# GENETIC BASIS OF HEREDITARY COLORECTAL CANCERS:

## HEREDITARY NONPOLYPOSIS COLORECTAL CANCER AND FAMILIAL ADENOMATOUS POLYPOSIS

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# ABBREVIATIONS

| | |
|---|---|
| AAPC | Attenuated adenomatous polyposis coli |
| ACI | Amsterdam criteria I |
| ACII | Amsterdam criteria II |
| AFAP | Attenuated familial adenomatous polyposis |
| APC | Adenomatous polyposis coli |
| bp | base-pair |
| C | Codon |
| cDNA | complementary DNA |
| CHRPE | Congenital hypertrophy of the retinal pigment |
| DGGE | Denaturing gradient gel electrophoresis |
| DNA | Deoxiribonucleic acid |
| FAP | Familial adenomatous polyposis |
| gDNA | genomic DNA |
| HNPCC | Hereditary nonpolyposis colorectal cancer |
| IDL | Insertion/deletion loop |
| InSiGHT | International Society for Gastrointestinal Hereditary Tumors |
| LOH | Loss of heretozygosity |
| MAP | MYH-associated polyposis |
| MCR | Mutation cluster region |
| MLH1, 3 | MutL homolog 1, 3 |
| MLPA | Multiplex Ligation-dependent Probe Amplification |
| MMR | Mismatch repair |
| MSH2, 3, 6 | MutS homolog 2, 3, 6 |
| MSS | Microsatellite stable |
| MYH | Human Mut Y homolog, a base excision repair gene |
| NMD | Nonsense-mediated mRNA decay |
| PCR | Polymerase chain reaction |
| PTT | Protein truncation test |
| RNA | Ribonucleic acid |
| RT | Reverse transcriptase |
| SNP | Single nucleotide polymorphism |
| SNuPE | Single nucleotide primer extension |
| SSCP | Single-stranded conformational polymorphism assay |
| UV | Ultraviolet |
| Wnt | Wingless type |

# ABSTRACT

Hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) are the two most common types of hereditary colorectal cancers (CRC) that are inherited in an autosomal dominant manner. HNPCC is associated with germline mutations in one of the four mismatch repair (MMR) genes: MLH1, MSH2, MSH6 and PMS2. A defective MMR system is unable to recognize and repair base/base mismatches and insertion/deletion loops that arise during DNA replication. Thus, the mutation rate is increased more than 100 fold and tumors with MMR deficiency display genetic instability of microsatellites (MSI).

A majority of FAP cases are due to germline mutations of APC, causing a visible clear phenotype during early adulthood by the development of multiple colorectal polyps. Among these, one or several inevitably progress to cancer by mid-age. In addition to colorectal polyps, various extracolonic manifestations, such as desmoid tumors, duodenal adenomas, mandibular osteomas, hypertrophic pigmentary lesions of the retina and dental abnormalities are observed in FAP families. Fewer than 100 adenomas, later onset of adenomas and cancer, and lower lifetime risk of cancer are associated with attenuated FAP (AFAP).

To date, more than 450 MMR gene mutations and more than 800 APC mutations have been identified for HNPCC and FAP/AFAP predisposition, respectively. Most of these are either frameshift or nonsense mutations leading to a truncated protein, easily detected by conventional mutation detection methods such as the protein truncation test (PTT), single-stranded conformational polymorphism assay (SSCP), and cDNA sequencing. However, in more than 30% of HNPCC, about 30% of FAP, and about 90% of AFAP families, mutations remain unknown. This is partly a consequence of the methods used, which are unable to detect all kinds of mutations in known predisposition genes, but may also be due to mutations that lie in other susceptibility gene(s).

We aimed to clarify the genetic basis of mutation negative HNPCC and FAP families, and determine genotype-phenotype correlations in such families. We applied a panel of advanced mutation detection methods to detect large genomic rearrangements, mRNA and protein expression alterations, promoter mutations, phenotype linked haplotypes, and tumoral loss of heterozygosity. We also aimed to estimate the frequency of HNPCC in Uruguayan CRC patients.

Our expression based analysis of MMR gene mutation negative HNPCC divided these families into two categories: 1) 42% of families linked to the MMR genes, MLH1, MSH2 and/or MSH6, with a phenotype resembling that of mutation positive, and 2) 58% of families likely to be associated with other susceptibility genes. Unbalanced mRNA expression of

MLH1 was observed in two families. Further studies revealed a nonsense MLH1 mutation, R100X, co-segregating with the HNPCC phenotype, and associated with aberrant splicing of exons not related to the mutation. An MLH1 deletion (AGAA) at nucleotide 210 was associated with multiple exon skipping, without an overall increase in the frequency of splice events.

By advanced mutation detection methods, APC mutation negative FAP/AFAP families were divided into four groups according to the genetic basis of their predisposition. Four (14%) families displayed a constitutional deletion of APC with profuse polyposis, early age of onset and frequent extracolonic manifestations. Three of these had a deletion of an entire allele of APC, and one had a deletion of APC exon 4. Reduced or absent mRNA expression of one allele was observed in seven (24%) families and these had later onset and less frequent extracolonic manifestations. In 52% (15/29) of families the involvement of APC could neither be confirmed nor excluded. The phenotype of this group was variable, suggesting genetic heterogeneity. In three (10%) of the families a germline mutation was detected in genes other than APC: AXIN2 in one family, and MYH in two families. The families with undefined genetic basis and especially those with AXIN2 or MYH mutations frequently displayed AFAP or atypical polyposis with lesser than 100 polyps, and often presented as solitary affected cases.

Of the Uruguayan CRC patients, 2.6% fulfilled the diagnostic criteria for HNPCC and 5.6% (26/461) were associated with increased risk of cancer. These frequencies resemble those reported from non-Southern American populations. Genetic analysis revealed an unexpectedly low frequency of molecularly defined HNPCC cases, despite advanced methodology used for mutation detection, which may suggest the involvement of novel susceptibility genes.

Accurate genetic and clinical characterization of families with hereditary colorectal cancers, and the definition of the genetic basis of "mutation negative" families in particular, facilitate proper clinical management of such families.

# REVIEW OF THE LITERATURE

## CANCER

A major feature of higher eukaryotes is the defined life span, a property that extends to the individual somatic cells, whose growth, division and death is highly regulated. A notable exception is a cancer cell, which has become independent of growth factors (small molecules usually needed for cell growth), and apoptotic signals (controlling transformation and immortalization) and gained the ability to invade normal tissue and move away from the tissue of origin and establish a new tumor elsewhere in the body (metastasis).

Different types of human cancers are divided into three groups according to the tissue from which the cancer originates: 1) carcinomas, cancers of the cells of epithelia or sheet, 2) sarcomas, cancer of supporting tissue, and 3) leukemias and lymphomas, cancers of cells that produce the circulating cells of the blood and immune system. Over 90% of all human cancers are carcinomas (Henderson et al. 1991).

International data show that cancer is one of the leading causes of death, and more than 50% of deaths are attributable to lung, breast and colon carcinomas in Western countries (Parkin 2001). Colorectal cancers rank third in incidence (9.4% of the world total) and also in mortality (7.9%), with similar numbers in men and women (Paker et al. 1996, Parkin 2001). The Finnish Cancer Society has estimated that 25% of Finns are diagnosed with cancer during their lifetime, approximately half of which are cured by medical treatment available today (www.cancerregistry.fi). In Finland, over 24 000 individuals were diagnosed with cancer in 2003, and more than 2300 of whom were diagnosed with a primary tumor in the colon or rectum (Table 1).

## CANCER GENETICS

Cancer is a genetic disease in the sense that the development of tumor tissue is a result of genetic alterations that are transferred from one cell to its daughter cells (reviewed by Knudson 2002). Complex DNA repair mechanisms (see: DNA repair mechanisms, below) operate continuously to keep DNA and genes intact, however, genetic alterations occur as a result of defective function of the DNA replication mechanism through DNA damage caused by environmental mutagens (carcinogens), or naturally with increasing age after countless cell divisions (reviewed by Christmann et al. 2003). Cancer manifests after accumulation of

several genetic changes, such as point mutations, deletions, insertions, chromosomal translocations, chromosome rearrangements, and gene amplifications (Bishop 1987), that can be either somatic or inherited. In most cases cancer-related mutations have arisen in a somatic cell, and only few are inherited. These changes target proto-oncogenes, tumor suppressor genes, and DNA repair genes that are essential to maintain the normal function of the cell and suppress tumor formation (Knudson 2002).

**Table 1:** Incidence of the 5 most common cancers in Finland in 2003.

**MALES**

| Primary location | ICD-10 | # of cases | % of total |
|---|---|---|---|
| Prostate | C61 | 4234 | 33.9 |
| Lung | C33-34 | 1556 | 12.5 |
| Colon | C18 | 659 | 5.3 |
| Ureter | C66-68 | 613 | 4.9 |
| Rectum | C19-21 | 492 | 3.9 |
| Colon & Rectum | C18-21 | 1151 | 9.2 |
| Total | | 12489 | 100.0 |

**FEMALES**

| Primary location | ICD-10 | # of cases | % of total |
|---|---|---|---|
| Breast | C50 | 3787 | 31.5 |
| Corpus uteri | C54 | 756 | 6.3 |
| Colon | C18 | 748 | 6.2 |
| Central nervous system | C70-72 | 541 | 4.5 |
| Lung | C33-34 | 530 | 4.4 |
| Colon & Rectum | C18-21 | 1496 | 9.7 |
| Total | | 12039 | 100.0 |

**Proto-oncogenes and Oncogenes**

The products of proto-oncogenes are highly conserved, generally situated in the cytoplasm or the plasma membrane and regulate cascades of events that maintain the ordered progression through the cell cycle, cell division, and differentiation. Proto-oncogene products have been shown to function at critical steps in signaling pathways and include proteins such as extra-cellular cytokines, growth factors and transmembrane growth factor receptors. Oncogenes are

mutated forms of proto-oncogenes. A mutation that transforms a proto-oncogene to an oncogene is dominant since mutation of one allele is sufficient. Transformation can happen by four different pathways, which all lead to gain-of-function of the gene. 1) Retroviral transduction activates an oncogene (Bishop 1982); 2) Deletion or a point mutation occurs in the coding sequence of the gene, in a way that increases the activity of the protein (Olivero et al. 1999); 3) The proto-oncogene becomes amplified, which can be seen as a homogenously staining chromosomal regions or double-minute chromosomes, and thus produces more protein than normally (Alitalo et al. 1983); 4) A higher level of protein is produced by chromosomal rearrangement that places the proto-oncogene under the influence of a strong enhancer sequence (reviewed by Boxer and Dang 2001).

