets for instability, although most presumed target genes show low mutation frequencies (Duval *et al.* 2001). However, it has been argued that, since control genes with structurally similar repeat tracts are free from alterations, the somatic repeat-mutations may be selected during tumor development rather than simply reflecting a general instability (Duval *et al.* 2002). Mutations in target genes, i.e. mutations that are caused by MMR-deficiency and important for neoplastic growth, may be difficult to distinguish from genes that simply undergo "passenger mutations", which probably outnumber the target mutations in MMR-deficient cancers (Zhang *et al.* 2001). Among the repeat-containing genes that are generally considered as possible targets in MMR defective tumorigenesis are the pro-apoptotic genes *BAX* and *Caspase-5*, the MMR genes *MSH3* and *MSH6*, the growth factor receptor genes *IGFIIR* and *TGFBRII*, and the transcription factor gene *TCF4* (Duval and Hamelin 2002; Markowitz *et al.* 1995). Recently, the genes *GRB14*, which is involved in signal transduction, *RAD50*, which is implicated in DNA repair, and *RHAMM*, a mediator of cell motility, were reported as new target genes (Duval *et al.* 2001). Another repeat-containing candidate tumor suppressor is the pro-apoptotic gene *RIZ*, which shows a high frequency of mutations, including biallelic inactivation, in gastric, endometrial, and colorectal cancers with MSI (Chadwick *et al.* 2000). Moreover, the defective MMR system probably also influences tumor development through additional mechanisms such as proliferation, DNA-damage induced cell death, and alterations at cell cycle checkpoints (Lengauer *et al.* 1998).

CIN and MSI are biologically specific tumor pathways in colorectal cancer, although, they partly develop in parallel. *APC* mutations are found in both pathways, but at a somewhat lower frequency in MMR deficient tumors, where mutations in β-*catenin* might be found instead, which functionally affects the same pathway as the *APC* mutations. Furthermore, the TGF-β-signaling pathway is commonly affected in both CIN and MSI tumors, with mutations in *SMAD2/SMAD4* and *TFGβRII*, respectively. Also, *TP53* mutations occur at somewhat lower frequencies in MSI tumors, whereas *BAX* mutations are more frequently seen (Zhang *et al.* 2001).

In MSI tumors, conflicting results have been reached regarding the pattern of somatic alterations in other genes frequently mutated in colorectal cancer development; activating mutations have been demonstrated at frequencies similar to that of MSS tumors in some studies (Duval *et al.* 1999; Fukushima *et al.* 2001), whereas low rates of these alterations in MSI tumors have been reported by other investigators (Konishi *et al.* 1996; Losi *et al.* 1997; Olschwang *et al.* 1997). A reduced frequency of *TP53* mutations in MSI colorectal tumors has been found in several studies (Konishi *et al.* 1996; Losi *et al.* 1997; Olschwang *et al.* 1997; Salahshor *et al.* 1999). Mutations of the *APC* gene predominantly occur through alterations in repetitive tracts of the *APC* gene (Huang *et al.* 1996) and seem to occur at a reduced frequency in MSI colorectal cancers (Konishi *et al.* 1996; Olschwang *et al.* 1997), although mutations in the *CTNNB1* gene that encodes for β-catenin may provide an alternative mechanism in these tumors (Shitoh *et al.* 2001). As mentioned above, mutations of the repetitive

tract of the *TGFβRII* gene occur at a considerably higher frequency in colorectal tumors of the MSI phenotype than in MSS tumors (Markowitz *et al.* 1995).

MMR gene inactivation provides the cells with a proliferative advantage and recent studies have demonstrated that cell lines deficient in MLH1, MSH2 or MSH6 have an increased resistance to alkylator-induced apoptosis. Furthermore, MLH1 and MSH2 are components of apoptotic pathways (Zhang *et al.* 1999). These findings have led to the proposal of an alternative model for MMR function in tumorigenesis, in which the MMR proteins act as signaling molecules that may either exert DNA repair or directly signal an apoptotic response. Another proposed function of the MMR genes is in the G2/M cell cycle checkpoint, during which DNA mispairs cause G2 arrest in MMR-proficient cells, whereas MMR-deficient cells are not sensitive to this mechanism (Hawn *et al.* 1995). Moreover, MLH1 has been shown to interact with the protooncogene product MYC, whereas MSH2 interacts with MAX, the heterodimeric partner of MYC (Partlin *et al.* 2003). *MYC* is involved in cell cycle arrest, apoptosis induced by DNA damage, polypoloidization, and inappropriate progression through the cell cycle (Obaya *et al.* 1999).

The heavy mutation burden that results from defective MMR has been proposed to be unfavorable to the tumor cells and could thereby explain the improved prognosis observed in HNPCC patients (Sankila *et al.* 1996). Hence, defective MMR may have a prognostic value also in sporadic MMR defective tumors, but the findings are contradictory (Clark *et al.* 2004; Colombino *et al.* 2002; Gryfe *et al.* 2000; MacDonald *et al.* 2000; Samowitz *et al.* 2001; Wright *et al.* 2000). Since the MMR machinery plays a role in the correction of damage induced by alkylating agents, MMR deficient cells are resistant to these drugs (Karran and Hampson 1996), presumably through ecsape from apoptosis. The most extensively documented agent with resistance associated with MMR deficiency is cisplatin (Fink *et al.* 1998). Resistance to 5-fluorouracil, the most widely used cytostatic drug in colorectal cancer, has been demonstrated in MMR deficient cell lines, and an uncertain survival benefit in patients with MSI tumors treated with 5-fluorouracil has been suggested (Carethers *et al.* 2004; Clark *et al.* 2004; Meyers *et al.* 2001), although, MSI *per se* may be an independent favorable prognostic factor (Lim *et al.* 2004). An increased sensitivity to topoisomerase-inhibitors has recently been demonstrated in MMR deficient colorectal cancer cell lines (Jacob *et al.* 2001) and the combined observations suggest that MSI status may be a possible predictive marker.

Cancer of the small intestine

Prevalence and pathology

Cancer of the small intestine accounts for 3% of cancers in the gut and annually affects about 200 individuals in Sweden (with a yearly incidence of about 2/100 000) (National Board of Health and Welfare). Adenocarcinoma is the most frequent tumor type, but also carcinoids, sarcomas, and lymphomas develop in the small bowel (Hamilton and Aaltonen 2000). There are many suggestions as to why cancer of the small intestine, comprising three fourths of the gastrointestinal tract, is rare in comparison to cancer of the colorectum. The structure of the small intestine involves both villus and crypts with base columnar cells and stem cells in the crypts and the epithelial cells produced in the crypts migrate upwards, enters cell cycle arrest at the crypt-villus junction and are eventually shed at the top of the villus, which results in a high cell turnover rate and shedding of transformed cells (Kariv and Arber 2003; Sancho *et al.* 2004). Also, the stem cells of the epithelium are located deeply in the crypts, and thus protected from carcinogens within the bowel content (Booth and Potten 2000). Absence of bacteria may be another factor since bacteria are required for certain carcinogens to be "active" (Neugut *et al.* 1998). Rapid transit time minimizes exposure time for carcinogens, and the alkaline environment prevents production of nitrosamines, known to be carcinogenic in the acid environment of the stomach. Certain enzymes present in the small intestine have also been suggested to reduce the cancer risk through inactivation of carcinogens (Kariv and Arber 2003).

Adenocarcinomas are located in the duodenum in 50–60%, jejunum in 20%, ileum in 10–15%, and at unspecified sites in the remainder (Howe *et al.* 1999). The risk factor profile of cancer of the small intestine is similar to that of colon cancer with an increasing incidence in the Western world, and an association with high intake of animal fats, red meat, salt-cured and smoked foods, and low intake of fibers (Kariv and Arber 2003). Also, alcohol, smoking, and bile acids have been suggested as disease promoting factors (Neugut *et al.* 1998). Patients with Crohn's disease who have a chronic inflammation of the intestinal mucus have an increased risk for cancer in the small intestine, and have a poorer prognosis (Howe *et al.* 1999). Both FAP and HNPCC confer an increased risk of cancer of the small intestine (Björk *et al.* 2001; Vasen *et al.* 1996). In FAP patients cancer of the small intestine is the most common extracolonic cancer, and most commonly develop in the periampullary region, but since FAP is rare, these patients constitute only a small fraction of individuals with cancer of the small intestine (Björk *et al.* 1999; Björk *et al.* 2001). Cancer of the small intestine was first reported to occur in association with HNPCC by Lynch in 1989 (Lynch *et al.* 1989), and is now included in the clinical criteria for HNPCC (Vasen *et al.* 1999). HNPCC patients have a 25–100-fold risk increase, which translates to a 1–4% lifetime risk of cancer of the small intestine (Aarnio *et al.* 1995; Rodriguez-Bigas *et al.* 1998; Vasen *et al.* 1996). In line with clinicopathological data on HNPCC-associated colorectal carcinoma, HNPCC patients with carcinomas of the small intestine have a lower age of onset, a higher male to female ratio, a high incidence of metachronous tumors, and a partly different site distribution within the small bowel with an even distribution of tumors in HNPCC, compared to a predilection for the duodenum among the sporadic cases.

Tumor biology

The adenoma-carcinoma sequence has been suggested to apply also to cancer of the small intestine (figure 5) (Esposito *et al.* 2001; Sellner 1990). Inactivation of the *APC* gene has been reported in 14-18% of sporadic carcinomas of the small intestine, thus at a lower frequency than in colon cancer (Blaker *et al.* 2002; Rashid and Hamilton 1997). *KRAS* mutations have been found in 40% of the adenomas and in 36-67% of the carcinomas (Arber *et al.* 2000; Mitomi *et al.* 2003; Rashid and Hamilton 1997; Sutter *et al.* 1996), suggesting that *KRAS* inactivation is an early but not indispensable event. p53 is reported to be overexpressed in 45% of adenomas and in 47-65% of carcinomas (Arber *et al.* 1999; Rashid and Hamilton 1997). LOH at 18q21-22 has been reported at a frequency of 80% and the *SMAD4* gene located here seems to play a central role in tumorigenesis of the small intestine (Blaker *et al.* 2002).

Defective MMR has been found in 27/120 (23%) of cancers of the small intestine (Hibi *et al.* 1995; Kim *et al.* 2003; Muneyuki *et al.* 2000; Murata *et al.* 2000; Park *et al.* 2003; Rashid and Hamilton 1997; Wheeler *et al.* 2000). Somatic frameshift mutations are associated with MSI tumor development and the target genes are *TGFβR2* and *IGFR2,* resulting in loss of its tumor suppressing function (Murata *et al.* 2000; Nagai *et al.* 1999). Studies on the prognostic significance of MSI in carcinomas of the small intestine suggests that patients with MSI tumors have a longer cancer-specific survival, which is in concordance with the findings in colon cancer (Brueckl *et al.* 2004; Lim *et al.* 2004).

Upper urothelial cancer

Prevalence and pathology

Cancer of the urinary organs constitutes 4–5% of the cancer cases in Sweden and the lifetime risk of developing the disease is 4.6% in males and 1.3% in females (National Board of Health and Welfare). The majority of the tumors are transitional cell carcinomas and adenocarcinomas and two thirds of the tumors develop within the bladder (Lynch and Cohen 1995). Worldwide, the most common etiologic factor for development of urologic malignancy is schistosomiasis, but urothelial cancer is also related to smoking and diet. Smoking has been found to increase the risk of cancer of the ureter

and the renal pelvis about 6 times, and intake of laxatives and analgesics have also been associated with an increased risk (Pommer *et al.* 1999).

Also, an inverse association between consumption of vegetables and fruit and the risk of urothelial cancer has been found (Zeegers *et al.* 2001). A familial risk of urinary tract cancer has been reported with a slightly increased risk among first-degree relatives of bladder cancer patients (Goldgar *et al.* 1994; Kiemeney *et al.* 1997; Kramer *et al.* 1991; Plna and Hemminki 2001) and a higher risk if the parent was young (<60) at diagnosis (Aben *et al.* 2002; Goldgar *et al.* 1994; Watson and Lynch 2001). Patients with HNPCC have been estimated to have a 2–5% lifetime risk of developing upper urothelial cancer (UUC) (Aarnio *et al.* 1999; Sijmons *et al.* 1998) and these tumors are characterized by an early age at onset and predominance of females. The relative risk of transitional cell cancer of the upper urinary tract is 14 in HNPCC patients whereas the risk of bladder cancer is not increased (Sijmons *et al.* 1998).