Chromosomal breakpoints have been useful in identifying oncogenes especially in lymphomas and leukemias, in which the activation of oncogenes commonly occurs as a primary event. To date, more than 100 proto-oncogenes have been identified through a variety of experimental strategies (Bishop 1991, Rabbitts 1994). In inherited cancers, oncogenes play a predisposing role in only a few rare cases. The best known examples are the RET gene where specific activating mutations cause an autosomal dominant cancer syndrome known as multiple endocrine neoplasia type 2 (MEN2) (Mulligan et al. 1994, Koch 2005), and the MET gene whose germline mutations predispose to familial papillary renal carcinoma (Schmidt et al. 1997, Olivero et al. 1999).

**Tumor Suppressor Genes**

Tumor suppressor genes encode proteins that are essential for the normal function of the cell and are generally located in the nucleus or in contact with cell membranes (reviewed by Oliveira et al. 2005). They participate in transcription and cell adhesion events. These proteins function in growth regulatory or differentiation pathways, and loss of their function contributes directly to the altered phenotype of cancer cells. Identification of tumor suppressor genes has been more difficult than identification of oncogenes. However, more than a dozen tumor suppressor genes have been localized and identified. The best-known tumor suppressor gene is probably p53 (Oliveira et al. 2005). Its cellular functions are well understood while those of many other tumor suppressor genes remain poorly understood. Loss or inactivation of one tumor suppressor allele probably increases cancer risk, but contrary to dominant oncogenes, a mutation in a tumor suppressor gene is recessive because both alleles have to be inactivated before tumorigenesis. However, recent observations suggest that mutations in tumor suppressor genes are not always completely recessive, since dosage-dependence (haploinsufficiency: one wild-type allele is unable to maintain the function of two wild-type alleles) and qualitative differences (gain of function and dominant negative mutations) may be sufficient for inactivation (Payne and Kemp 2005). According to the multi-hit mechanism of

carcinogenesis (Knudson 1985, Knudson 1993), the inactivation of several different tumor suppressor genes can occur in one tumor and one tumor suppressor gene can be inactivated in many different tumor types (Coleman and Tsongalis 2006).

Most tumor suppressor genes are inactivated by somatic mutation arising during tumor development, however, tumor suppressors can be inactivated in the germline. In such case their inactivation strongly predisposes to cancer. Inactivation of tumor-suppressor genes is responsible for many different hereditary and sporadic cancer forms, including colon cancer (APC) (Groden et al. 1991), kidney cancer (WT1) (Haber and Housman 1992), and skin cancer (PTCH) (Lacour 2002).

Tumor suppressors can be roughly divided into two groups, named gatekeepers and caretakers (Kinzler and Vogelstein 1997). Gatekeepers are genes that directly regulate growth of tumors by inhibiting their growth or by promoting their death (Oliveira et al. 2005). The functions of these genes are rate limiting for tumor growth, and both maternal and paternal copies of these genes must be inactivated for tumor development. The identity of gatekeepers varies with each tissue. Gatekeepers tend to be often somatically mutated in sporadic cancers as well as in the germline of predisposed individuals, such as APC in sporadic and hereditary colon cancers (Kinzler and Vogelstein 1997).

In contrast, inactivation of caretakers does not directly promote the growth of tumors. Rather, inactivation of caretakers leads to genetic instability that indirectly promotes growth by causing an increased mutation rate (Levitt and Hickson 2002). The increase in genetic instability greatly accelerates the development of cancer. Caretaker mutations in the germline occur in two different forms. In dominantly inherited diseases, like HNPCC, only one mutant allele of the caretaker is inherited and the remaining allele must acquire a somatic mutation for a phenotypic defect (Peltomäki 2001). In recessively inherited cases, as xeroderma pigmentosum (XP), both alleles of the gene must be inherited in a mutant form to cause susceptibility (Norgauer et al. 2003).

**DNA Mismatch Repair Genes**

DNA mismatch repair (MMR) genes resemble tumor suppressor genes since both alleles need to be inactivated before tumorigenesis begins. For that reason these genes are often grouped together with tumor suppressor genes (Chung and Rustgi 1995). However, many DNA repair proteins are likely to have a more passive role in regulating cell growth than do products of tumor suppressor genes. The inactivation of MMR proteins in tumor cells results in increased rates of mutations in other genes, including proto-oncogenes and tumor suppressor genes (Peltomäki et al. 2001). Because the accumulation of mutations in these genes appears to be

rate determining in tumorigenesis, the process of tumor progression is greatly accelerated (Peltomäki et al. 2001). The role of different MMR proteins in MMR is explained in more detail below.


## DNA REPAIR MECHANISMS

DNA in cells suffers a wide variety of damage: purine bases are lost by spontaneous fission of the base-sugar link, many chemicals form adducts with DNA bases, ultraviolet light causes adjacent thymines to form a stable chemical dimer, and mistakes during DNA replication and recombination occur resulting in mismatched bases and strand breaks in DNA. DNA repair mechanisms seldom involve simply undoing the change that caused the damage. Almost always a stretch of DNA containing the damaged nucleotide(s) is excised and the gap filled by resynthesis (Marti and Fleck 2004). To cope with all of these forms of damage, cells must be capable of several different types of DNA repair pathways. To date, more than 100 genes associated with DNA repair are identified in humans (Christmann et al. 2003).

### Direct Repair

Direct repair reverses the DNA damage, for example in bacteria thymine dimers can be removed in a photoreactivation reaction that depends on visible light and an enzyme called photolyase (Van der Horst et al. 1999).

### Base Excision Repair

Base excision repair (BER) uses glycosidase enzymes to remove abnormal bases or repair spontaneous depurination (reviewed by Christmann et al. 2003). An AP endonuclease cuts the sugar-phosphate backbone at the position of the missing base. Thereafter, an exonuclease removes a few nucleotides and a DNA polymerase refills the gap and the remaining nick is sealed by DNA ligase (Blaisdell et al. 2001). BER is also responsible for repair of oxidative DNA damage causing substitution of a thymine-adenine base pair for a guanine-cytosine base pair. Three human proteins work together in BER to remove modified base pairs: MTH1 hydrolyzes oxidized purine nucleoside triphosphates to monophosphates (Sakumi et al. 1993), OGG1 detects and removes incorporated guanosines (Shinmura et al. 1997), and an adenine-specific DNA glycosylase MYH scans the daughter strand after replication and excises mispaired adenosines (Slupska et al. 1999). According to recent studies (Al-Tassan et al. 2002, Sieber et al. 2003), biallelic germline mutations of MYH are associated with a subgroup of multiple colorectal polyposis, MYH-associated polyposis (MAP). Recent data provided strong evidence for a causative role of BER defects in colorectal cancer susceptibility, and

inherited biallelic MYH mutations increase the risk of colorectal cancer and coexist with adenomatous polyps (Farrington et al. 2005).

**Nucleotide Excision Repair**

Nucleotide excision repair (NER) removes thymine dimers and large chemical adducts. The mechanism of NER involves more than 30 proteins and consists of two distinct pathways: global genomic repair (GGR) and transcription-coupled repair (TCR) (Christmann et al. 2003). GGR removes lesions, such as sysclobutene pyrimidine dimers, from the non-transcribed strand of the genome whereas TCR focuses on removal of different RNA-blocking lesions from the transcribed strand of active genes. Common stages of both NER repair pathways include (Christmann et al. 2003): 1) recognition of damage (by XP-protein complex in GGR and by blockage of RNA polymerase II, 2) unwinding of the DNA double helix by helicases, 3) cutting the sugar-phosphate backbone on both sides of the damaged site, 4) removal of the damaged strand, 5) resynthesis by DNA polymerase $\varepsilon$ or $\delta$, and 6) ligation by DNA ligase I (Lehmann 1995). Defects in NER cause UV-hypersensitive disorders such as the autosomal recessive disease, XP (Lambert et al. 1998) and Cockayne's syndrome (CS) (van Hoffen et al. 1993).

**DNA Double-Strand Break Repair**

DNA double-strand break (DSB) repair mechanisms are recombinational repair mechanisms that include: homologous recombination (HR), single strand annealing (SSA), and non-homologous end-joining (NHEJ) repair pathways (Thompson and Schild 2003). The HR is considered as error-free pathway whereas NHEJ and SSA are error-prone (Christmann et al. 2003) The HR and SSA require DNA sequence homology whereas the NHEJ repairs DSBs without sequence homology between the DNA ends (Christmann et al. 2003). The HR and SSA mechanisms repair replication errors by provoking a DNA strand exchange by physical contact of the undamaged homologous DNA and the damaged DNA molecule followed by DNA resynthesis, ligation, branch migration and resolution of Holliday junctions (Christmann et al. 2003). The HR requires RAD51 to assist gene conversion and crossing over the SSA works in RAD51-independent manner (Cousineau et al. 2005). The NHEJ recognizes and binds to damaged DNA by Ku70-Ku80 heterodimer which forms a DNA-PK holoenzyme that activates ligation of the broken DNA ends together (Christmann et al. 2003). The breast cancer susceptibility genes BRCA1 and BRCA2 are strongly linked with DSB repair mechanisms (HR and HNEJ), thus playing an important role in maintaining genomic integrity (review by Thompson and Schild 2003 and Zhang and Powell 2005).

**DNA Mismatch Repair (MMR)**

DNA MMR is an evolutionarily conserved postreplicative repair mechanism which eliminates mistakes in the newly synthesized DNA strand during DNA replication (reviewed by Kunkel and Erie 2005). Such biosynthetic mistakes include base/base mismatches and insertion/deletion loops (IDLs). The latter mistakes arise during the slippage of the primer against the template strand, especially in repeated sequence motifs like microsatellites. A model for the eukaryotic MMR mechanism is provided by Saccharomyces cerevisiae (Strand et al. 1993) where mutations in three genes lead to 100-700 fold increases in mutation levels at poly (GT) sequences. The discovery of defects in MMR that co-segregate with certain cancer predisposition syndromes (for example, HNPCC) highlights the essential role of MMR in mutation avoidance.

MMR consists of five major steps: 1) mismatch recognition, 2) assembly of the repair complex, 3) strand discrimination, 4) degradation of the mismatch containing strand, and 5) resynthesis of the excised strand (reviewed by Kunkel and Erie 2005). Depending on the type of mismatch on the newly synthesized DNA strand, either an MSH2-MSH6 (MutSα) or MSH2-MSH3 (MutSβ) dimer recognizes the mismatch. The MSH2-MSH6 complex is involved in the repair of single base pair mismatches and IDLs of one or two nucleotides, whereas the MSH2-MSH3 complex preferentially targets mismatches from two up to 13 nucleotides (Sia et al. 1997). The recognition complex also involves proliferating cell nuclear antigen (PCNA), until it stumbles upon the mismatch site. After the PCNA has exited the complex, MutSα/ß uses ATP to form a lariat of model DNA and assembly of repair complex begins following the addition of a MutLα (MLH1-PMS2) heterodimer (Kolodner and Marsischky 1999). The function of MutLα can be partially replaced by MutLß (MLH1-PMS1) or MutLγ (MLH1+MLH3) but their role in MMR in humans are less clear (Li and Modrich 1995). Next, the newly synthesized strand is discriminated by DNA helicase(s) and DNA nuclease(s) begin to remove one nucleotide at time. Finally, resynthesis of the excised strand occurs by DNA polymerase(s) with a few assistant proteins, such as replication protein A (RPA), PCNA, DNA ligase and DNA methylase (Kolodner and Marsischky 1999).


**GENE EXPRESSION AND QUALITY CONTROL**

Expression of a protein-coding gene is a complicated but highly accurate process, however, DNA repair mechanisms are prone to errors involving approximately one incorrect amino acid per 10 000 peptide bonds formed (Yarus 1979). Such a high level of precision is achieved by the pre- and co-translational quality control (QC) system of gene expression (Custodio and Carmo-Fonseca 2001, Fasken and Corbett 2005). This QC system begins at the

level of gene transcription and has physical interactions also with the components of pre-mRNA processing, mRNA transport, and mRNA translation systems (Custodio and Carmo-Fonseca 2001). If interactions between these components are delayed or abnormal, the transcript will be degraded or otherwise prevented from entering into protein synthesis either in the nucleus or cytoplasm (Fasken and Corbett 2005).
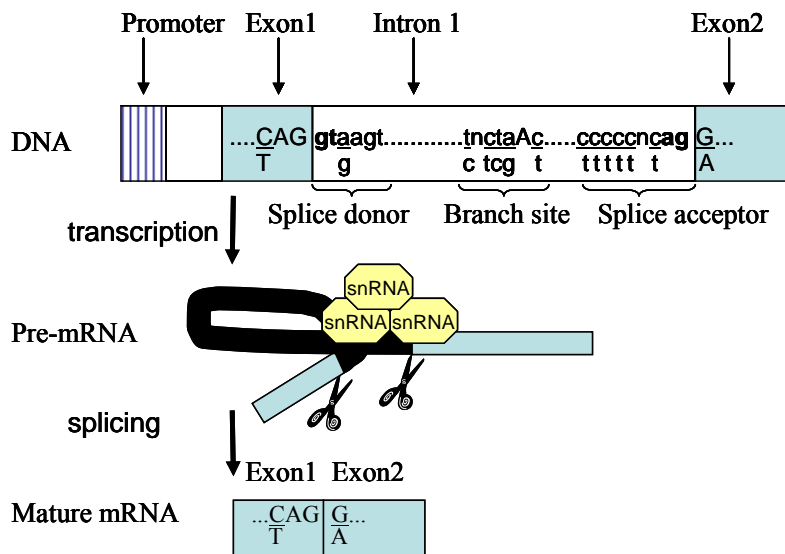


**Figure 1**: RNA splicing removes intronic RNA sequences from a pre-mRNA transcript and fuses exonic (protein coding) sequences together. Splicing starts at the assembling of a large RNA-protein complex called a spliceosome, which consists of five different types of small nuclear RNA (snRNA) and more than 50 assistant proteins (Staley and Guthrie 1998). First, splice donor is cleaved, following a lariat formation by the terminal G of the splice donor site and invariant A nucleotide at branch site. Finally, cleavage of the 3' splice junction leads to release of the intronic RNA lariat and fusion of exonic RNA sequences can take place.

**Processing of mRNA**

One of the important stages in processing RNA is RNA splicing, where internal regions that do not code for protein (introns) are removed and RNA with a continuous open reading frame (ORF) is created (Figure 1). Other important processing events are modification of the 5' and 3' ends (Custodio and Carmo-Fonseca 2001). The 5' end of RNA is modified by the addition of a "cap" virtually as soon as it appears. This replaces the triphosphate of the initial transcript with a nucleotide in reverse (3'-5') orientation, thus "sealing" the end. The 3' end is modified by the addition of a series of adenylic acid nucleotides (poly(A)-tail) immediately after its cleavage. Transcription and processing of RNA occur in the nucleus (Custodio and Carmo-

Fonseca 2001). Pre-mRNA processing offers a number of opportunities for transcription to affect the quality and quantity of mRNA. Changes in promoter structure may strongly affect splice site selection, as observed for human fibronectin (Cramer et al. 1997).

The role of the carboxyl terminal domain (CTD) is essential. The CTD is the largest subunit of RNA polymerase II (RNAP II) and functions to regulate gene transcription, especially initiation, but also functions in association with factors required for pre-mRNA capping, splicing, and 3' end formation to coordinate pre-mRNA synthesis and progress on throughout transcription initiation, elongation and termination (Proudfoot 2001). As a consequence of mRNA QC, errors in these processes can lead to RNA nuclear retention, degradation or both (Custodio and Carmo-Fonseca 2001, Fasken and Corbett 2005).

**Export of mRNA**

After pre-mRNA processing, mRNAs are exported to the cytoplasm and screened one more time by the mRNA QC mechanism. Efficient mRNA export requires 1) binding of shuttling proteins, including splicing-dependent messenger ribonucleoproteins (mRNPs), 2) proper 5' end capping and 3' end poly(A) formation, and 3) removal of non-shuttling proteins, spliceosomes and other possible blocking proteins (Maquat and Carmichael 2001, Custodio and Carmo-Fonseca 2001, Aguilera 2005). Transport is an active and selective process in which immature and mature mRNPs are discriminated. Export is promoted by many proteins which have bound to the mRNA during pre-mRNA processing (Aguilera 2005). When QC discovers an incompletely processed pre-mRNA, many different blocking proteins attach to the mRNA and inhibit its export to the cytoplasm. Degradation begins by deadenylation and decapping procedures (Gu and Lima 2005).

In successful export, a general mRNA export factor Aly (also called REF) is recruited to pre-mRNA during spliceosome assembly and subsequently becomes tightly associated with spliced mRNPs in a way that promotes mRNP export (Zhou et al. 2000) Then Aly shuttles between the nucleus and cytoplasm (Zhou et al. 2000). Aly and another mRNA export protein, Y14, have been shown to be components of a splicing-dependent complex (Kataoka et al. 2000) placed 20-24 nucleotides upstream of the exon-exon junctions of mRNPs (Le Hir et al. 2000). mRNAs derived from genes that naturally lack introns might co-opt the same factors used for the export of spliced mRNAs, or they may use a different pathway (without Y14) or other pathway not yet discovered (Kataoka et al. 2000).

**Translation of mRNA**

The next stage of gene expression is the translation of the mRNA into protein. This occurs in cytoplasmic ribosomes, and can occur only after all modification and processing events have

been accomplished. The ribosomes are large RNA-protein complexes composed of two subunits. In eukaryotes, cytoplasmic ribosomes have a large 60S subunit and a smaller 40s subunit (Pestova et al. 2001). Translation consists of three different stages: initiation, elongation and termination. Initiation of translation is the rate-limiting step of proteins synthesis and is tightly regulated (reviewed by Preiss and Hentze 2003).

Less is known about the control of elongation. There is evidence that protein elongation is also carefully monitored (Wower et al. 2001). For instance, the elongation rate appears to be variable: a ribosome may pause at rare codons (Ramachandiran et al. 2002) or at the formation of stable structures in certain regions of the mRNA (Fernandez et al. 2005). Sometimes a ribosome can become stalled because of a premature termination codon in the mRNA. It will then be recycled by a QC mechanism called trans-translation (Wower et al. 2001). All details of trans-translation in eukaryotes are not yet fully understood, but it is known that it requires two adaptor molecules, tRNA and transfer-messenger RNA (tm-RNA: a tRNA and mRNA hybrid, also called SsrA RNA). The latter resumes elongation and provides an ORF followed by two stop codons. This ORF encodes a short peptide that signals a number of proteases to digest the truncated tagged protein (Wower et a. 2001).