Tumor biology

Molecular LOH studies of transitional cell cancer have revealed deletions of several chromosomal arms, most commonly 3p, 6q, 9q, 11p, 17p, and 18q (Sandberg and Berger 1994). Chromosome 9q has been lost in both low-grade and high-grade lesions, suggesting an early inactivation (Tsai *et al.* 1990). LOH at 11p and 18p is also found in superficial lesions, suggesting early inactivation, whereas LOH at 17p (*TP53*) occur frequently only in high-grade lesions, which suggests a late inactivation. Studies that have compared papillary transitional carcinomas with flat tumors (carcinomas *in situ*, CIS) have found LOH at chromosome 9 in 34% of papillary tumors compared to 12% in CIS, and *TP53* mutations in 3% of the papillary cancers versus 65% in CIS (Spruck *et al.* 1994). Van Rhijn *et al.* (van Rhijn *et al.* 2004) found mutations in the fibroblast growth factor 3 *(FGFR3)* gene in 59% of urothelial cancers, which was associated with a favorable prognosis, and *TP53* mutation in 24% of urothelial cancers, which was associated with an unfavorable prognosis. *FGFR3* and *TP53* mutations occurs concordantly only in 8% of the investigated tumors, which supports the two-pathway theory (van Rhijn *et al.* 2004; van Rhijn *et al.* 2003). The tumor suppressor gene *RB1* is inactivated only in 10% of superficial urothelial cancers, compared to 34% in muscle-invasive tumors. Tumors with positive p21 expression analysis are associated with a better prognosis compared to those with negative immunostaining (Al-Sukhun and Hussain 2003). COX-2 is commonly expressed in bladder cancer, and over-expression is associated with aggressive disease, which suggests a role for COX-2 in bladder cancer development and invasion (Shariat *et al.* 2003).

The MSI pathway of tumor development has been investigated in urothelial carcinomas through MSI analysis, MMR protein expression, and MMR gene mutations. In carcinomas of the urinary bladder, MMR defects have identified in <10% of the tumors (Gonzalez-Zulueta *et al.* 1993; Saetta *et al.* 2004). However, the risk increase in HNPCC mutation carriers is mainly conferred to urothelial carcinomas of the upper urinary tract and assessments of MMR defects in UUC have indicated a high frequency, 15–45%, of MMR defects (Amira *et al.* 2003; Blaszyk *et al.* 2002; Hartmann *et al.* 2002; Roupret *et al.* 2004). A difference in the fraction of MSI tumors depending on tumor location has been shown with MSI in 33% of the uretral tumors and in 10% of tumors in the renal pelvis (Amira *et al.* 2003; Catto *et al.* 2003; Hartmann *et al.* 2002).

Soft tissue sarcoma

Prevalence and pathology

Soft tissue sarcoma (STS) is a rare tumor type that accounts for less than 1% of the cancer cases in Sweden and affects 30–35/1 000 000 individuals annually. 1/3 of the patients develop metastases, most of whom die from the disease. 3/4 sarcomas develop in the extremities, most commonly in the thigh. STS is a heterogeneous tumor type with over 50 different histological entities identified (Fletcher *et al.* 2002). Malignant fibrous histiocytoma (MFH), pleomorphic sarcoma, leio-

myosarcoma, liposarcoma, synovial sarcoma, and malignant peripheral nerve sheath tumor are the most common STS. Since local recurrences and metastases are common, prediction of prognosis is relevant for the choice of therapy. The prognostic markers include tumor size, depth, histotype, malignancy grade, mitotic rate, necrosis, vascular invasion, DNA-ploidy, S-phase fraction, molecular markers, and development of local recurrences (Coindre *et al.* 1996; Gustafson 1994; Pisters *et al.* 1996; Pisters and Pollock 1999).

Risk factors for developing STS include a family history of cancer, certain genetic syndromes, exposure to ionizing radiation, and certain chemicals such as vinyl chloride, for review, see (Olsson 2004; Zahm and Fraumeni 1997). Also, lymphedema, immunosuppressive drugs, alkylating agents, human immunodeficiency virus, and exposure to human herpes virus may increase the risk (Olsson 1999, 2004; Zahm and Fraumeni 1997). Sarcomas have been associated with several hereditary syndromes, including Werner syndrome due to mutations in the *WRN* gene, Li-Fraumeni syndrome due to germline *TP53* mutations, and other rare syndromes such as Rothmund-Thomson syndrome and Bloom syndrome (Garber *et al.* 1991; Goto *et al.* 1996; Lynch and de la Chapelle 2003; Vennos *et al.* 1992). In studies that assess the cancer risk among patients with HNPCC, a slightly increased risk for sarcomas, of about 1–2%, has been demonstrated (Aarnio *et al.* 1995; Mecklin and Jarvinen 1991).

Tumor biology

Studies of the genetic basis in sarcomas have revealed two main groups of genetic aberrations; tumor-specific reciprocal translocations sometimes together with structural and/or numerical aberrations, and complex aberrations with distinct intratumor cytogenetic variation. Cytogenetic aberrations result in deregulated or rearranged genes and the tumor type-specific fusion genes that often include transcription factors, e.g. the *EWSR1* gene that has been found to fuse with several sarcoma-associated genes in Ewing sarcoma, myxoid liposarcoma, clear cell sarcoma, extraskeletal myxoid chondrosarcoma, and desmoplastic small round cell tumor (Aman 1999; Mandahl *et al.* 1999; Mitelman *et al.* 2004). In pleomorphic sarcomas, such as MFH, leiomyosarcoma, and pleomorphic liposarcoma the karyotypic picture is generally complex with multiple numerical as well as structural rearrangements (Fletcher *et al.* 2002; Mandahl *et al.* 1999). MSI have been assessed in a few sarcomas, for instance in 11/44 gynecological sarcomas where two cell lines from a uterine mixed mesodermal tumor was further analysed revealing both MSI and *MSH2* mutation (Risinger *et al.* 1995). MSI has also been assessed in a series of mixed sarcoma subtypes, with MSI in 3/39 tumors (Suwa *et al.* 1999), and in 6/36 dermatofibrosarcomas (Takahira *et al.* 2004). There are a few reports of sarcomas that have developed in HNPCC-patients (den Bakker *et al.* 2003; Lynch *et al.* 2003; Sijmons *et al.* 2000), but sarcomas have not been considered part of the HNPCC-associated tumor spectrum.

Materials and Methods

Patients and tumor materials

The Swedish Cancer Registry

Cancer registration in Sweden is based on mandatory reports from both clinicians and pathologists of all cancer diagnoses on surgically removed tissues, biopsies, cytological aspirates, and autopsies. The registry was started in 1958 and is further divided into 6 regional registries located at the oncological centers in Sweden. If a person develops more than one primary tumor, each tumor is registered separately, whereas tumor recurrences are not. Information from death certificates is available to the registry. The registry uses unique personal identification numbers and contains information on sex, domicile, hospital and department, pathology department, specimen number, date, tumor site and the source of diagnosis. In studies I–IV the patients were identified through the regional cancer registry of the southern Swedish health care region (about 1.5 million inhabitants) and data on family history of cancer or blood samples for mutation analysis were not available, since the studies were retrospective and register-based. In study V, we used the national registry.

Tumor materials (table 3)

We used paraffin-embedded tumor blocks (studies I–IV) for the analyses. The original histological reports were retrieved for confirmation of all diagnoses. A 4-μm section was made from the block and stained with hematoxylin and erythrosine (H&E) for routine morphology to verify the diagnosis and that representative; non-necrotic tumor tissue was present in the tumor block. A pathologist reviewed the stainings in studies I-III and selected cases in study IV. If several paraffin-embedded tumor blocks were available, the one with best-preserved and largest amount of tumor material was used. The studies were approved by the ethics committee at Lund University.

Study I

All patients diagnosed with double primary colorectal cancer, either synchronous or metachronous, with at least one additional primary malignancy (n=264) during the time period 1958–2000 were identified. We further selected the 47 patients who had developed at least 4 primary malignancies (at least 2 of which were colorectal cancers) for studies of defective MMR. The material included 30 men and 17 women with a median age at first diagnosis of 65 (26–91) years. These patients had developed 209 tumors, including 133 colorectal cancers. We successfully retrieved 156 tumors, including 114 colorectal cancers, 6 prostate cancers, 10 urothelial cancers, 4 skin cancers, 3 melanomas, 2 endometrial cancers, 4 breast cancers, 3 gastric cancers, 2 cancers of the small intestine, and 8 single tumors of various other types.

Study II

Two partly overlapping tumor series were used; I) a population-based series was studied in order to determine the overall frequency of defective MMR in adenocarcinomas of the small intestine, and II) a series of tumors from patients younger than 60 years at diagnosis was studied regarding the contribution of defective MMR to the development of cancer of the small intestine in younger patients. The H&E stainings were re-evaluated by a gastrointestinal pathologist to confirm a primary tumor origin within the small intestine and to exclude other tumor types than adenocarcinomas. A primary tumor origin within the small intestine was defined as presence of an adenoma-carcinoma transition or of mucosal dysplasia. Furthermore, we required that the diagnosis should be preceded

Table 3. Tumor materials in studies I–IV

	Multiple primary tumors Study I	Cancer of the small Intestine Study II		Cancer of the upper urinary tract Study III	Soft tissue sarcomas Study IV
Year of diagnosis	1958–2000	Series I 1989–1999	Series II 1958–1988	1992–1999	1986–1994
Number of identified individuals	47 patients (209 tumors)	149	54	262	208 [a,b]
Male:female ratio	1.8:1	1:1	1.4:1	1.8:1	1.1:1
Median age (range)	65 (26–91)	69 (21–90)	50 (28–59)	70 (34–90)	71 (19–96)
Number of excluded individuals					
Not successfully retrieved	53	19	20	27	0
Primary origin not verified	0	33	2	0	0
Adenocarcinoma within 2 years of diagnosis	0	2	2	0	0
Autopsy cases/autolysis	0	6	0	19	0
Poor IHC staining quality	0	0	0	0	5
MSI analysis inconclusive	0	0	11	0	0
Number of analyzed tumors	156 from 45 individuals	89	24 from series I and 19 additional	216	204
Tumor location	114 CRC, 10 urothelial cancers, 6 prostate cancers, 4 skin cancers, 3 melanomas, 2 endometrial cancers, 4 breast cancers, 3 gastric cancers, 2 cancers of the small intestine, 1 lung cancer, 1 mesothelioma, 1 ovarian cancer, 1 sarcoma, 1 meningeoma, 1 thyroid cancer, 1 cervical cancer, and 1 cancer of the vagina	46 duodenal carcinomas, 33 tumors of the jejunum or ileum, and 10 tumors of unspecified site	15 duodenal carcinomas, 25 tumors of the jejunum or ileum, and 3 tumors of unspecified site	154 tumors of the renal pelvis, 60 tumors of the ureter, and unspecified location within the urinary tract in 2 cases	136 upper extremity, 48 lower extremity, and 24 trunk wall
MSS	91 (59%)	73 (82%)	33 (77%)	180 (93%)	N/A
MSI-high MSI-low	59 (38%) 4 (3%)	12 (13%) 4 (4%)	6 (14%) 4 (9%)	9 (5%) 5 (3%)	1 (0.5%)
Retained MMR expression	97 (62%)	80 (90%)	36 (84%)	205 (95%)	202 (99%)
MMR protein loss	MLH1: 24 MSH2: 33	MLH1: 2 MLH1/PMS2: 7 MSH2/MSH6: 0	MLH1/PMS2: 4 MSH2/MSH6: 3	MLH1/PMS2: 2 MSH2: 1 MSH6: 2 MSH2/MSH6: 6	MSH2/ MSH6: 2

[a] reviewed by the Scandinavian Sarcoma Group
[b] one individual with two primary tumors

by at least two years free of other adenocarcinomas and, on autopsy cases, no evidence of other tumors, in order to avoid adenocarcinoma metastasis to the small intestine. Neither data on family history of cancer nor blood samples for mutation analysis were available. Patients with HNPCC, FAP or

inflammatory bowel disease were not excluded.