**Nonsense Mediated Decay of mRNA**

Nonsense-mediated decay (NMD) of mRNA is also called mRNA surveillance. The main function of this translational QC mechanism is to rapidly degrade mRNAs that contain errors impeding full-length translation, such as transcripts that contain premature stop codons (Cheng and Maquat 1993, Alonso 2005). Because of this down regulation of mRNA translation and activation of mRNA decapping and degradation (Gu and Lima 2005), truncated proteins are seldom made and their accumulation is avoided and a deleterious impact on the function of the cell is prevented (Fasken and Corbett 2005).

NMD is thought to survey translated mRNAs in order to prevent synthesis of proteins from those that prematurely terminate translation as a consequence of errors in gene expression. These errors include aberrant transcription initiation, inaccurate or inefficient splicing, the failure to incorporate selenocysteine at specific UGA codons, and frameshift or nonsense mutations within germline or somatic DNA. Base substitution causes premature polypeptide chain termination whenever a sense codon is changed to a stop codon (UUA, UAG, UGA). In almost every gene multiple stop codons reside in the alternative reading frames in AT-rich genomes. For this reason, most frameshift mutations lead to a premature stop codon. A nonsense mutation can also cause chain termination. The mRNA containing these mutations is called nonsense mRNA and is usually highly unstable and is degraded. However, not all nonsense mutations induce NMD, because of the dependency of exon-exon junction in

mammals that distinguishes mammalian NMD from non-mammalian NMD systems (reviewed by Maquat 2005).

The mRNA surveillance in S. cerevisiae is thought to take place in the cytoplasm. In contrast, the fact that many mammalian nonsense mRNAs are reduced in abundance to the same extent in nuclear and cytoplasmic fractions suggests that NMD takes place either prior to or concomitant with nuclear mRNA export.

At least for human nonsense triosephosphate isomerase (TPI) transcripts, the possibility of decay concomitant with nuclear mRNA export has been put forth as a logical mechanism considering that 1) cytoplasmic ribosomes constitute an integral component of the only known means by which nonsense codons are recognized and 2) nuclear mRNA may be simultaneously translocated across the pore and translated by cytoplasmic ribosomes (Zhang and Maquat 1996). Nonsense codons upstream of position 192 of the human TPI gene have been found to reduce the abundance of TPI mRNA to ~25% of normal. TPI mRNA that co-purifies with cytoplasm is immune to NMD. Mehlin et al. (1992) support a theory that NMD occurs together with export and translation because, in Chironomus tentants, mRNA is invariably exported from the nucleus 5' end first and becomes associated with cytoplasmic ribosomes before the 3' end transits the nuclear pore.

Evidence that cytoplasmic ribosomes may not be involved in nonsense-mediated mRNA decay is derived from reports that nonsense codons can be associated with an increased level of mRNA that lacks the nonsense codon-containing exon or an increased level of mRNA that lacks part of an exon or contains part or all of an intron (Maquat 1995, Maquat 1996). These reports have led to a proposal that nonsense codon recognition can take place in the nucleus either prior to or concomitant with splicing. This nucleus-associated NMD reflects assessment of the translational reading frame either during the export of nuclear mRNA to the cytoplasm by cytoplasmic ribosomes or that it is somehow related to cytoplasmic translation. This proposal is based on the finding that nonsense mutations inhibit the splicing of immunoglobulin pre-mRNA in intact B cells.

Cytoplasmic NMD is somehow related to late stages of export or function of cytoplasmic ribosomes, and it functions at least for TPI transcripts, whose nonsense codon recognition appears after splicing, since nonsense codons that span two exons reduce the abundance of TPI mRNA to the same extent as nonsense codons that are confined to a single exon (Zhang and Maquat, 1996).

In addition to QC mechanism of nonsense mRNAs, NMD is also a conserved cellular mechanism that downregulates the expression of a wide variety of naturally occurring mRNA transcripts without nonsense codons (Mendell et al. 2004). These NMD substrates belong to a wide variety of gene classes including protein kinases, phosphatases, splicing factors, genes of amino acid metabolism and transposable elements, such as transposons and retroelements (Mendel et al. 2004).

**mRNA Stability**

Although most analyses of gene expression focus on transcriptional regulation, mRNA stability is an important factor in the control of gene expression (Hollams et al. 2002). The major mRNA degradation pathway in yeast involves initial deadenylation, followed by decapping and 5'→ 3' exonucleolytic degradation. mRNA deadenylases remain poorly characterized, but their activity in human cells has been identified (Mitchell and Tollervey 2000, Gu and Lima 2005).

The stability of individual mRNAs vary widely. Very short half-lives are observed for transiently expressed genes, including early response genes such as lymphokines, cytokines and transcription factors (Ford et al. 1999). Features of an mRNA molecule that impact stability includes the cap and the poly(A) tail that synergistically stimulate translation initiation (Wells et al. 1998). Generally, the cap and poly(A) tail of an mRNA contribute to cytoplasmic stability, presumably by stimulating translation and thereby physically protecting the mRNA from the action of nucleases (Mitchell and Tollervey 2000).

**MUTATION DETECTION**

Despite the continuously working DNA repair mechanisms and QC system in normally functioning cells, genetic changes may accumulate resulting in malignant transformation of cells. Detecting mutations is an essential part of the molecular analysis of inherited human diseases, providing diagnosis and a possible correlation between genotype and phenotype. Detection of a mutation can, in the best case, lead to more efficient treatment and care of mutation carriers. In family members who turn out mutation negative, predictive genetic testing may alleviate unnecessary worry and make the need for regular, life-long surveillance unnecessary (Aktan-Collan et al. 2001).

In the first step of mutation detection, the proband DNA is tested for differences against a reference (wild-type) DNA. At present, DNA sequencing is the best method of detecting and identifying single-base mutations with high certainty and precision. However, because

sequencing hundreds of bases of DNA is expensive and slow, many different methods have been developed to preliminarily identify mutations, hence reducing sequencing to a minimum. The existing methods differ widely in regard to sensitivity, accuracy, speed, and cost, and each detects a subset of mutations (Csako 2006). Therefore, the detection of uncharacterized mutations or polymorphisms often requires a combination of overlapping tests, followed by confirmatory DNA sequencing or direct mutation detection (see below).

The strategy of mutation detection depends on the molecular genetic background of an inherited disease. When a single (or only a few) mutation(s) is associated with the majority of families, direct mutation detection is the best option. When the genetic background is known to be heterogenous, more extensive mutation screening followed by mutation detection becomes a more efficient and more economic choice. Methods detecting genomic changes can be divided into two categories: mutation specific methods and mutation screening methods.

**Direct Mutation Detection**

A direct test is useful when all or a majority of the mutation carriers are expected to share the same predisposing mutation. A sample (DNA, RNA, or protein) is tested to see whether or not it has a certain genotype – typically a pathogenic mutation in a certain gene. The methods of testing for a specific mutation may include: 1) restriction mapping when a mutation creates or abolishes a natural restriction site, 2) utilization of allele-specific oligonucleotides (**ASO**) for allele-specific primer extension, such as minisequencing (Syvänen 1999), ARMS (nowadays called real-time PCR), and applications of microarray for multiplex high-throughput genotype analysis, 4) OLA (oligonucleotide ligation assay), and 5) a PCR-based test combined with polyacrylamide gel electrophoresis or Southern blotting to detect repeat expansions, such as extension of (CAG)n in Huntington disease (Nance et al. 1999).

**Mutation Screening**

Mutation screening is an indirect way to pinpoint the existence of genetic alterations. Screening methods are used to search segments of DNA for uncharacterized alterations and these tests are divided into four subtypes according to the basis of the test.

Tests based on differences in electrophoretic migration between mutant and reference DNAs include denaturing gradient gel electrophoresis (DGGE) (Fischer and Lerman 1983), single-stranded conformational polymorphism (SSCP) (Orita et al. 1989), and non-denaturating heteroduplex analysis (HA) and protein truncation test (PTT) (Roest et al. 1993). These tests are simple, cheap, and are typically used to localize mutations in segments of DNA of around 200 bp in length. Nowadays DGGE is often replaced by denaturing high-performance liquid chromatography (DHPLC) because of better throughput (Underhill et al. 1996). DGGE,

DHPLC and HA tests are based on heteroduplex formation. PTT (also known as in vitro synthesized protein (IVSP) test) is a RNA- or DNA-based method specifically designed to detect truncations (Roest et al. 1993), and can be used to identify the majority of mutations in cancer associated genes (including APC, BRCA1, BRCA2, and the MMR genes) that cause truncation of the respective protein products.

Tests based on the detection of sequence changes in concert with electrophoretic migration differences include two fingerprinting strategies that both use SSCP to resolve differences generated either by the creation or loss of a dideoxy termination site or by the loss or creation of a restriction endonuclease recognition site. The latter method is also known as restriction endonuclease fingerprinting (REF) (Buchman et al. 1978). In general these fingerprinting methods are more difficult and expensive, but they are capable of mapping mutations with a resolution of 10 bp and the alterations are thus easily confirmed by sequencing.

A third subclass consists of methods that are based on chemical cleavage of mismatched bases (CCM) in heteroduplexes formed between the reference and mutant nucleic acids (Cotton et al. 1988). Such mismatches can be cleaved by enzymes such as RNAses, DNA repair enzymes and resolvases or by chemicals (i.e. osmium tetroxide). CCM is dependable, but requires toxic chemicals. CMM can be used to scan segments of nucleic acids up to 1.5 kb in length, and it can ascertain the position of the mutation with accuracy.