Series I. Between 1989 and 1999, 149 adenocarcinomas of the small intestine were diagnosed in the southern Swedish health care region. We successfully retrieved 130 paraffin-embedded tumor blocks. Of these 41 were excluded; 33 tumors because a primary tumor origin within the small intestine could not be established, 6 because of autolysis or lack of MSI results and 2 cases because of adenocarcinomas at other sites (one case with a hepatobiliary cancer and one case with a colon cancer and a renal cancer) within 2 years of the diagnosis of small bowel cancer. The mean age at diagnosis in the whole series was 69 (21–90) years and among the 89 cases analyzed 68 (21–89) years. The male: female ratio was 1:1. Tumor location was duodenum in 46 tumors (52%), jejunum or ileum in 33 tumors (37%), and an unspecified site within the small intestine in 10 tumors (11%).

Series II. We extended the study to include all individuals diagnosed with adenocarcinomas of the small intestine before age 60 during the period 1958–1988, a total of 54 individuals. Of these, 20 tumor blocks were not possible to locate, 2 cases could not be confirmed to be primary within the small intestine, MSI analysis failed in 11 tumors and 2 cases were excluded because of a diagnosis of adenocarcinoma within 2 years of the small intestinal cancer, which left an additional 19 successfully analyzed samples. The total series, combining the 24 patients from the population-based series and the 19 patients in the extended study, thus included 43 patients diagnosed before age 60. The mean age among the cases analyzed was 50 (21–59) years, the male to female ratio was 1.4:1, and the tumor location was duodenum in 15 tumors (35%), jejunum/ileum in 25 tumors (58%), and an unspecified site within the small intestine in 3 cases (7%).

Study III

All carcinomas of the upper urothelial tract, defined as cancer of the renal pelvis and the ureter that had developed between 1992 and 1999 were identified; 262 patients with a median age of 70 (34–90) years and a male: female ratio of 1.8:1. For further analyses, 27 patients were excluded because of lack of tumor blocks, and 19 were excluded because of autopsy-based diagnosis with autolysis that prevented good quality immunostaining. Hence, 216 patients with a median age of 69 (34–89) years were analyzed. Tumor location was for the whole material (cases analyzed within parenthesis) was: renal pelvis 173 (154), ureter 75 (60), and an unspecified tumor location in 14 (2) patients. Synchronous tumors of the urinary tract developed in 11 patients.

Study IV

The Scandinavian Sarcoma Group (SSG) has since 1986 maintained a sarcoma registry. The registry contains approximately 90% of all diagnosed STS of the extremities and trunk wall in Sweden and Norway. From 1986 through 1994, 682 MFH had been registered and 545 of these were histologically reviewed by the SSG pathology review group. Of these, 164 were excluded; 98 were reclassified as another type of sarcoma, 30 cases were excluded due to insufficient or non-representative material, 21 patients with metastases at diagnosis, and 15 patients with cutaneous tumors, incomplete follow-up or suboptimal treatment were excluded. From the remaining 381 patients, 209 primary tumors from 208 patients were succesfully retrieved. The 209 tumors studied have been re-evaluated by the SSG Pathology Review Group with access to the clinical history and previous pathology reports. When necessary, electron microscopy and extensive immunohistochemical stainings were performed to exclude a demonstrable lineage of differentiation (Meis-Kindblom *et al.* 1999). The antibody panel included muscle specific actin, smooth muscle actin, desmin, S-100, epithelial membrane antigen, EMA, cytokeratin, and several markers specific for melanoma and lymphoma. Malignancy grading was based on an IV-tiered grading system, including the factors cellularity, pleomorphism, nuclear atypia, tumor necrosis, vascular invasion, and mitotic activity. Among the 208 patients included in the study, 109 were men and 99 women. Mean age was 71 (19–96)

years, the male:female ratio was 1.1:1, tumor location was trunk wall in 24 patients, lower extremity in 48, and upper extremity in 136 patients. Of the tumors, 124 were of the storiform-pleomorphic subtype and 82 tumors were classified as myxoid MFH (Fletcher *et al.* 2002).

Study V

In paper V, we used the national Swedish Cancer Registry, through which we identified all potential probands, i.e. individuals who had developed at least one HNPCC-associated cancer, as defined in the Amsterdam II criteria (Vasen *et al.* 1999). These were identified through the international classification of diseases (ICD), version 7, codes. We included the following codes: 152 (small intestine), 153 (colon), 154 (rectum), 172 (corpus uteri), 174 (uterus, part unspecified), 180.1 (renal pelvis), 180.9 (renal pelvis, part unspecified), 181.1 (ureter), 181.8 (urinary organs, multiple locations), and 181.9 (unspecified location within the urinary organs). Anal cancer, intestinal lymphoma, and carcinoid tumors of the small intestine/colorectum were not included. This material was matched to the Swedish Fertility Register and the Swedish National Censuses. The Swedish Fertility Registry contains data on all births in Sweden since 1961 with identification number of mothers, children and fathers who were married to the mother at the time of birth. The Swedish National Censuses contains information on offspring born before 1961 and children to unmarried fathers, thus about relationships in individuals sharing the same household. Accordingly, children born before 1961 and children to unmarried male probands were identified through the National Censuses, whereas the Swedish Fertility Registry identified children who were born after 1960, with female or married male probands. Through these registers, all offspring to individuals with HNPCC-associated cancer were identified, and probands were thus selected on the basis of having offspring. If an individual had two affected parents, the child was only included once in the study cohort. Information regarding malignant tumors, emigration and deaths among the offspring were obtained from the Cancer Registry and from the Swedish Population Registers. Since the Swedish Censuses could not identify children who died before 1961, the follow-up started in January 1961, or at birth for children born thereafter. Follow-up was terminated at death or emigration, or in December 1999, when the study was closed. 102 814 probands, and their 204 358 offspring were identified.

Methods

MSI analysis (studies I–IV, figure 6, table 4)

DNA was extracted from 3 sections (10-µm) of formaline fixed, paraffin-embedded tumor tissue. The sections were incubated at 65 °C with proteinase K (50mM Tris, pH 8.4, 1mM EDTA, 0.5 % Tween-20, 200 µg/mL proteinase K) for at least two hours, followed by 10 minutes of boiling for enzyme inactivation. The samples were then centrifuged for 5 minutes and the aqueous phase was transferred to a new tube. We analyzed the MSI markers BAT25 (an intronic $(T)_{25}$ sequence in the *KIT* gene), BAT26 (an intronic $(A)_{26}$ sequence in the *MSH2* gene), BAT40 (an intronic $(T)_{40}$ tract in the 3-β-hydroxysteroid dehydrogenase gene), BAT34C4 (a $(T)_3C(T)_6C(T)_{17}C(T)_5C(T)_3$ tract in the 3´ non-translated part of the *TP53* gene) and the dinucleotide markers D2S123 (a $(CA)_{13}TA(CA)_{15}(T/GA)_7$ repeat in the *MSH6* gene) and D5S346 (a $(CA)_{26}$ repeat in the *APC* gene) with some variations between the different studies (for primer sequences and additional data see table 4 and studies I–IV). The markers BAT34C4, BAT25, and BAT26 are quasi-monomorphic with allelic size variations rarely exceeding 2 bp. The marker BAT40 rarely shows size variations (-6 to -16 bp, and a -16 bp polymorphism has been described) (Zhou *et al.* 1997). All markers used are among those recommended in the National Cancer Institute reference panel for MSI analysis and have been shown to assess MSI with high accuracy (Boland *et al.* 1998). In order to classify a tumor as MSS, data from at least three markers were required. A tumor was classified as MSI-high if at least two of the markers were instable and MSI-low if only one marker was

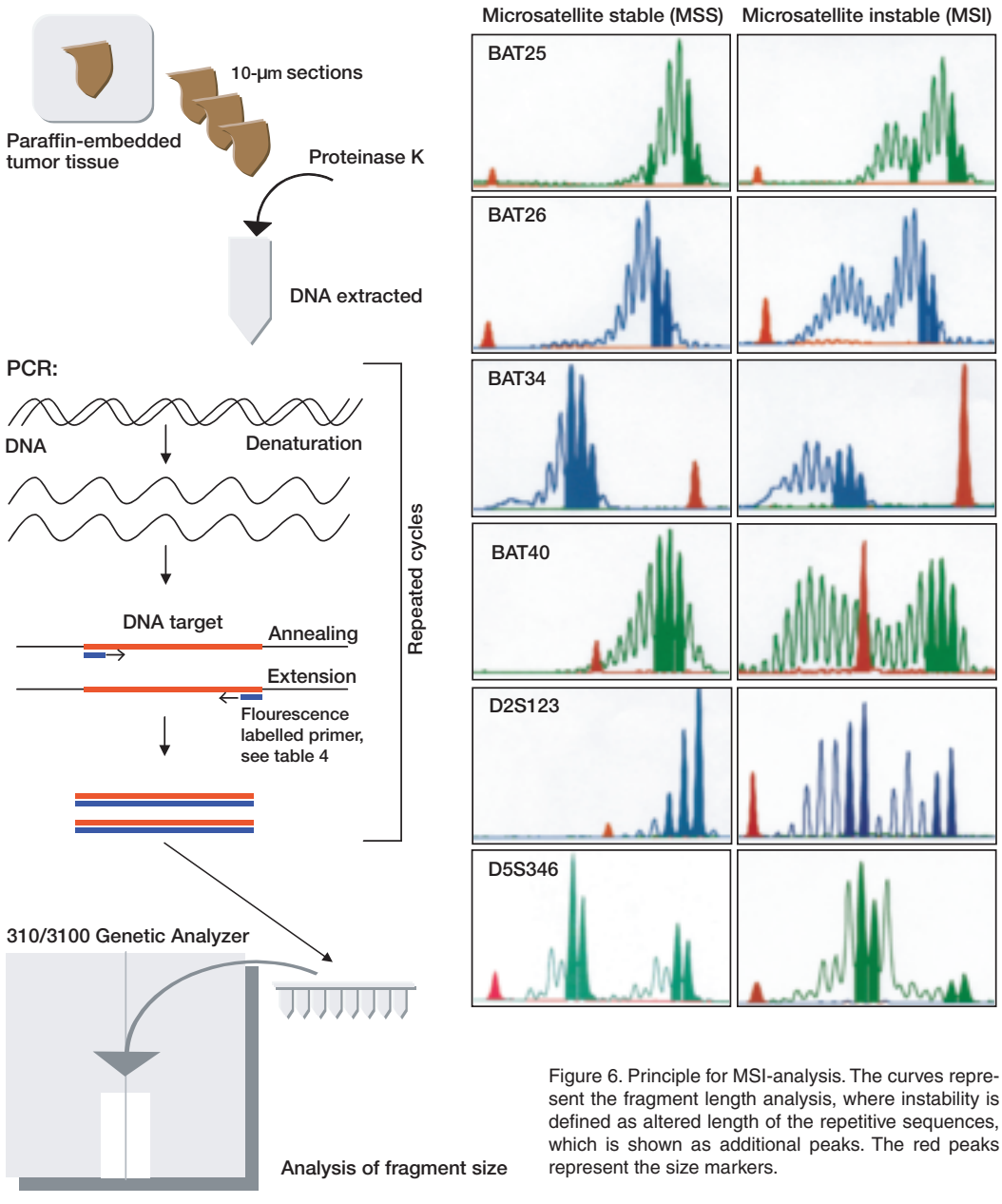


Figure 6. Principle for MSI-analysis. The curves represent the fragment length analysis, where instability is defined as altered length of the repetitive sequences, which is shown as additional peaks. The red peaks represent the size markers.

instable. We aimed at collecting data from at least 5 markers for most of the tumors. The PCR amplifications were performed using AmpliTaq Gold® DNA polymerase from Applied Biosystems Roche (Foster City, CA, USA). The PCR reactions were performed with an initial denaturation at 94 °C for 7 minutes, 10 cycles of denaturation at 94 °C for 15 seconds, annealing at 45 °C or 50 °C for 15 seconds and extension at 72 °C for 15 seconds, followed by 23 cycles with denaturation at 89 °C for 15 seconds, annealing at 45 °C or 50 °C for 15 seconds and extension at 72 °C for 15 seconds. Finally, the