A fourth subclass consist of methods that are based on physical separation of alleles, thus allowing individual assessment of the maternal and paternal allele, such as monoallelic mutation analysis (MAMA) (Papadopoulos et al. 1995, Laken et al. 1999), Conversion technology (Yan et al. 2000), and applications of primer extension methods (Cama et al. 1995), such as SNuPE combined with cDNA cloning and sequencing (Renkonen et al. 2003).

Most mutation screening methods are designed to detect all sequence differences including phenotypically silent changes, which makes the analysis of large, multi-exonic genes time consuming and laborious. Therefore, methods that selectively detect translation-terminating mutations, such as PTT, and do not require special equipment are widely applicable diagnostically in any disease where early terminations contribute a substantial disease cause.

**Single Nucleotide Polymorphisms**

Single nucleotide substitutions have been estimated to occur on average at one in every thousand nucleotides in the human genome (Cooper et al. 1985, Wang et al. 1998). Analysis of genetic variation caused by single nucleotide polymorphisms (SNPs) is an important tool in the search for genes underlying multifactorial diseases by association studies or genome wide

linkage disequilibrium mapping (Schafer and Hawkins 1998). Most known human genetic diseases are caused by point mutations. Consequently, improved technology for screening SNPs on a large scale will be required in the near future for both basic research and routine diagnostics. SNPs can be screened by the same methods as other genetic changes and by recently developed applications of high-throughput SNP array that can be used for detecting LOH and, further more, to identify genomic regions that may carry tumor suppressor genes. In addition, they may be applied to discriminate paternal and maternal alleles, like in this study, to evaluate their relative expression levels for possible imbalances.

## HEREDITARY CANCERS

The observation that cancer can run in a family had already been recognized before the Mendelian laws of inheritance were discovered 100 years ago. Without any knowledge of genetic etiology, a family prone to cancer was earlier identified by three typical clinical signs: a family history with several affected close relatives, early age of onset compared with sporadic cases of the same disease, and multiple primary tumors.

In 1971 fundamental theory of hereditary cancers, Knudson's two hit hypothesis (Knudson 1971), was published. It suggested that two successive mutations are required to turn a normal cell into a cancer cell. In hereditary cancers, the first allelic inactivation is inherited and the second hit is a somatic mutation in the other allele of the same cancer susceptibility gene. The first mutation is usually a point mutation while the other often involves loss of all or part of a chromosome, causing loss of heterozygosity (LOH). Therefore, a loss of one allele but retention of the other one is often seen in both sporadic and inherited disease. This means that the oncogenesis is recessive, because one normal allele is enough to suppress cancer formation (Knudson 1971), but the predisposition to cancer is inherited in a dominant way. This general rule is somewhat modified by the fact that the penetrance is reduced for many cancer-causing mutations (Garber and Offit 2005). Therefore, the disease may not manifest in gene carriers, but can be transmitted to the next generation.

Hereditary colon cancer syndromes can be classified into four groups according to the types and patterns of precursor lesions, adenomas (reviewed by Abdel-Rahman and Peltomäki 2004, Burt and Neklason 2005). The first group consists of syndromes that cause countless adenomatous polyps with the capacity to become malignant. These syndromes include familial adenomatous polyposis (FAP) and Turcot's syndrome. The second group includes MYH-associated polyposis (MAP) that exhibits both the phenotype of FAP and multiple adenomatous polyposis with less than 1000 polyps per patient, The third group consists of syndromes with only a few adenomas with the possibility to turn malignant, such as

hereditary nonpolyposis colorectal cancer (HNPCC), also referred to as Lynch syndrome. Syndromes with hamartomatous polyps, such as Peutz-Jeghers syndrome, familial juvenile polyposis and Cowden syndrome, form the fourth group (reviewed by Schreibman et al. 2005). Hamartomatous polyps are beyond the present thesis and are therefore not described in greater detail. Hamartomatous polyps are much less aggressive than adenomatous polyps. Most hereditary colon cancer syndromes are rare and only two diseases, HNPCC and FAP, may account for more than 1% percent of all colorectal cancers (Bisgaard et al. 1994, Bishop and Hall 1994, de la Chapelle 2005).

## HNPCC

### History and Characterization of HNPCC

The hereditary nonpolyposis colorectal cancer syndrome (HNPCC) was first described by Warthin in 1913 and further described by Lynch and Krush in 1971 (Lynch and Krush 1971). The characterization of medical history of several large families strongly suggested the involvement of an autosomal dominant disease (Boland 1983).

Prior to the identification of HNPCC predisposition genes, ascertainment was limited by the need for large families with several affected members and recognition of the typical characters of HNPCC families: familial accumulation of colorectal cancer, early onset, and multiple primary tumors. These families were divided into two subgroups according to the type of cancer(s) detected: Lynch syndrome I (site-specific colorectal cancer), and Lynch syndrome II (manifestation of extra colonic tumors) (Lynch et al. 1991).

HNPCC is considered to be the most common hereditary CRC syndrome comprising approximately 5% of all CRCs (Lynch et al. 1993, Lynch et al. 1996, de la Chapelle 2005). International epidemiologic studies of HNPCC suggest that environmental agents, probably of dietary origin, are the most important factors that influence the higher prevalence of this disease in certain countries (Boland 1995).

In addition to familial accumulation of colorectal cancer, the tumor spectrum of HNPCC also includes cancers of endometrium, stomach, small intestine, ovary, ureter, renal pelvis, brain, hebatobiliary tract, and skin (Lynch et al. 1993, Watson and Lynch 1993, Vasen et al. 1999). Among HNPCC patients, the estimated overall lifetime risk of colorectal cancer varies between 70-100% depending on the mutated MMR gene (Table 2). The risk of endometrial cancer (EC) is estimated to be around 50-60% (Watson and Lynch 1993, Aarnio et al. 1999),

but in some female members of HNPCC families, the risk of EC even exceeds the risk of CRC (Aarnio et al. 1999). For other cancers of the HNPCC tumor spectrum the risk is estimated to be less than 15% (Vasen et al. 1996), but higher than in age-matched controls in the general population.

**Table 2**: Lifetime risks (%) of colorectal cancer (CRC) and endometrial cancer (EC) in HNPCC according to the mutated MMR gene.

| The risk for | CRC | | EC | by age of 70. |
|---|---|---|---|---|
| | males | females | females | |
| **MLH1/MSH2** | 100 | 50 | 60 | (Aarnio et al. 1999) |
| **MSH6 mutated** | 69 | 30 | 71 | (Hendriks et al. 2004) |

The specific feature of HNPCC patients is a tendency to develop multiple primary tumors. Typically the tumors are located on the proximal side (right-side) of the splenic flexure of colon, whereas in about two-thirds of the patients with sporadic CRC tumors occur in the distal colorectum (left-side of splenic flexure) (Mecklin and Järvinen 1986, Lynch et al. 1993). These tumors occur both synchronously and metachronously. The mean age of onset in HNPCC is 40-45 years, sometimes even during early adulthood, while the corresponding age of onset in sporadic CRCs is 60-65 years, (Mecklin and Järvinen 1986, Vasen et al. 1990, Mecklin and Järvinen 1991, Lynch et al. 1993). A few adenomatous polyps may be detected in HNPCC patients, but clear polyposis such as detected in FAP is not a feature of HNPCC. These adenomas occur earlier and may be more frequent in HNPCC patients than in healthy controls (Halvarsson et al. 2005).

In 1991, prior to the knowledge of the genetic basis of HNPCC, the lack of uniform diagnostic features prompted the International Collaborative Group on HNPCC (ICG-HNPCC) to develop the Amsterdam criteria 1 (ACI) for uniformity in collaborative studies (Table 3) (Vasen et al. 1991). In 1999, the Amsterdam criteria II (ACII) were introduced to include the extracolonic cancers of the HNPCC spectrum (Vasen et al. 1999). HNPCC diagnosis can be confirmed by demonstration of a predisposing mutation in one of the MMR genes.

**Table 3:** Amsterdam criteria I and II (ACI and ACII) for the diagnosis of the HNPCC syndrome.

**Amsterdam criteria I (Vasen et al. 1991)**

Requires the presence of histologically verified colorectal cancer in at least three relatives:

◆ one of whom is a first-degree relative of the other two
◆ manifestation of the disease in at least two successive generations
◆ one or more of the relatives diagnosed with CRC before the age of 50
◆ various polyposis syndromes, especially FAP, must be ruled out

**Amsterdam criteria II (Vasen et al. 1999)**

Requires the detection of at leas three relatives with cancer of HNPCC tumor spectrum (colorectal, endometrial, stomach, ovary, ureter, renal pelvis, brain, small intestine, hepatobiliary tract of skin):

◆ one of whom is a first-degree relative of the other two,
◆ manifestation of the disease in at least two successive generations,
◆ one or more of the relatives diagnosed with HNPCC-related cancer before the age of 50,
◆ the various polyposis syndromes, especially FAP, must be ruled out in any relative with
◆ the presence of tumors should be verified whenever possible


## Mutations and Polymorphisms in HNPCC

The first HNPCC-associated DNA MMR gene, MSH2, was identified by genetic linkage analysis (Peltomäki et al. 1993). To date, six human MutS homologues and four MutL homologues are known to participate in DNA MMR, but not all are associated with HNPCC predisposition (Peltomäki et al. 2005). According to recent knowledge, HNPCC predisposition is a consequence of an inherited mutation in one of four MMR genes (MLH1, MSH2, MSH6 and PMS2) resulting in defective DNA MMR. The roles of MLH3, MSH3 and PMS1 in HNPCC predisposition is less clear (Liu et al. 2001, Huang et al. 2001, Peltomäki and Vasen 2004). Estimates of the relative proportions of mutations in the different MMR genes vary depending on the country and population (Liu et al. 1994, Han et al. 1995, Tannergård et al. 1995, Vasen et al. 1996, Kane et al. 1997, Herman et al. 1998).