Table 4. Data on markers used for the microsatellite instability analysis

MSI-marker	Repeat	Primer sequence	Gene	Fluorescence
BAT25 mononucleotide 90–125 bp	TTTT.T.TTTT. $(T)_7$.A$(T)_{25}$	F: TCG CCT CCA AGA ATG TAA GT R: TCT GCA TTT TAA CTA TGG CTC	*c-KIT* 4q12	TET (study I–II) NED (study III–IV)
BAT26 mononucleotide 95–121 bp	$(T)_5$.....$(A)_{26}$	F: TGA CTA CTT TTG ACT TCA GCC R: AAC CAT TCA ACA TTT TTA ACC C	*MSH2* 2p22-21	FAM
BAT40 mononucleotide 80–100 bp	TTTT.TT..$(T)_7$ ..... TTTT.$(T)_{40}$	F: ACA ACC CTG CTT TTG TTC CT R: GTA GAG CAA GAC CAC CTT G	*3-β-hydroxylsteroid dehydrogenase* 1p13.1	HEX
BAT34C4 mononucleotide 130–140 bp	$(T)_3C(T)_6C(T)$ $17C(T)_5C(T)_3$	F: ACC CTG GAG GAT TTC ATC TC R: AAC AAA GCG AGA CCC AGT CT	*TP53* 17p13	FAM
D2S123 dinucleotide 197-227 bp	$(CA)_{13}TA(CA)_{15}$ $(T/GA)_7$	F: AAA CAG GAT GCC TGC CTT TA R: GTT TGG ACT TTC CAC CTA TGG GAC	*MSH6* 2p16	FAM
D5S346 dinucleotide 96–122 bp	$(CA)_{26}$	F: ACT CAC TCT AGT GAT AAA TCG R: AGC AGA TAA GAC AGT ATT ACT AGT T	*APC* 5q21-22	HEX

PCR products were subjected to 7 minutes of elongation at 72 °C, followed by a cooling step at 4 °C. The annealing temperature was 45 °C for BAT25 and 50°C for BAT26, BAT40, BAT34C4, D2S123 and D5S346. To verify presence of the correct PCR product these were subjected to electrophoresis in a 7.5% acrylamide gel and stained with ethidium bromide. For the MSI analysis, the PCR products were combined with Hi-Di formamide (Applied Biosystems, Foster City, CA) and Gene Scan™ 500 ROX™ Size Standard, denatured at 95 °C for 2 minutes, chilled on ice and separated in Performance Optimized Polymer-4 (POP-4™, Applied Biosystems) on a ABI PRISM™ 310 or 3100 Genetic Analyzer (Applied Biosystems) for fragment analysis. The two tumors with MMR protein expression loss in study IV were analyzed with the Multiplex System Prototype Kit (Promega, USA) at the department of Clinical Genetics, Karolinska Hospital, Stockholm,

MMR protein immunostaining (studies I–IV, figure 7)

Immunohistochemical staining was performed using 4-µm sections of formaline fixed, paraffin-embedded tissue, mounted on DAKO ChemMate™ Capillary Gap Microscope Slides (Dako Cytomation Norden A/S, Glostrup, Denmark) and dried at room temperature overnight followed by incubation at 60 °C for 1–2 hours. The tissue sections were deparaffinized in xylol and rehydrated through descending concentrations of alcohol. Antigen retrieval was achieved by microwave-treatment in 1 mM EDTA, pH 9.0, at 900 W for 8 minutes followed by 15 minutes at 350 W. The slides were then allowed to cool for at least 20 minutes in the EDTA-solution. Immunohistochemical staining was performed in an automated immunostainer (TechMate™ 500 Plus, DAKO), according to the manufacturer's instructions. The main steps were as follows: mouse monoclonal IgG antibodies to MLH1 (clone G168-15, dilution 1:100, BD Pharmingen, San Diego, CA, USA), MSH2 (clone FE 11, dilution 1:100, Oncogene Research Products, San Diego, CA,, USA), MSH6 (clone 44, dilution 1:1000, BD Transduction Laboratories) and PMS2 (clone: A16-4, dilution 1:500, BD Pharmingen) were applied and the sections were incubated at room temperature for 25 minutes, followed by incubation with biotinylated link antibody (DAKO) for 25 minutes. The MLH1 antibody used recognizes the full-length human MLH1 protein. The epitope for the antibody used for the

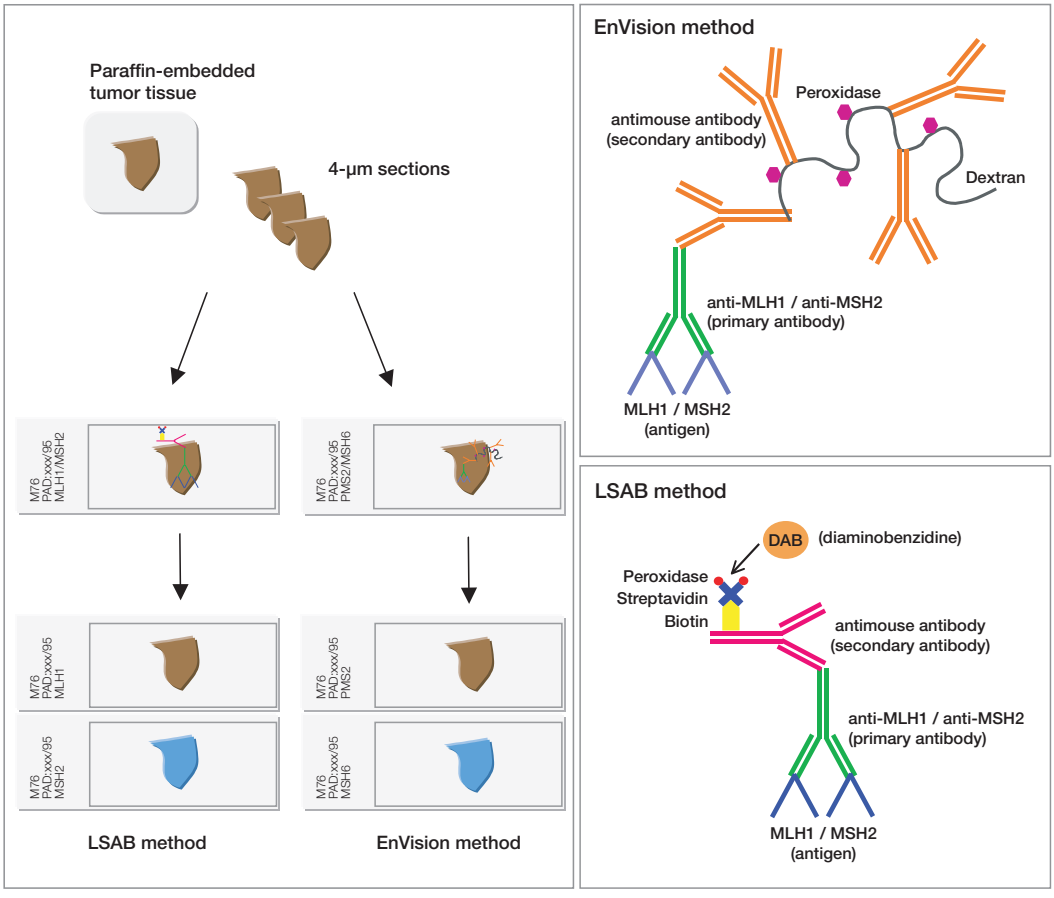


Figure 7. Principles behind the two different methods used for immunohistochemistry in the studies; the LSAB method and the EnVision method.

MSH2 stainings is the carboxyl terminal region of the protein. The immunogenic region of the MSH6 protein is a region close to the N-terminal between codons 225 and 333. For the PMS2 antibody, the immunogenic region is the C-terminal half of the protein.

For MLH1 and MSH2, two staining methods/kits have been used, Linked Streptavidin Biotin Method (LSAB) in studies I and II and EnVison™ in studies III and IV (figure 7). For the MSH6 and PMS2 stainings we always used the EnVision method. The LSAB method for MLH1 and MSH2 included incubation with biotinylated link antibody (DAKO) for 25 minutes, blocking of endogenous peroxidase activity in hydrogen peroxide solution for 3 × 2.5 minutes, followed by incubation with streptavidin conjugated with peroxidase for 25 minutes. Finally, the slides were treated with diaminobenzidine, counterstained with hematoxylin, rinsed in running tap water, dehydrated in ascending concentrations of alcohol and cover slips were mounted. Tris-buffered saline, pH 7.6, with Triton-x-100 was used to rinse the slides between each step. Bovine serum albumine was added to the buffer before the antibody binding step in order to block nonspecific protein binding.

The EnVision method included an extra enhancing incubation step after incubation with the primary antibody in Rabbit-anti-Mouse immunoglobulins (Dako, dilution 1:400) for 20 minutes.

Endogenous peroxidase activity was blocked in Peroxidase-blocking solution (DAKO) for 3 × 2.5 minutes, whereafter the slides were incubated with Dako EnVision™/HRP Rabbit/Mouse for 25 minutes. Finally, the tissue sections were treated with diaminobenzidine (DAB) for 3 × 5 minutes, counterstained with hematoxylin for 1 min, rinsed in running tap water for 10 min, dehydrated in ascending concentrations of alcohol and coverslips were mounted. After each step, the sections were rinsed in Tris-buffered saline, pH 7.4, and Tween-20. Two investigators, who were blinded regarding the MSI status, independently evaluated all stained sections, and difficult cases were reviewed by a pathologist. Sections without nuclear staining in the tumor cells, in the presence of normal nuclear staining in lymphocytes and normal epithelial or stromal cells in the same section, were considered to have a lost expression (figure 6). The expression was classified as present, absent or non-evaluable, thus without grading of the staining intensity.

Correlation between MSI and MMR protein immunostaining

Studies that compare MSI and MMR protein immunostaining have shown a high degree of correlation between these methods. The sensitivity for an abnormal MMR protein staining to detect MMR defects has in larger studies generally been reported to be between 80% and 95%, and the specificity has reached 100% in most studies (Halvarsson *et al.* 2004; Lindor *et al.* 2002; Moslein *et al.* 1996; Rigau *et al.* 2003; Ruszkiewicz *et al.* 2002). Both MSI and MMR protein immunostaining may give inconclusive results due to poor quality of the tumor material such as necrotic or irradiated tissue and use of extensive formalin fixation at high concentration or of long duration. Immunostaining also has the added value of pinpointing the MMR gene affected, and thereby directs the mutation analysis. Several studies have described normal MMR protein expression using immunohistochemistry in about 10% of the MSI tumors and the genetic background to this observation is unknown (Halvarsson *et al.* 2004; Lindor *et al.* 2002; Ruszkiewicz *et al.* 2002). Retained IHC expression has been demonstrated in a small number of tumors from patients known to carry germline MMR gene mutations. Indeed, the sensitivity for an immunohistochemical loss differs between the different MMR proteins; whereas virtually all *MSH2* mutant tumors and most *MSH6* mutant tumors loose expression, *MLH1* mutations may more often be accompanied by a normal immunostaining (de Leeuw *et al.* 2000), which is probably due to a higher number of HNPCC-associated missense mutations in *MLH1*

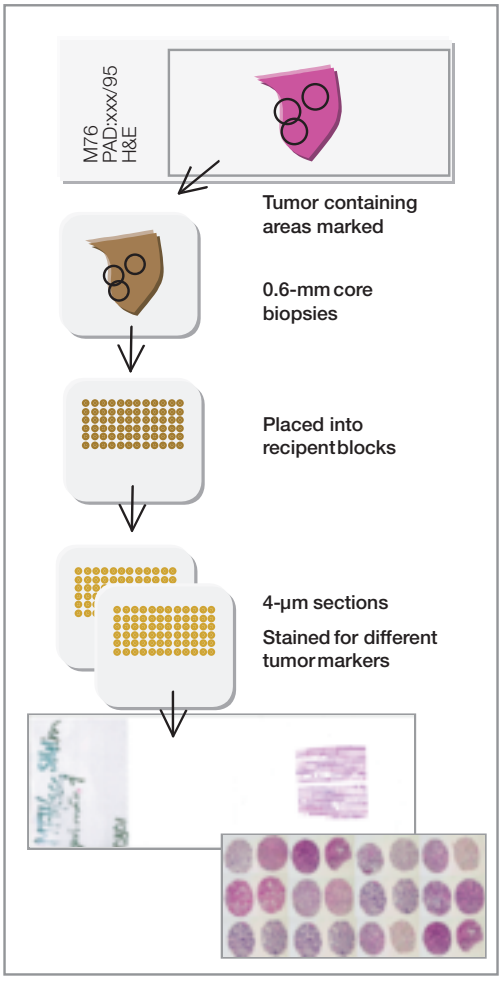


Figure 8. The tissue microarray technique. Multiple biopsies from tumor paraffin blocks are placed into a recipient block, which allows analysis of multiple samples on a single slide.

than in the other genes. Certain mutations may affect the enzyme activity, but still allow production of a stable and immunoreactive, albeit non-functional MMR protein. Generally MSS and MSI-low tumors show retained expression, although occasional such tumors with loss of immunostaining have been reported (Müller *et al.* 2004; Müller *et al.* 2004). Thus, MMR protein immunostaining is a valuable complement to the MSI analysis in the diagnosis of HNPCC, and identifies MMR defects with high sensitivity and specificity. Immunostaining can be used as the first screening method, at least in families fulfilling the Amsterdam II criteria. However, tumors with retained, normal expression will also need to be characterized using MSI analysis until the genetic, clinical and technical background to the discrepant cases with MSI and normal immunohistochemical expression for the MMR proteins have been characterized. However, most data are derived from studies in HNPCC-associated tumors and in colorectal tumors and only a few studies have applied MSI analysis to the rare tumor types studied herein (Amira *et al.* 2003; Blaszyk *et al.* 2002; Goodfellow *et al.* 2003; Hartmann *et al.* 2002; Kariv and Arber 2003; Pal *et al.* 1998; Rashid and Hamilton 1997). We therefore chose to use MSI in combination with MMR protein immunostaining in order to obtain as high accuracy as possible in the determinations of MMR-defective tumors.

Tissue microarray (study IV, figure 8)

The tissue microarray (TMA) technique was developed in 1998 for high-throughput analysis of multiple tumor samples in single experiments (Kononen *et al.* 1998). Viable and representative tumor areas are marked and 0.6-mm core biopsies are punched out from the corresponding area in the paraffin-embedded tumor block using an arrayer (Beecher Instruments, MD, USA) and are positioned in a recipent paraffin array block. The TMA slides were immunostained using antibodies to the MMR proteins MLH1, MSH2 and MSH6, and immunostaining for the MMR proteins has previously been validated (Hendriks *et al.* 2003). However, tumors that showed inconclusive staining or suspected loss of staining in the TMA sections were subjected to immunostaining using whole-tissue sections in order to confirm the results. Tumors that showed loss of the MMR proteins were selected for MSI analysis.

Standardized incidence ratios and cumulative risks (study V)

Standardized incidence ratios, SIRs (the observed number of cancer cases divided by the expected number of cases), were calculated for individuals whose parents were affected with HNPCC-associated tumors in study V. The expected number of cases was calculated by multiplying the person years at risk (total observation time added over subjects) with the Swedish cancer incidence, stratified by sex, calendar year, and 5-year age groups. Individuals may thus have been included in the analysis both directly as cohort members contributing to the follow-up time and, indirectly, as part of the exposure. We assumed that the relative risk was the same before and after the dates of the parents'/siblings' diagnoses. The method for choosing time scale, and for constructing the cohort of individuals with a parent and at least one sibling with HNPCC-associated cancer, has earlier been described in Anderson *et al.* (2000). Confidence intervals and p-values for the SIRs were calculated by assuming that the observed cases were independent and followed a Poisson distribution. However, since there is dependence between siblings, the assumption about independence is not valid, and we have therefore also calculated confidence intervals and p-values accounting for dependence (Anderson *et al.* 2000).

The cumulative incidence of HNPCC-associated tumors was calculated using the life table method. The follow-up period of observations was divided into a series of time intervals, the conditional interval-specific survival proportion for each interval was estimated, and the cumulative survival function was given at the end of a certain interval by the product of the interval-specific survival proportions of that interval and the preceding

Results and Discussion

Study I

Development of multiple primary tumors, including colorectal tumors, are in 1/3 associated with MMR defects and 2/5 of these patients may carry HNPCC.

HNPCC patients have an increased cancer risk in several organs and approximately 1/3 of the patients develop multiple primary malignancies (Aarnio *et al.* 1995; Beck *et al.* 1997; Bittorf *et al.* 2001; Brown *et al.* 1998; Ueno *et al.* 2003). In the national Swedish Cancer Registry, 91% of the patients have developed one neoplasm, 8% two, and 1% of the patients developed at least three neoplasms (National Board of Health and Welfare; Talbäck 2002). This is also in accordance with estimates that 5.2% of cancer patients develop a second primary cancer, among which gastrointestinal tumors were the most common irrespective of the primary cancer site (Ueno *et al.* 2003). Regarding colorectal cancer, 98% of the patients in the national Swedish Cancer Registry developed one colorectal cancer, 2% developed two colorectal cancers (synchronous or metachronous) and about 0.1% of the patients had three or more colorectal cancers (National Board of Health and Welfare; Talbäck 2002). Other studies have reported that 1–3% of colorectal cancer patients develop metachronous or synchronous tumors (Chiang *et al.* 2004; Wang *et al.* 2004), that 4% of all cancer patients develop other malignancies (Bittorf *et al.* 2001), and a risk of 0.1% for a third primary cancer (Bittorf *et al.* 2001). Colorectal cancer patients are at increased risk of developing also cancers of the endometrium, urothelium, small intestine, ovary, stomach, i.e. tumor types associated with HNPCC (Aarnio *et al.* 1995; Brown *et al.* 1998; Cali *et al.* 1993; Cederquist *et al.* 2001; Enblad *et al.* 1990; Shih *et al.* 2002; Vasen and Wijnen 1999). Male sex, a previous history of colorectal cancer or colorectal adenoma, proximally located tumors, mucinous carcinomas, and a family history of gastric carcinoma have been identified as general risk factors for development of second primary colorectal cancers (Pinol *et al.* 2004). Indeed, several of these features occur in HNPCC (Jass 2000).

MSI status range from 21–32% in synchronous colorectal cancers (Dykes *et al.* 2003; Norrie *et al.* 2002; Pedroni *et al.* 1999) and 25–89% in metachronous colorectal cancers (Masubuchi *et al.* 1999; Pedroni *et al.* 1999; Sengupta *et al.* 1997). MSI has also been assessed in patients who have developed double primary tumors of the stomach and colorectum with MSI found in 18–50% of the cases (Kim *et al.* 2001; Ohtani *et al.* 2000). Among patients with endometrial cancer, 18% of those who had developed MSI tumors without *MLH1* promotor methylation, had synchronous or metachronous HNPCC-associated cancer, compared to 4.5% of patients with MSI tumors with *MLH1* promotor methylation, and 2.1% with MSS endometrial cancers (Buttin *et al.* 2004). Investigations of patients with double primary tumors of the colorectum and endometrium have revealed an association with defective MMR in over 40% of the patients (Cederquist *et al.* 2004; Planck *et al.* 2002) and mutations have been found in 60% of patients with MSI tumors of the colorectum and the endometrium (Cederquist *et al.* 2004).

We assessed the contribution of defective MMR in a population-based series of patients who had developed multiple primary tumors, including two colorectal cancers. The tumors were investigated using MSI analysis and immunostaining for MMR protein expression. From the regional cancer registry, we identified all individuals (n=264) with at least three primary tumors, including at least two metachronous and/or synchronous colorectal cancers during 1958–2000. The most frequent additional tumor types were cancer of the prostate, skin

and the urothelium (study I, figure 1). We selected the 47 patients who had developed at least 4 primary tumors, including two colorectal cancers, for further analysis. Of the 209 tumors in this group, 156 tumors from 45 patients were successfully retrieved and analyzed. Two tumors were not evaluable for MSI analysis, and two tumors were not evaluable for MMR protein expression. MSI-high was present in 59/154 (38%) of the tumors and affected tumors of the colon, rectum, endometrium, urothelium, small intestine, and stomach, i.e. tumor types associated with HNPCC. Among the evaluable MSS tumors 87/89 (98%) showed retained MMR protein expression, whereas two tumors showed loss of MSH2 expression. In 5 patients occasional tumors showed MSI and/or loss of MLH1 expression, which suggests somatic inactivation (study I, table 3). Retained MMR protein expression of MLH1 and MSH2 was seen in 8/63 (13%) MSI tumors, although staining for MSH6 and PMS2 was not available at the time for the study. In total, 57/154 (37%) of the tumors showed loss of one of the MMR proteins, and 87% of the MSI tumors showed loss (study I, table 1). Among the MSI tumors with MMR protein expression loss, MLH1 was affected in 24 cases and MSH2 in 33 cases. The equal distribution of loss of expression in MLH1 and MSH2 in this patient group is in accordance with mutations in these genes, being found at approximately equal frequencies in HNPCC (Beck *et al.* 1997; Mitchell *et al.* 2002; Peltomäki and Vasen 2004).

A concordant pattern with MSI and IHC loss affecting the same MMR protein in several tumors was found in 17/45 patients and suggests that these cases may be associated with HNPCC (study I, table 2 and figure 2). Concordant MMR protein loss affected MSH2 in 9 cases and MLH1 8 cases. These 17 patients developed the first tumor at mean age of 54 (26–80) years compared to a mean age of 72 years among the remaining 28 patients and a mean age of 65 years at first diagnosis among all 47 patients. Indeed, 10/17 had their first cancer diagnosis after age 50, which suggests that development of metachronous cancers is an independent hallmark of HNPCC and that such patients should not be deferred from genetic analysis because of high age of onset. Since the general lifetime risk of colorectal cancer is 3–5%, sporadic tumors will appear also in families with HNPCC. We found 12 MSS tumors with retained MMR protein expression in 8 patients, whose other tumors were MMR deficient, which suggests that these MSS tumors were not associated with HNPCC, although they developed in patients likely to carry HNPCC. Although likely to be a rare finding these cases suggest that when HNPCC is strongly suspected and the genetic analysis does not show MSI and/or immunohistochemical loss, investigation of an additional tumor from the family may be worth considering.

In summary, this study demonstrates a high frequency of MSI and immunohistochemical loss of MMR protein expression in patients with multiple primary tumors including at least two colorectal cancers. Multiple MSI tumors with a concordant loss of MMR protein expression in the tumor tissue, a phenotype that strongly suggests HNPCC, developed in 17/45 (38%) evaluable patients, 10/17 of whom developed the first tumor after age 50. The Amsterdam II criteria for the classificiation of HNPCC might exclude patients with multiple primary tumors but a weak family history and a higher age at onset, although our findings suggest that the development of multiple HNPCC-associated tumors in an individual is a strong indicator of HNPCC, irrespective of age at onset.

Study II

Cancer of the small intestine display defective MMR at a frequency equal to colon cancer. We found 18% MSI, which is in concordance with previous findings. The majority of cases were caused by MLH1 inactivation, although MSH6 and MSH2 were inactivated at higher frequencies in young individuals.

The knowledge of genetic mechanisms in cancer of the small intestine is limited, although characterization of e.g. *APC*-mutations (lower frequency

compared to CRC), *KRAS* mutations (similar frequency), *TP53*-alterations, (similar frequency) and other alterations found in colorectal cancer have been reported (Muneyuki *et al.* 2000; Murata *et al.* 2000). Data on MSI and MMR-deficiency have been confined to smaller studies that have totally identified 27/120 (23%) MSI tumors within the small intestine (Hibi *et al.* 1995; Kim *et al.* 2003; Muneyuki *et al.* 2000; Murata *et al.* 2000; Park *et al.* 2003; Rashid and Hamilton 1997; Wheeler *et al.* 2000). However, these studies have not evaluated MMR protein expression and therefore the relative contribution to the different MMR proteins has not been described. Kim *et al.* evaluated MSI and methylation status of the *MLH1* promotor in 12 duodenal carcinomas, and found 4 tumors with MSI, 3 of which showed *MLH1* promotor methylation (Kim *et al.* 2003). Partly contradictory results have been found in cancer of the biliary tract, Nagai *et al.* identified MSI among 70% (16/23) of analyzed tumors (Nagai *et al.* 1999), whereas Kim *et al.* did not find any MSI tumors among 18 cancers of the biliary tract (Kim *et al.* 2003).

We assessed the MSI frequency in a population-based series of 89 tumors of the small intestine and in a partly overlapping series of 43 tumors that developed before age 60. Since study II was performed, we have also assessed MSH6 expression in all tumors and PMS2 expression in tumors with MSI or MMR protein expression loss.