The International Society for Gastrointestinal Hereditary Tumors (InSiGHT) maintains a database of HNPCC-associated deleterious mutations and polymorphisms. To date, more than 440 predisposing mutations of MMR genes have been identified in over 740 families (http://www.insight-group.org). According to the relative proportions of defective MMR genes, MLH1, MSH2 and MSH6 are "major" and the remaining MMR genes are "minor" susceptibility genes (Table 4). MLH1 and MSH2 account for nearly 90% of all mutations, whereas MHS6 mutations occur in 7% of HNPCC families, and the remaining genes are

together responsible for under 5% of HNPCC families. In Finland, the MLH1 accounts for more than 90% of Finnish HNPCC families with a known predisposing mutation (Nyström-Lahti et al. 1996). This may be explained by the predominance of two common founding mutations of MLH1 (a 3.5kb genomic deletion of exon 16 and a splice acceptor site mutation of exon 6) which account for 63% of all mutations detected in Finnish HNPCC kindreds (Nyström-Lahti et al. 1995, Moisio et al. 1996).

In general, MMR gene mutations are spread equally over the coding sequences of the three major susceptibility genes and their exon-intron boundaries. Among them, five mutation hot-spots are detected in MLH1 exons 1 and 16, MSH2 exons 3 and 12, and MSH6 exon 4 (Peltomäki and Vasen 2004). The majority (81%) of inherited MLH3 mutations is clustered in exon 1 (Liu et al. 2003). Most MLH1 and MSH2 mutations are nonsense (11% and 49%), and frame-shift mutations (44%, 19%) that cause the truncation and loss-of-function of the respective protein product. Missense mutations account for 32% of MLH1 and 18% of MSH6 mutations. Often, these mutations, especially missense mutations of MSH6, are associated with small or atypical HNPCC families, sometimes with milder phenotypes, thus their pathogenicity is more difficult to determine (Kariola et al. 2002, Kariola et al. 2004). Due to the rarity of mutations in the remaining genes, the analysis of mutation distribution and frequency of different types of mutations remains unknown.

More than 100 unclassified sequence variants and polymorphisms in MMR genes have been reported. The majority (40%) has been found in MSH6, 26% in MLH1, 25% in MSH2, and 4.5% in PMS2 and MLH3 (Peltomäki and Vasen 2004, http://www.insight-group.org).

**Table 4:** The different human MMR genes, and the number of pathogenic germline mutations and non-pathogenic genetic alterations reported. ND = not determined.

| Gene | Location | Length of cDNA (kb) | Number of exons | Genomic size (kb) | Number of mutations | % | Number of non-pathogenic variants |
|------|----------|---------------------|-----------------|-------------------|---------------------|-----|-----------------------------------|
| MLH1 | 3p31-23 | 2.3 | 19 | 58-100 | 225 | 50 | 27 |
| MSH2 | 2p21 | 2.8 | 16 | 73 | 175 | 39 | 28 |
| MSH6 | 2p21 | 4.2 | 10 | 20 | 32 | 7 | 43 |
| MLH3 | 14q24.3 | 4.4 / 4.9 | 12 | | 16 | 3 | 5 |
| PMS2 | 7p22 | 2.6 | 15 | 16 | 5 | 1 | 5 |
| PMS1 | 2q31-33 | 3.2 | ND | | 1 | <1% | - |

**Genotype-Phenotype Correlation in HNPCC**

The majority of inherited mutations of MLH1 and MSH2 genes are associated with classical HNPCC families that meet the ACI. In addition, tumors in these families are MSI positive (Nyström-Lahti et al. 1996). Inherited mutations of MSH6 are associated by families that fulfill ACI or ACII criteria or with putative or atypical HNPCC families (Kolodner et al. 1999). This reflect the roles of the different proteins in MMR, where MSH6 can be partially replaced by the MSH3 protein, whereas the functions of MLH1 and MSH2 proteins are essential and not known to be compensated for by other proteins when defective.

The association of malignant central nervous system tumors and primary brain tumors (often glioblastomas) with multiple colorectal adenomas is referred as the Turcot syndrome. This syndrome co-segregates with homozygous or compound heterozygous mutations in APC, MLH1, MSH2, PMS2 and MSH6 genes in an autosomal dominant or recessive fashion (Hamilton et al. 1995, Hegde et al. 2005). PMS2 mutations are more frequent in Turcot syndrome than in HNPCC. Another phenotypic variant of HNPCC is an autosomal dominant Muir-Torre syndrome (MTS) in which internal malignancies including gastrointestinal tumors occurs together with sebaceous skin tumors. MTS is a consequence of germline mutations in the MLH1 and MSH2 genes, the majority of which occur in MSH2 (Kruse et al. 1998, Ponti and Ponz de Leon 2005).

**Microsatellite Instability**

MMR deficiency leads to a mutator phenotype, in which the accumulation of somatic mutations is accelerated, especially in short tandem repeats, resulting in microsatellite instability (MSI) (Lynch and Chapelle 1999). MSI tumor cells display at least a 100-fold increased mutation rate at (CA)n repeats compared to microsatellite stable (MSS) tumor cells, and the increased mutability of MSI cells has been shown to associate with a profound defect in strand-specific MMR (Parson et al. 1993). When the MSI status of a tumor is defined with a Bethesda panel (a panel of five markers: BAT25, BAT26, D2S123, D5S346, and D17S250, Boland et al. 1998), tumors can be divided into MSI-high (MSI-H, two or more markers show instability) and MSI-low (MSI-L, one marker shows instability) subgroups. A total of 85-90% of HNPCC patients show MSI, and this proportion is even higher in mutation positive families, whereas only 10-15% of sporadic colorectal tumors are MSI positive (Aaltonen et al. 1993). Therefore, MSI may help to establish the diagnosis of HNPCC and should be tested at specific occasions in individuals with CRC tumors (Table 5) (Rodriquez-Bigas et al. 1997, Umar et al. 2004). Up to 90% of sporadic MSI positive cases have transcriptional silencing of MLH1, and the remainder is consistent with inactivation of MSH2 or MLH1 by somatic mutation (Kane et al. 1997, Herman et al. 1998).

**Table 5:** Original and revised Bethesda guidelines for testing colorectal tumors for microsatellite instability (MSI). (CRC = colorectal cancer; EC = endometrial cancer)

**Original guidelines (Rodriguez-Bigas et al 1997)**

Tumors from individuals should be tested for MSI in the following occasions:

- Individuals in a family meet Amsterdam criteria I
- Individuals with two HNPCC-related cancers, including synchronous metachronous CRCs or associated extracolonic cancers
- Individuals with CRC and first-degree relative with CRC and/or HNPCC –related extracolonic cancer and/or colorectal adenoma; one of the cancers diagnosed before 45 y., and the adenoma diagnosed before 45 y.
- Individuals with CRC or EC diagnosed before 45 y.
- Individuals with right-sided CRC with undifferentiated pattern (solid/cribiform) on histopathology diagnosed before 45 y.
- Individuals with signering-cell-type colorectal cancer diagnosed before 45 y.
- Individuals with adenomas diagnosed before 45 y.

**Revised Bethesda Guidelines (Umar et al. 2004)**

Tumors from individuals should be tested for MSI in the following occasions:

- CRC diagnosed in a patient under 50 y.
- presence of synchronous, metachronous CRC, or other HNPCC-related tumors despite age
- CRC with high MSI histology diagnosed in a patient under 50 y.
- CRC diagnosed in one or more first-degree relative with an HNPCC-related tumor, with one of them being diagnosed before age of 50 y.
- CRC diagnosed in two or more first-degree or second degree relatives with HNPCC-related tumors, despite age.

## Management and Treatment of HNPCC

Mutation testing in HNPCC families reduces unnecessary worry among family members who turn out mutation negative and offers a possibility to cancer prevention, early detection and treatment through cancer surveillance programs among mutation carriers (Ponder 1997). The lifetime risk of CRC is as high as 80-90% (Järvinen et al. 1995). The surveillance programs therefore primarily consist of colonoscopies or sigmoidoscopies and are recommended to start at the age of 20-25 years, repeated at two or three year intervals throughout life (Vasen 2000). This screening reduces both morbidity and mortality of CRC among mutation carriers in HNPCC families (Järvinen et al. 2000). The observation suggests that adenomatous polyps in HNPCC may be more prone to malignant transformation than adenomas in the general population. Therefore, removal of benign and malignant tumors from HNPCC family members is an important part of a prevention program. Further, the life-expectancy is

increased by seven years with surveillance screens and the cost without surveillance among the mutation positive is higher than the cost of CRC screening (Vasen et al. 1998). The screening of cancers of the HNPCC tumor spectrum is complex. Recent studies showed no benefits in screening for EC (Dove-Edwin et al. 2002), despite the fact that biannual check-ups by aspiration biopsy are recommended. The value of screening for other related cancers is ambiguous, since screening for all of these is practically impossible and/or ineffective (Mecklin and Järvinen 2005).

For mutation positive individuals in HNPCC families, prophylactic surgery (e.g. colectomy, hemicolectomy, hysterectomy) offers an option for cancer prevention instead of life-long CRC screening (Lynch and Chapelle 1999). The risk for CRC is decreased significantly by removing the high risk tissues/organs, and the number of inconvenient endoscopic screens is minimized.

Recently, a decreased risk of colorectal adenomas both in sporadic and hereditary colorectal cancer patients was observed in association with regular use of non-steroidal anti-inflammatory drugs (such as aspirin) and specific medications (such as NSAIDs and COX2-inhibitors) (Baron et al. 2003). In addition, in some countries, eating a lot of starchy food appears to reduce the risk of developing polyps and bowel cancer. However, it is unknown whether daily ingestion of aspirin and/or resistant starch will reduce adenoma initiation and progression in hereditary colorectal cancer syndromes. The Concerted Action Polyp Prevention trials (CAPP1 for FAP and CAPP2 for HNPCC) will hopefully give some answers to those questions within three to four years (Mecklin and Järvinen 2005). To date, no prophylactic medical treatments exist for patients with HNPCC and no other interventions, except diagnostic screening and prophylactic surgery, have been demonstrated to have a beneficial effect in preventing cancers in HNPCC patients.