In the population-based series, 73 (82%) tumors were MSS, 4/89 (4%) MSI-low and 12 (13%) were MSI-high. 73 MSS tumors in series I showed retained expression of MSH2 and MSH6, whereas one MSS tumor showed expression loss of MLH1 and one MSS tumor showed expression loss of both MLH1 and PMS2. Among the 16 MSI tumors, 7 tumors showed loss of expression for MLH1 and PMS2, one tumor showed loss of MSH6 expression, one tumor showed loss of PMS2 expression and 6 tumors showed retained expression of all 4 proteins (including 3 MSI-low cases) (table 5). One MSI-high tumor was not evaluable for MMR protein expression.

Furthermore, we assessed the contribution of defective MMR in tumors from younger patients in an extended series which included 24 patients from series I, and an additional 19 patients who developed cancer of the small intestine before age 60. Among the young patients, 10/43 (23%) tumors showed MSI and 9/43 tumors showed MMR protein loss, which affected MLH1/PMS2 in 4 cases, MSH6 in 2 cases, and MSH2/MSH6 in 3 cases (table 5). Thus, defective MMR occurs in a somewhat higher frequency among younger patients.

Altogether, in series I and II, MSI and IHC staining pattern were discordant in 9 tumors; 2 MSS tumors showed loss of MMR expression (which affected MLH1 in 1 case and MLH1/PMS2 in 1 case) and 4 MSI-low tumors and 3 MSI-high tumors showed retained MMR protein expression (table 5). Normal immunohistochemical staining patterns and lack of MMR gene mutations have previously been reported in the majority of MSI-low tumors, indicating that these different degrees of MSI also reflect separate tumorigenic mechanisms (Dietmaier *et al.* 1997; Pawlik *et al.* 2004; Young *et al.* 2001). The significance of MSI-low tumors have been questioned since many tumors will have a MSI-low phenotype if large number of MSI markers is investigated (Tomlinson *et al.* 2002). MSI-low status has previously been associated with *MSH6* mutation (Wu *et al.* 1999), but we found retained MSH6 expression in 4/5 evaluable MSI-low cases. 4/6 MSI-low tumors were positive for BAT40 only, a marker where polymorphisms are common in the population. Loss of MSH2 expression is almost exclusively associated with HNPCC (Young *et al.* 2001), whereas loss of MLH1 is associated either with mutation or with sporadic hypermethylation of the promotor (Kane *et al.* 1997; Marcus *et al.* 1999). A subset of the small intestinal tumors in this study is probably associated with HNPCC, and thus a family or individual history of cancer should always be obtained from these patients. Our findings suggest that MLH1 silencing is, like in other types of gastrointestinal tumors, the main mechanism behind defective MMR in cancers of the small intestine, but also, based on the 3 MSI-high tumors with retained MSH2 and MLH1 expression in our study, indicate that other mechanisms or other genes may be causative in the

Table 5. Tumors with MSI and/or immunohistochemical MMR protein loss in study II

Series	Pat No	MSI	MLH1	PMS2	MSH2	MSH6	Age	Sex	Tumor location
I	X12	high	-	-	+	+	74	f	jejunum
	X15	high	+	-	+	+	66	m	duodenum
	X18	low*	+	+	+	+	57 [a]	f	ileum
	X33	high	-	-	+	+	80	m	duodenum
	X42	high	+	+	+	♦	48 [a]	f	ileum
	X53	MSS	-	-	+	+	52 [a]	f	duodenum
	X57	low*	+	+	+	+	77	f	duodenum
	X65	low	-	-	+	IC	57 [a]	m	duodenum
	X68	high	IC	IC	IC	IC	85	f	jejunum
	X74	high	+	+	+	+	63	m	duodenum
	X79	high	+	+	+	+	77	m	duodenum
	X88	high	-	-	+	+	69	f	jejunum
	X90	low	+	+	+	+	65	f	jejunum
	X91	high	-	-	+	+	54 [a]	m	NOS
	X95	MSS	-	+	+	+	71	f	duodenum
	X97	high	-	-	+	+	74	f	duodenum
	X98	high	+	+	+	+	65	f	NOS
	X99	high	-	-	+	+	63	m	jejunum
II	X115	low*	+	+	+	♦	57 [a]	m	jejunum
	X123	high	+	+	-	-	45 [a]	m	duodenum
	X131	low*	+	+	+	+	57 [a]	m	ileum
	X134	high	+	+	-	-	54 [a]	f	jejunum/ileum
	X138	high	-	-	+	+	58 [a]	f	jejunum
	X139	high	+	+	-	-	56 [a]	f	jejunum/ileum

Abbreviations:
* positive for BAT40 only
+ = retained MMR protein expression, – = loss of MMR protein expression
MSS = Microsatellite stable
IC = inconclusive
♦ = overall loss, but reduced expression in a few cells
m = male, f = female
[a] individual below age 60 at diagnosis
NOS = Not otherwise specified

remaining MSI tumors of the small intestine. The MSH6 and PMS2 stainings revealed 3 cases with MMR protein loss that have not been identified by the MLH1 and MSH2 stainings (table 5).

In summary, we have in a population-based series of carcinomas of the small intestine demonstrated MSI in 18% of the tumors and the contribution of defective MMR to the carcinogenesis in the small intestine is thus similar to that observed in the large intestine. However, whereas *MLH1* is found to be defective in the vast majority of MSI colorectal cancers, our results indicate that only about half of the MSI tumors show loss of MLH1 expression. In patients with carcinomas of the small intestine before age 60, MSI was detected in 23% of the tumors and in these tumors immunohistochemistry revealed loss of MSH2/MSH6 at higher frequency compared to MLH1/PMS2. Since somatic mutations are rare in *MSH2*, these findings suggest that a subset of these tumors are HNPCC-associated. Thus, in patients with early onset small bowel cancer and/or a family history suggesting HNPCC, a combined analysis of MSI and immunohistochemical MMR protein staining may be a valuable diagnostic tool.

Study III

Defective MMR occur at a low frequency in tumors of the upper urinary tract. We found MSI in 5% of

UUC, and loss of MMR protein expression, which most often affected MSH2, in 5% of the tumors. Our data suggest that MMR defects represent a minor tumorigenic pathway in the development of UUC.

Several studies describe familial clustering of urothelial cell carcinoma (Kiemeney *et al.* 1997; Kiemeney and Schoenberg 1996; Kramer *et al.* 1991) and a two-fold increased risk has been described among first-degree relatives to patients with urothelial cell carcinoma (Aben *et al.* 2002). Patients with HNPCC are at 2–5% lifetime risk of developing cancer of the upper urinary tract, and we have in a population-based series assessed the contribution of defective MMR to UUC. Sijmons *et al.* have, in HNPCC-patients from the Dutch HNPCC registry, calculated relative risks of developing cancer of the renal pelvis and the ureter; to be 14 (95% CI 6.7–30, with a mean age of 58 years) and bladder cancer; to be 1.5 (95% CI 0.6–3.6, thus not significantly increased) (Sijmons *et al.* 1998). This suggests that these sites may differ in susceptibility, and indicate an increased risk of cancer in the renal pelvis and the ureter in HNPCC individuals (Sijmons *et al.* 1998). Most studies of MMR in urothelial cancer have analyzed transitional cell carcinoma of the bladder, whereas data on tumors in the upper urinary tract are scarce.

We applied the southern Swedish part of the cancer registry to assess the contribution of defective MMR to the development of UUC in a population-based patient material. The results are based on 216/262 (82%) of the tumors that occurred in the southern Sweden health care region between 1992 and 1999. A MSI-high phenotype was found in 9/194 (5%) evaluable tumors and a MSI-low phenotype in 5/194 (3%). The overall frequency of MSI in UUC tumors in our study (with 5% MMR defects in renal pelvis tumors), is in accordance with the 5–8% previously reported, although we identified MMR defects in a lower fraction, 5%, of the urothelial tumors compared to the 25–41% previously reported (Amira *et al.* 2003; Catto *et al.* 2003; Hartmann *et al.* 2002). In other tumor types, methylation of the *MLH1* promoter causes the majority of the sporadic cases, whereas *MSH2* and *MLH1* contribute at about equally to the HNPCC cases (Cunningham *et al.* 1998; Gurin *et al.* 1999; Kane *et al.* 1997; Peltomäki and Vasen 2004; Salvesen *et al.* 2000). Methylation of the *MLH1* promotor has not been reported in sporadic cancers of the upper urinary tract, and thus the fraction of UUC tumors with MSI that are dependent on heredity in unknown. In this study, loss of MMR protein expression was found in 11/216 of the cases, and affected MSH2 and MSH6 in 6 tumors, MSH2 only in one tumor, MSH6 only in two tumors, and MLH1 and PMS2 in two cases (study IV, table 1 and figure 1). Thus the majority of MMR protein expression losses affect MSH2, which suggests germline mutations rather than somatic inactivation. Multifocal tumors occur within the urinary tract (Jones and Droller 1993), and different theories exist for their development; the first indicates that the urinary tract shares the same carcinogenic insults and that independent clones transform into tumors—field cancerization—and the second implies intraepithelial migration or intraluminal dispersion of tumor cells (Habuchi *et al.* 1993). In 11/216 patients synchronous tumors occurred within the urothelial tract and one patient had synchronous MSI-high tumors, all of which displayed a concordant immunohistochemical loss of MLH1. Thus, the vast majority of synchronous UUC does not display MMR defects and do not develop within the HNPCC syndrome.

An increased incidence of metachronous tumors has been observed in patients with MMR-defective UUC (Roupret *et al.* 2004). Of the 8 patients with MSI tumors in our study, 5 had developed metachronous malignant tumors, including two cancers of the urinary bladder, one colon cancer, one rectal cancer, one endometrial cancer, one soft tissue sarcoma, and one patient had developed myelofibrosis (study III, table 1). Among these neoplasms, 5 could be retrieved and assessed for MMR protein immunostaining. The leiomyosarcoma, the colon tumor, the endometrial cancer and two bladder tumors also showed loss of expression for MSH2 and MSH6 (figure 9). About 1/3 of HNPCC patients develop metachronous primary

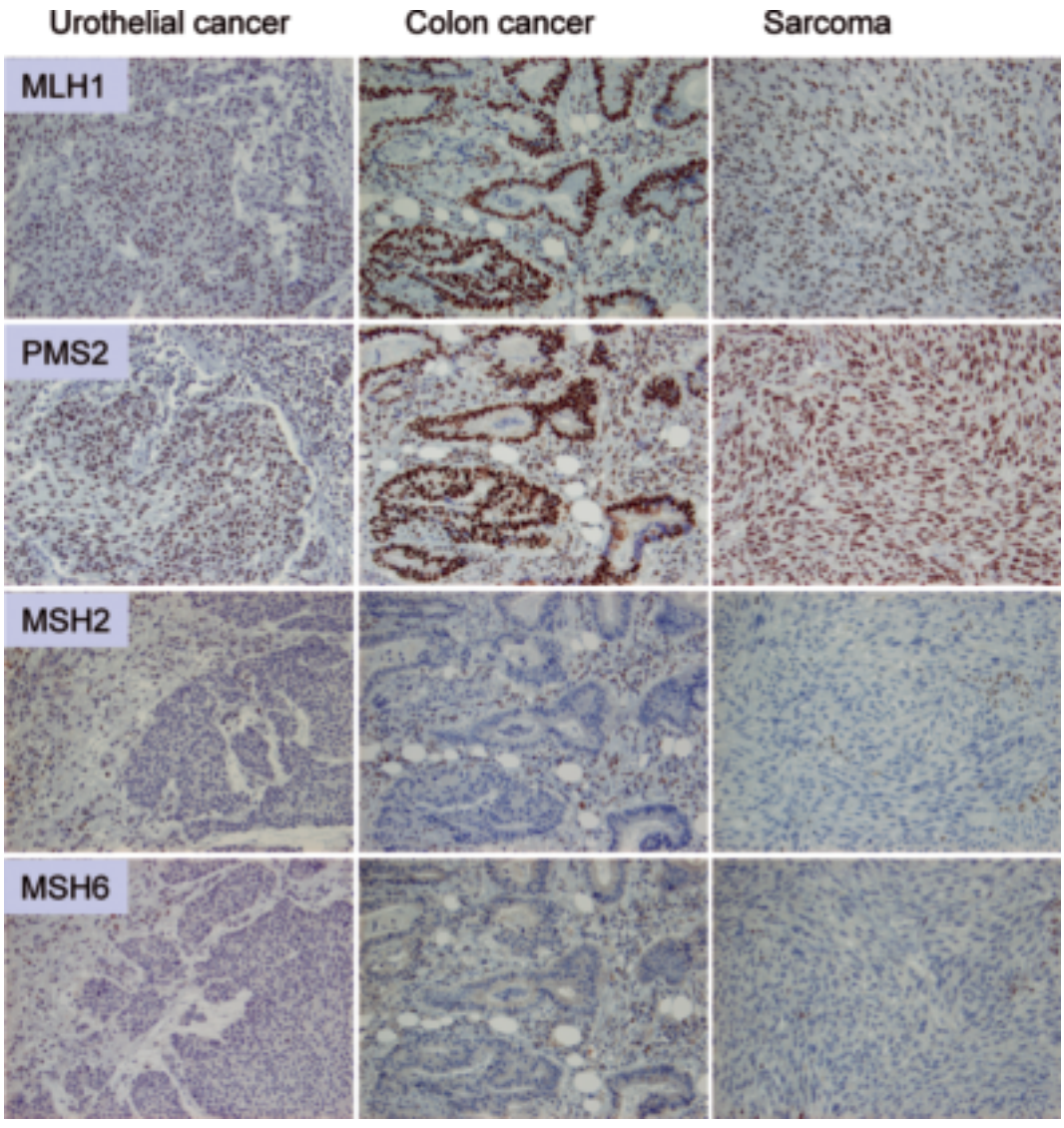


Figure 9. Immunostaining in case RP1-101 (study V). The patient had developed metachronous cancer of the upper urinary tract, the colon, and a sarcoma, all of which showed loss of nuclear expression of MSH2 and MSH6, and retained expression of MLH1 and PMS2.