## FAMILIAL ADENOMATOUS POLYPOSIS (FAP)

### History and Characterization of FAP

Familial adenomatous polyposis (FAP) is an autosomal dominant syndrome first described in 1925 (Lockhart-Mummery 1925). It is characterized by early onset of multiple (often hundreds to over a thousand) adenomatous polyps throughout the entire colorectum (Groden et al. 1991). In general, (tubular) adenomas are spread throughout the whole large bowel, but in over one-third of the cases their density is greatest in the proximal (cecum, appendix, ascending colon, hepatic flexure, transverse colon and splenic flexure) colon. Usually,

colorectal adenomas become visible at endoscopic examination between the ages of 10-20 years. Over time adenomas increase in number and grow in size.

If untreated, a portion of adenomas eventually progress to cancer commonly along the adenoma-carcinoma sequence (Figure 2), therefore the distribution of cancer follows that of adenomas (Bülow 1986, Caspari et al. 1994). The risk of CRC is associated with the number of polyps (Debinski et al. 1996) and is estimated to be 1-6% already at early adulthood, but CRC is also reported in children with FAP. The mean age of development of CRC is approximately 40 years. FAP accounts for 0.5-1.5% of all colorectal cancers with an incidence of around 1/8000 (Bisgaard et al. 1994).
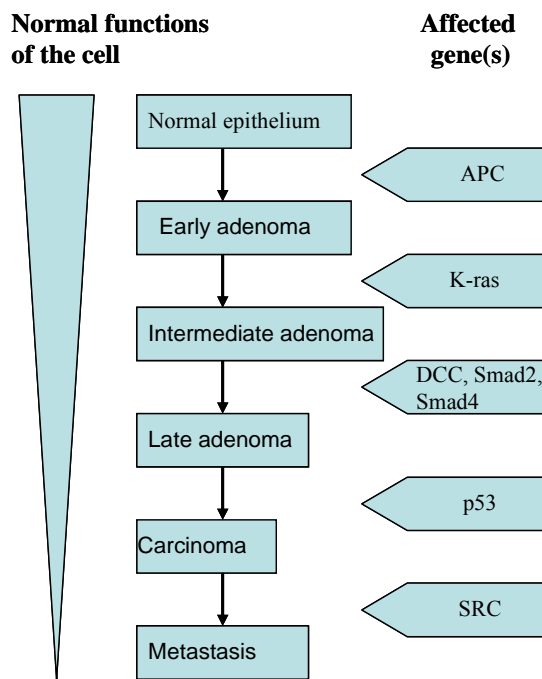
**Normal functions of the cell**

**Affected gene(s)**

Normal epithelium

APC

Early adenoma

K-ras

Intermediate adenoma

DCC, Smad2, Smad4

Late adenoma

p53

Carcinoma

SRC

Metastasis

**Figure 2**: A model of adenoma-carcinoma sequence of colorectal carcinogenesis (modified from Kinzler and Vogelstein 1996).

In addition to predisposition to colorectal carcinoma, FAP is often accompanied by extracolonic features such as upper gastrointestinal adenomas and tumors of the small intestine and stomach (Offerhaus et al. 1992) and upper gastrointestinal endoscopy is therefore recommended. Additionally, several extra-gastrointestinal organs are involved. Desmoid tumors (benign fibromas) are detected in approximately 10% of FAP patients (Clark and Phillips 1996). Dental abnormalities (up to 80% of FAP patients) (Carl and Sullivan 1989) and congenital hypertrophy of retinal pigment epithelium (CHRPE) (in 60-80% of FAP patients) are even more frequent (Blair and Trempe 1980, Chapman et al. 1989). Epidermal cysts, bone lesions (exostoses and endostoses) and tumors of the endocrine and nervous

systems are rare (in only a few percent), but they may serve as markers of FAP, and be present in children.

Tumors of the nervous system, mostly brain tumors (medulloblastomas), are associated with Turcot syndrome, a phenotypic variant of FAP that may also be a consequence of germline mutation of APC (Hamilton et al. 1995). Another phenotypic variant of FAP is Gardner syndrome with colorectal polyps and specific extracolonic manifestations, such as epidermoid skin cysts, benign osteomas, dental anomalies and desmoid tumors (Gardner 1962). Desmoid tumors cause significant morbidity and mortality in FAP patients (Jones et al.1986). Hereditary desmoid disease is also due to a germline mutation of APC that is inherited in an autosomal dominant manner (Eccles et al. 1996) and is considered as a subtype of FAP.

**Attenuated FAP (AFAP)**

The most common phenotypic variant of FAP is attenuated FAP (AFAP, AAPC). AFAP is distinguished from classical FAP by the presence of fewer than 100 adenomas in the colon, but still carries a significantly increased risk of colorectal cancer. In AFAP, the age at onset of adenomas (35-40 years) and cancer (in mid-50s) is higher and thus the lifetime risk of cancer remains lower (Friedl et al. 1996). In addition, extracolonic features and family history of colon adenomas and cancer are less frequently described. While the true incidence and frequency of AFAP is unknown (Lynch and Watson 1998), it may account for up to 10% of adenomatous polyposis families (Vasen 2000).

**APC Mutations and Polymorphisms in FAP and AFAP**

High-penetrance germline mutations of the adenomatous polyposis coli (APC) tumor suppressor gene are responsible for most FAP and AFAP cases (Groden et al. 1991, Joslyn et al. 1991). APC was localized on chromosome 5q21-22 by positional linkage analysis and subsequently cloned (Groden et al. 1991). The APC gene encompasses 8535 bp divided into 23 exons (16 translated exons and 7 alternatively expressed) that spread over a region of 120 kb. The largest exon, exon 15, comprises more than 75% of the coding sequence and is the most frequent target for both germline and somatic mutations. APC encodes a large protein that interacts with several cytoplasmic proteins. It plays an integral role in Wnt-signaling, cellular adhesion, intercellular interactions, the intracellular transmission of signals of the epithelium, and cell cycle regulation; it may also have an indirect role in regulation of apoptosis (Goss and Groden 2000). The functionally different domains of APC are shown in Figure 3.
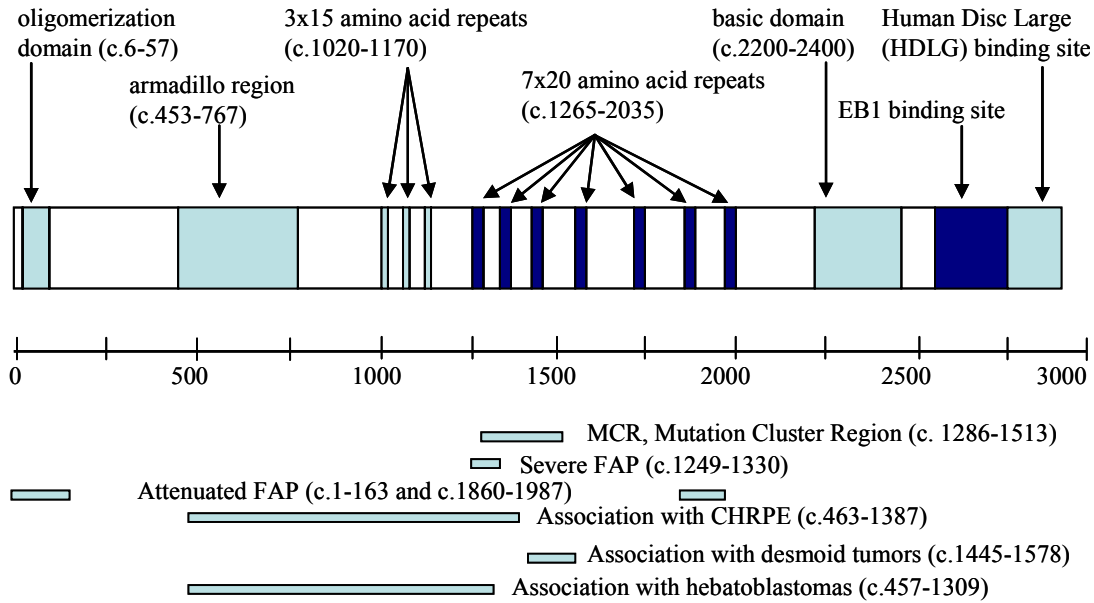
**Figure 3:** Schematic picture of functional domains of the APC protein. APC is functionally active as a homodimer via binding to itself, which occurs by oligomerization domain at the N-terminus of the protein. Lack of this domain may interfere with the function of wild-type APC and cause a dominant negative effect (Su et al. 1993). The armadillo region consists of seven highly conserved repeats, and it nearly always remains in mutated proteins (Miyoshi et al. 1992a). This region plays a role in ß-catenin degradation via its binding to the subunit of protein phosphatase 2A (PP2A) (Seeling et al. 1999), but is also suggested to have an alternative role in stabilization and motility of the actin cytoskeleton network (Kawasaki et al. 2000). Both 15 and 20 amino acid proteins bind to ß-catenin, but only the latter ones mark ß-catenin for subsequent ubiquitin-mediated degradation in the cytoplasm (Ozawa et al. 1989). Most of the 15 amino acid repeats are retained in mutated APC protein, whereas minimum of three of the seven 20 amino acid repeats are required to provide downregulation of ß-catenin (Rubinfeld et al. 1997). Because most of the truncated proteins lack all or most of the 20 amino acid repeats, the degradation of ß-catenin is prevented, and this gives rise to tumorigenesis. Three SAMP (Ser-Ala-Met-Pro) repeats lie within the 20 amino acid repeats and bind to AXIN1 protein and thus participate in stimulation of degradation of ß-catenin (Nakamura et al. 1998). The basic domain consists of an usually high proportion of arginine, lysine, and proline residues, suggesting that it is a binding site for microtubules. This domain is rarely retained in mutated APC (Smith et al. 1994). According to its name, the EB1 site binds to the end-binding protein EB1, which facilitates the interaction of APC with microtubules and other sites at the cell membrane thus regulating microtubule dynamics, cell polarity, and chromosome stability (Tirnauer and Bierer 2000). The human disc large tumor suppressor protein (HDLG) binds to a similarly named protein and participates in cell cycle progression independently of ß-catenin (Ishidate et al. 2000). Below the scale (codons), the mutation cluster region of somatic mutations is shown together with regions of germline APC mutations associated with different FAP and AFAP. Figure modified from Fearnhead et al. 2001.