tumors, and the concordant MSI and loss of MMR protein expression in these cases strongly suggests HNPCC although mutation analysis could not be performed. Although our data suggest that MMR defects represent a minor tumorigenic pathway in the development of UUC. The high frequency of MSH2/MSH6 loss in MSI tumors found in this study should caution clinicians to obtain an individual and a family history of cancer in patients with carcinomas of the renal pelvis and the ureter.

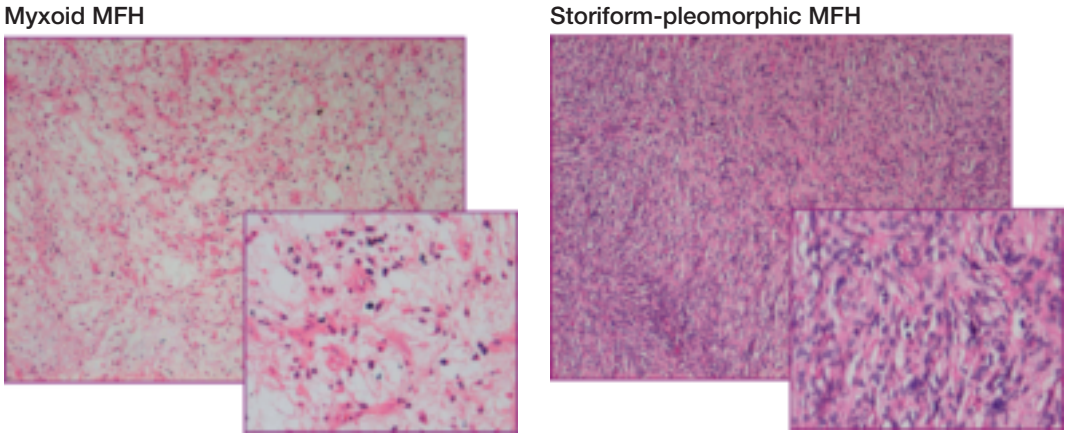


Figure 10. Hematoxylin and erythrosine staining of the two sarcomas (study IV) that were found to have expression loss of MSH2 and MSH6.

Study IV

Occasional soft tissue sarcomas develop due to defective MMR and may be associated with HNPCC. MMR deficiency was demonstrated in 2/209 malignant fibrous histiocytomas.

Soft tissue sarcomas have been associated with rare genetic syndromes such as Li-Fraumeni syndrome, Werner syndrome, Rothmund-Thompson syndrome, and Bloom syndrome (Goto *et al.* 1996; Li *et al.* 1991; Lynch *et al.* 2003; Mecklin and Jarvinen 1991; Vennos *et al.* 1992). There are reports of 5 cases of sarcomas in HNPCC patients, including two pleomorphic rabdomyosarcomas, two malignant histiocytomas and one osteosarcoma. Of these patients, three were known *MSH2* mutation carriers, one had a *MSH2* mutation in the family but was not tested herself, and one tumor evolved in a 19-year old individual from a family fulfilling the Amsterdam II criteria (den Bakker *et al.* 2003; Lynch *et al.* 2003; Sijmons *et al.* 2000).

The lifetime risk of sarcoma development has been estimated to 1–2% in HNPCC patients (Aarnio *et al.* 1995; Mecklin and Jarvinen 1991). We investigated the involvement of defective MMR in soft tissue sarcoma through immunohistochemical stainings for the MMR proteins in a TMA series of 209 MFH from 208 patients. Totally, 202 tumors were classified to show retained expression of all three proteins, 5 cases showed inconclusive immunostainings and two cases showed nuclear expression loss of MSH2 and MSH6 with retained expression of MLH1. The two tumors with loss of MSH2 and MSH6 were both high-grade MFH (figure 10 and study IV, figure 1). The 20-cm storiform-pleomorphic MFH developed in the left thigh of a 96-year-old woman who had not previously been affected by cancer. However, both her father and her brother died from rectal cancer in their seventies, but their tumors were not available for analysis. The myxoid MFH was a 7-cm tumor that developed in the right biceps muscle in a 77-year-old man, who had previously been diagnosed with a prostate cancer. A family history of cancer was not possible to obtain since he was of non-Scandinavian origin and had no children. MSI analysis showed MSI for the markers NR21 and D5S346 in the myxoid MFH, whereas the storiform-pleomorphic MFH did not show MSI for the markers analyzed. Our findings, together with the previous reports of MMR-defective sarcomas with loss of MSH2, suggest that sarcomas may represent a rare tumor type in HNPCC-families. So far, the cases described have specifically been associated with inactivation of MSH2/MSH6 and, indeed, the MSH2 gene has been postulated as the preferred site of germline mutations in HNPCC families with a high frequency of extraintestinal tumors (Wagner

et al. 2001). After performing this study, we have found one additional case of MMR protein expression loss in a leiomyosarcoma (study III, case RP1-101, figure 9).

In summary, a subset of sarcomas develop through defective MMR, and sarcomas may occur as a rare tumor within the HNPCC tumor spectrum. This implicates that HNPCC-testing can be performed in family members who have developed STS and suggests that genetic counseling should be offered to patients with soft tissue sarcomas who reveal a family history of HNPCC-associated tumors.

Study V

We assessed the risk of cancer in a nationwide, population-based cohort of individuals whose parent developed at least one of the tumor types included in the Amsterdam II criteria for diagnosis of HNPCC. HNPCC-associated malignancy in a parent conferred highly increased risks for several cancer types, with the highest risks attributable to young age at parental diagnosis, occurrence of more than one cancer in a parent, or cancer in both a parent and a sibling.

In many families with a familial aggregation of cancer, the underlying genetic mechanism remains to be identified. Concerning colorectal cancer, the most well-characterized syndromes, HNPCC and FAP, account for only 3–6% of the cancers (Aaltonen *et al.* 1998; Lynch and de la Chapelle 1999). In the families where the underlying genetic mechanism is unknown, cancer risk assessments are based on pedigree information, using epidemiological data that have demonstrated various cancer risks calculated in large cohorts. Familial cancer risks have been calculated in previous epidemiological studies, which have assessed the familial risk in first-degree relatives of colon cancer patients to 2 (Carstensen *et al.* 1996; Fuchs *et al.* 1994; Hemminki and Vaittinen 1999), and endometrial cancer to 1.5–3 (Gruber and Thompson 1996; Parazzini *et al.* 1994). Hemminki *et al.,* who in a previous study had assessed increased cancer risks at concordant sites for offspring to parents with various cancers, calculated the proportion of cases attributable to a family history of colorectal cancer to be 5% in the population. Based on information on proximal cancer site within the colon, age at colorectal cancer, and occurrence of extraintestinal HNPCC-associated tumors, they estimated that HNPCC accounted for 20–50% of those familial cases, which thus correlated to an overall HNPCC-frequency of 1–2.5% of all colorectal cancer cases (Hemminki and Li 2001).

We made a population-based risk estimate of familial cancer risks of tumors associated with HNPCC, and have through different Swedish population registries identified the children of individuals who had developed HNPCC-associated cancer, as defined in the revised Amsterdam criteria (Vasen *et al.* 1999), including cancer of the colon, rectum, endometrium, small intestine, and upper urinary tract. The cohort was further analyzed in six disjoint groups, determined by age at diagnosis in the parent, development of multiple malignancies in the parent, or occurrence of cancers among siblings, i.e. factors known to increase the probability of heredity. Since this thesis focus on tumors associated with HNPCC, we have stressed these aspects in the discussion below, but naturally, observed increased cancer risks in our study can also be explained by shared environmental factors, recessive mechanisms, modifier genes or other modes of familial aggregation.

Previously published Swedish population-based familial studies (Hemminki and Li 2001; Hemminki and Vaittinen 1999) have been based on the Swedish multi-generation registry, a registry of individuals born after 1931 and their parents. A shortcoming of the multi-generation registry in studies calculating familial risk of diseases is lack of information from parents to individuals who have died before 1999 and thus might have died from the outcome studied. With our study design we are not able to trace all the offspring to individuals with HNPCC-associated tumours, but, importantly, there is no correlation between the studied outcome and whether the offspring are identified.

Table 6. Overview of the cohort and selected subgroups

	The whole cohort	Parent diagnosed <50	Parent one tumor diagnosed <50	Multiple tumors in parent		Parent and sibling affected	
				≥50	<50	≥50	<50
Number of parents	102 814	8855	8421	2764	294	420	452
Number of offspring	204 358	18675	17677	5542	559	784	904
Number of offspring who developed cancer	8765	466	395	292	28	63	82

Altogether, the 102 814 parents with HNPCC-associated cancer in the cancer registry during the time period of 1958–1999 had 204 358 children, 8 765 of whom developed cancer (table 6). There were 48 141 colon cancers, 18 542 rectal cancers, 21 589 endometrial cancers, 1 473 cancers of the small intestine, and 5 013 cancers of the upper urinary tract among the parents. Altogether, 8 855 (9%) of the parents were diagnosed before 50 years of age, and 3 058 (3%) of the parents developed multiple HNPCC-associated malignancies (294 of whom had the first diagnosis before 50 years of age). The cohort contained 9 337 malignancies, of which 1 317 were classified as HNPCC-associated. In all, 18 675 individuals had parents diagnosed before the age of 50 (of whom 559 belong to the group with multiple tumors in parent and 904 belong to the group with both affected parent and sibling), and 466 of these children developed cancer. Thus, the disjoint group with one tumor in parent before age 50, consists of 17 677 offspring, of whom 395 developed cancer. Of the 559 children whose parent developed multiple HNPCC-associated tumors with onset before age 50, 28 developed cancer, and of the 904 individuals, whose parent and sibling were affected and of whom at lest one of whom was diagnosed under age 50, 82 individuals developed cancer (table 6). Most (>90%) of the follow-up time was confined to ages below 50. Thus, the assessment of cancer risks later in life is not valid in this study.

In the whole cohort, offspring cancer risks were significantly increased, with SIR 1.7 for colon cancer, SIR 1.6 for rectal cancer, SIR 1.4 for endometrial cancer, and SIR 1.6 for any HNPCC-associated cancer (table 7). Overall, diagnosis before age 50 in the parent conferred a higher risk for cancer in the offspring (tables 7–8).

Table 7. SIR and 95% CI in offspring by parent's age at diagnosis

Offspring cancer site	Parent any diagnosis at any age	Parent any diagnosis <50 years
Colon	1.7 (1.6–1.9)	3.6 (2.4–5.0)
Rectum	1.6 (1.5–1.7)	3.8 (2.3–5.8)
Endometrium	1.4 (1.3–1.6)	1.8 (0.9–3.4)
Small intestine	1.1 (0.7–1.6)	4.6 (1.0–13.5)
Upper urinary tract	1.2 (0.9–1.5)	1.0 (0.0–5.6)
Stomach	0.9 (0.8–1.0)	2.8 (1.5–4.8)
Ovary	1.1 (1.0–1.3)	2.3 (1.5–3.4)
Any HNPCC-associated cancer site	1.6 (1.5–1.7)	3.1 (2.4–3.9)

In the group whose parent was diagnosed with one HNPCC-associated tumors before age 50, thus in one of the six disjoint subgroups analyzed, the following significantly increased cancer risks were seen: SIR 3.6 for colon cancer, SIR 3.8 for rectal cancer, SIR 2.8 for gastric cancer and SIR 2.3 for ovarian cancer (table 7). Increased risks, although not significant, were seen for endometrial cancer (SIR 1.8) and cancer of the small intestine (SIR 4.6) (table 7). These risks were more pronounced if calculated in a cohort consisting of offspring to all individuals with diagnosis before age 50, thus including the groups with multiple tumors in parent at a young age and both sibling and parent affected at a young age (table 8), with significantly increased SIR 6.1 for colon cancer, 4.7 for rectal cancer, 3.7 for endometrial cancer, 2.8 for gastric cancer and 2.7 for ovarian cancer. Cancer of the upper urinary tract was significantly increased only in the groups with both sibling

Table 8. SIR and 95% CI for offspring cancer at concordant site with parent cancer, comparison to any cancer in parent. The group whose parent developed any HNPCC associated diagnosis includes, in this table, the different subgroups, i.e. those with multiple tumors in parent or both sibling and parent affected. This is to enable comparision with the concordant site-risks, which are calculated in the whole cohort and not in the disjoint groups

Diagnosis in offspring	Parent any HNPCC-associated diagnosis		Parent cancer site concordant with offspring cancer site	
	Parent ≥50	Parent <50	Parent ≥50	Parent <50
Colon cancer	1.6 (1.5–1.8)	6.1 (4.7–7.8)	1.9 (1.7–2.1)	8.9 (6.0–12.8)
Rectal cancer	1.5 (1.3–1.6)	4.7 (3.1–6.8)	1.7 (1.4–2.0)	4.2 (1.4–9.8)
Endometrial cancer	1.3 (1.2–1.5)	3.7 (2.3–5.6)	2.4 (1.9–2.9)	4.0 (1.9–7.4)
Cancer of the small intestine	1.0 (0.6–1.5)	4.3 (0.9–12.5)	0	0
Cancer of the upper urinary tract	1.2 (0.9–1.5)	0.9 (0.0–5.1)	2.0 (0.5–5.2)	0
Gastric cancer	0.8 (0.7–1.2)	2.8 (1.5–4.7)	NA	NA
Cancer of the ovaries	1.1 (1.0–1.2)	2.7 (1.9–3.9)	NA	NA

Abbreviations: NA: not applicable

Table 9. SIR and 95% CI in the high-risk groups by offspring age

	Parent any HNPCC–associated diagnosis <50		Multiple tumors in parent, at least one onset <50		Parent and sibling affected, at least one onset <50	
Offspring age	≥50	<50	≥50	<50	≥50	<50
Colon cancer	3.5 (1.7–6.5)	3.6 (2.3–5.3)	19.0 (3.9–55.5)	40.6 (21.6–69.5)	7.1 (2.3–16.7)	28.8 (17.8–44.0)
Rectal cancer	3.2 (1.2–7.0)	4.0 (2.2–6.8)	0	29.4 (9.6–68.6)	2.1 (0.1–11.9)	17.1 (6.9–35.2)
Endometrial cancer	1.3 (0.3–3.7)	2.3 (0.9–4.7)	16.8 (2.0–60.7)	25.6 (7.0–65.6)	4.0 (0.5–14.4)	26.6 (12.8–48.9)
Cancer of the small intestine	11.3 (1.4–40.8)	2.1 (0.1–11.9)	0	0	0	0
Cancer of the upper urinary tract	0	1.5 (0.0–8.5)	0	0	26.3 (3.2–95.0)	33.9 (4.1–122)

and parent affected (table 9). Modestly increased risks for other tumor types than those previously described in HNPCC were found significant for breast cancer, malignant melanoma and testicular cancer. We found no significantly decreased cancer risks in the cohort.

Colorectal cancer has been extensively studied regarding familial risks and our figures are well in line with these previous estimates (Burt 2000). When different diagnoses in the proband were investigated separately (table 8), a higher risk was found for concordant cancer sites in parent and offspring, compared to the risk in the whole cohort, e.g. the offspring's risk of developing colon cancer or endometrial cancer was higher if the parents had developed colon or endometrial cancer, respectively, compared to the risk among offspring whose parent had developed any HNPCC-associated cancer. This observation may reflect site-specific familial patterns, but may also, in part, reflect variability in tumor spectra between different HNPCC-families; extracolonic cancers are less frequent in families with mutations affecting the MMR gene *MLH1,* whereas *MSH6* mutations are associated with endometrial cancer (Aaltonen *et al.* 1998; Berends *et al.* 2002; Vasen and Wijnen 1999; Vasen *et al.* 1996). Colon cancer and endometrial cancer in the parent was linked to an increased risk of colon cancer, rectal cancer, and endometrial cancer in the offspring, whereas rectal cancer in the parent predisposed for cancer of the colon and rectum, but conferred no significantly increased risk for endometrial cancer (study V, table III). Several studies suggest that family history do not contribute to the etiology of rectal cancer to the same extent as in colon cancer (Wei *et al.* 2004).

In the groups with expected high risks, i.e. with either multiple tumors in parent or tumors in both sibling and parent, high SIR values were seen (table 9), with the highest risks if the first tumor had developed before age 50 in the parent. Moreover, the risks were higher before 50 years of age in offspring, which may be due to a larger probability of inheritance among young affected individuals. The families in the cohort where both a parent and sibling had developed HNPCC-associated cancer, with the first cancer before age 50, do, if offspring developed HNPCC-associated cancer, fulfill the Amsterdam II criteria. Notably, a parent with multiple HNPCC-associated diagnoses, conferred higher SIR-values compared to offspring whose parent and sibling had developed HNPCC-associated cancer. Indeed, in previous studies of epidemiological risks, MSI, and MMR protein expression loss have pointed out the importance of multiple malignancies as a marker for HNPCC (study I, Cederquist *et al.* 2001; Millar *et al.* 1999; Planck *et al.* 2002; Umar *et al.* 2004).

The cumulative incidences of HNPCC-associated tumors in the offspring were calculated with the ambition to provide age-specific and tumor-specific risk estimates for the offspring. These were aimed to be used in clinical oncogenetic counseling for individuals who report a familial aggregation of cancers, but not obviously fulfill clinical criteria for HNPCC, or any other characterized syndrome. However, since the cohort was too small to provide tumor-specific significant data and since most of the follow-up age in the offspring was before age 50, we calculated the cumulative incidences for any HNPCC-associated cancer at 5-year intervals up to age 65. The highest cumulative risks were found when multiple primary tumors developed in the parent or when both parent and sibling were affected, with a cumulative incidence of 7% by the age of 55 (compared to 0.5% in the general population) and 12.5% by the age of 65 (compared to 1.7% in the general population) (study V, table IV). These (compared to the high cumulative incidences among verified HNPCC mutation carriers) modestly elevated incidences reflect that our material contain sporadic tumors, as well as other, perhaps low-penetrant, not yet characterized inherited cancer syndromes.

In summary, we confirmed an increased risk for several tumor types among individuals whose parents developed HNPCC-associated tumors, with further increased cancer risks if the parents were diagnosed at a young age. No obvious over-risks were observed for tumor types not previously associated with HNPCC. Furthermore, no decreased risks were seen. Although the cancer risk among offspring was somewhat higher at the site of the parental tumor compared to other sites, the study demonstrated a familial clustering of HNPCC-associated tumor types overall. The risks were further increased if both a parent and a sibling were affected, although the highest cancer risks applied to the group whose parent had developed multiple primary tumors with the first diagnosis before the age of 50. The cancer risks also reflected offspring age at diagnosis, with higher SIRs at younger offspring ages.

Conclusions

The aims of this thesis were to characterize the contribution of defective MMR to the development of multiple tumors, cancer of the small intestine, cancer of the upper urothelial tract, and soft tissue sarcoma, and to study familial cancer risks among offspring to patients who developed tumor types associated with HNPCC. Briefly, the results can be summarized as follows:

- Development of multiple primary tumors, including colorectal cancer is associated with defective MMR in 41% of the tumors. Multiple MSI tumors with a concordant loss of MMR protein expression in the tumor tissue developed in 38% of the patients in our study. Of these, 10/17 developed their first tumor after age 50. Thus, development of multiple HNPCC-associated tumors in an individual is a strong indicator of HNPCC, irrespective of age at onset.
- MSI is present in 18% of the tumors of the small intestine. In the majority of tumors with MMR protein expression loss, MLH1 and PMS2 were affected. Among patients with cancer of the small intestine at a young age, MSI was detected in 23% with expression loss of MSH2 and MSH6 at higher frequency than loss of MLH1 and PMS2. Since somatic mutations are rare in *MSH2*, these findings suggest that a subset of these tumors are HNPCC-associated. Thus, cancer of the small intestine display MSI at a frequency similar to colon cancer, but a large fraction of the MSI tumors may be associated with HNPCC.
- Cancer of the upper urinary tract carries MMR defects in about 5% of the tumors with inactivation of MSH2/MSH6 in the majority of cases. Our data suggest that MMR defects represent a minor tumorigenic pathway in this tumor type.
- Occasional soft tissue sarcomas develop through defective MMR, mainly through somatic inactivation of *MSH2*. MFH may occur as a rare tumor within the HNPCC tumor spectrum, which implicates that HNPCC-testing can be performed in family members who have developed soft tissue sarcomas and suggests that genetic counseling should be offered such patients who reveal a family history of HNPCC-associated tumors.
- We confirm a familial clustering of HNPCC-associated tumors and demonstrate significantly increased risks of malignancy, compatible with high-penetrant inheritance in the pathogenesis of these cancer types. Increased risks were observed for cancers of the colorectum, endometrium, ovary, and stomach. The risks were further increased if the parent was diagnosed at a young age. High risks were seen among the offspring who had both a parent and sibling affected by cancer at a young age, and the highest risks were conferred to offspring whose parent had developed multiple primary tumors at a young age.

Acknowledgements

The studies presented herein were performed at the Department of Oncology, Lund University Hospital, Sweden. I would like to thank everyone who made this work possible, in particular:

Mef Nilbert, my supervisor, who is an immense source of inspiration, for her enthusiasm and competence, for showing me this field of research, and helping me off to further adventures.

Maria Planck, my co-supervisor, for help, good advice, fun moments, and support.

Dick Killander, for being the head of our department during most of my time there and for creating such a good working atmosphere.

All the personnel at the laboratory of the Department of Oncology, in particular *Eva Rambech* and *Kristina Lövgren,* for teaching me practical skills and for clear immunostainings, *Britta Halvarsson* for pathologic review, *Jacob Engellau* for collaboration on the sarcoma study, *Anna Bladström, Harald Andersson* and *Håkan Olsson* at the Department of Cancer Epidemiology, for important contributions to the epidemiological study, and *the principal investigators* at the department for encouraging discussions, and for generously sharing their knowledge.

All *co-authors* for help and advice.

My fellow PhD students, Karin, Anna, Princy, Josefin, Göran, Karolina, Sofia, Johan, Johan, Johan, Emma, and Susanne for friendship, for laughter and for creating a pleasant environment.

All colleagues at the Department of Oncology, for introducing and helping me in the clinic.

All my *friends*, whose love and loyalty made all the difference.

My brothers and sisters, *Eira Pernilla Ahlgren, Hannes Ahlgren* and his wife *Lina* and their children *Smilla* and *Ida, Matti Larsson* and *Clara Ericson,* for being my crazy and loving family.

My parents, *Gunnar Ericson* and *Birre Skoglund-Pråme* for always believing in me, for your love and tremendous help, *Agneta Hammer* and *Göran Pråme* for being part of the family and for your support.

This work has been supported by the Swedish Cancer Society, the Gunnar Nilsson Cancer Foundation, the Mrs. Berta Kamprad Foundation, the John and Augusta Persson Foundation, the Vera and Carl J Michaelsens Foundation for Donations, the King Gustaf V Jubilee Foundation, the Royal Physiographic Society of Lund, and the Lund University Hospital Foundations.

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