To date, more than 800 pathogenic mutations in APC have been described (Sieber et al. 2002) and a germline defect of APC is detected in 70% of FAP families and 10% of AFAP families. The majority (95%) of these mutations are frameshift and nonsense mutations resulting in truncated APC proteins with abnormal function. These mutations are typically easily identified by a conventional mutation detection method, PTT (Powell et al. 1993). In addition to an inherited predisposing APC mutation, tumorigenesis in FAP requires additional somatic mutations or LOH at the APC locus (Miyoshi et al. 1992a, Lamlum et al. 1999) to stimulate cancer development. Usually, somatic mutations in APC occur early in colorectal tumorigenesis and are found in the majority of colorectal adenomas and carcinomas. These mutations are scattered throughout the gene, with some hotspots in a mutation cluster region (MCR) between codons 1286 and 1513 (Laurent-Puig et al. 1998). Somatic mutations, as well as inherited mutations, show strong correlation between the location of the first (i.e. inherited) mutation and type of the second (acquired) mutation, which differs from Knudson's "two-hit hypothesis", which is based on random occurrence of the first and second hits (Knudson 1971). When the germline mutation lies between codons 1194 and 1392, the second mutation is typically LOH of the APC region, whereas a germline mutation outside of this region is usually subsequently followed by a sporadic truncating mutation in the MCR region (Lamlum et al. 1999, Rowan et al. 2000). Around 30% of the germline mutations occur in between the two most common germline mutations at codons 1061 and 1309 (Miyoshi et al. 1992b). Over 60% of somatic mutations are clustered in the MCR with two hotspots for somatic mutations at codons 1309 and 1450 (Miyoshi et al. 1992a).

Codon 1309 is the site of a germline APC missense variant, E1317Q, which is found in non-Jewish Caucasian populations at a very low frequency. It is associated with a significantly increased risk of multiple adenomatous polyps (Frayling et al. 1998, Lamlum et al. 2000). Another APC missense variant I1307K is found in Ashkenazi Jewish populations with an incidence of six percent and also confers a several-fold increased risk of developing multiple adenomas and colorectal cancer (Frayling et al. 1998, Lamlum et al. 2000). The region affected by these missense variants interacts with many other proteins and therefore they may result in the mild dominant negative effect on APC function (Frayling et al.1998).

Methylation of APC promoters (1A and 1B) is suggested as a possible second alteration causing inactivation of APC in cases with only one detected APC mutation (Hiltunen et al. 1997, Esteller et al. 2000). Heavy methylation of the major APC promoter 1A is detected after adenoma proceeds to carcinoma, although in some studies hypermethylation is also seen in adenomas. In comparison, hypermethylation of the APC promoter 1A is a normal condition in gastric mucosa, whereas the 1B promoter is protected from methylation in the stomach and

thus probably does not participate in this form of epigenetic APC inactivation (Tsuchiya et al. 2000).

Other genes of Wnt-signaling pathway are postulated as possible candidate genes for FAP predisposition, but to date, only a germline mutation of AXIN2 in a single family has been reported (Lammi et al. 2004).

**Genotype-Phenotype Correlation in FAP and AFAP**

FAP shows large intra- and interfamily variability in the age of onset, the number, distribution and histopathology of adenomas and tumors, and the frequency and type of extracolonic lesions, although clinical features of FAP correlate to some extent with the position of the APC mutation (Spirio et al. 1993, Varesco et al. 1994). For example, the AFAP phenotype is associated with mutations in the 5' end, in the alternatively spliced part of exon 9 (not shown in Figure 3), and in the 3' end of the APC (Figure 3). Severe FAP (over 5000 polyps) is associated with mutations between codons 1249 and 1464 (Nagase et al. 1992) – this region and especially codon 1309 is also associated with early onset (Caspari et al. 1994). Mutations associated with multiple extracolonic manifestations lie between codons 1444 and 1578 (Caspari et al. 1995, Miyoshi et al. 1992a), furthermore, some smaller regions are defined for specific extracolonic manifestations such as CHRPE, desmoid tumors and hepatoblastomas (Figure 3) (Caspari et al. 1995, Wallis et al. 1999).

**Management and Treatment of FAP and AFAP**

During early phase of FAP no clear symptoms exist, but usually beginning from the third decade of life adenomas cause specific symptoms such as rectal bleeding, diarrhea, and abdominal pain. At the time of symptom-based diagnosis, approximately two-thirds of patients have already developed colorectal cancer. Regular, preventive surveillance programs for asymptomatic FAP family members offer a possibility of early diagnosis, and timely prophylactic colectomy (Järvinen 1992), and thus prevent the development of cancer. Chemoprevention with nonsteroidal anti-inflammatory drugs can also be used to reduce adenoma number and size in FAP, but the effect appears to be incomplete (Benamouzig et al. 2003).

For AFAP, recommended surveillance includes full colonoscopy screens beginning at 20-25 years of age rather than flexible sigmoidoscopy as used in FAP, because adenomas of AFAP patients are often flat and thus more difficult to detect; more over they are more frequently located in the right colon (Soravia et al. 1998). Upper endoscopy of the gastrointestinal (UGI) tract is also recommended, because of the frequent occurrence of fundic gland polyps and duodenal adenomas (Soravia et al. 1998).

Once a predisposing mutation in an FAP family is identified, a preventive colectomy is recommended for mutation carriers. Therefore, a mutation specific genetic test should be offered to at risk family members on a voluntary basis and after obtaining informed consent. Genetic counseling is offered to ensure the patient gets appropriate information about the disease, purpose of the genetic test and understands the test results (Syngal et al. 1999). Difficulties of genetic testing include estimation of the need for surveillance in putative FAP families with no detectable germline mutations. In addition, a significant proportion (20%) of de novo mutations among FAP cases (Phillips et al. 1994) challenge the selection of at-risk individuals for surveillance.

**MYH-Associated Polyposis (MAP)**

Recently, biallelic mutations in the base excision repair gene MYH have been shown to be responsible for predisposition to multiple adenoma and colorectal cancer (Al-Tassan et al. 2002). While the incidence of MAP is unknown, biallelic mutations of MYH may account for 7-17% of APC mutation negative FAP families (Sieber et al. 2003, Russell et al. 2005) and approximately 4% of families with multiple adenomatous polyposis or AFAP phenotype (Sieber et al. 2003). The frequency of monoallelic MYH mutations in APC mutation negative FAP families may be even higher (Russell et al. 2005).

Typically, the colorectal polyps (3-1000 per patient) in MAP are synchronous and metachronous small, dysplastic tubular adenomas, with a minority of tubuvillous adenomas, and very few hyperplastic polyps (Sieber et al. 2003). The number of tumors in patients with biallelic MYH mutations exceeds that in those with monoallelic MYH mutations, supporting the observation that biallelic MYH mutations are associated with increased risks for tumor development (Sieber et al. 2003). The age of onset is higher in MAP than in carriers of pathogenic APC mutations (Sieber et al. 2003).

Because the phenotype of MAP closely resembles FAP and AFAP phenotypes, and clinical features alone cannot distinguish MAP from FAP or HNPCC, genetic analysis of MYH should be offered to patients with 1) a phenotype resembling FAP or AFAP, 2) no identifiable pathogenic mutation in APC in the family, and 3) a family history compatible with an autosomal recessive mode of inheritance. Predictive genetic testing should be offered to family members of carriers of biallelic MYH mutations.

# AIMS OF THE STUDY

A considerable fraction of families with HNPCC or HNPCC-like syndrome as well as families with FAP or AFAP fail to show mutations in the known susceptibility genes, the MMR genes and APC, respectively. This may be a consequence of mutation detection methods that are unable to detect all kinds of mutations or the fact that predisposing mutations may lie in other, known or as yet unknown, susceptibility genes.

The starting points for the present work were population based series of 81 HNPCC families (Nyström-Lahti et al. 1996, Holmberg et al. 1998) and 65 classical FAP families (Moisio et al. 2002) that screened negative for predisposing mutations by conventional mutation detection techniques. This thesis aimed to address the genetic basis of these mutation negative HNPCC and FAP families, and to clarify genotype-phenotype correlations in such families.

The specific aims of the thesis were to:
- clarify the frequency of "hidden" MMR gene mutations among HNPCC families that appeared mutation negative by conventional methods,
- diagnose truly MMR mutation negative HNPCC families for further searches for novel susceptibility genes,
- clarify the genetic epidemiology of HNPCC in the Uruguayan population by conventional and advanced mutation detection methods,
- clarify the genetic etiology of APC mutation negative FAP/AFAP,
- analyze the genotype-phenotype correlations between etiologically different FAP/AFAP families, and
- diagnose truly APC mutation negative FAP/AFAP families for further searches for novel susceptibility genes.

## MATERIALS AND METHODS

### PATIENTS AND PEDIGREE INFORMATION (I–IV)

Material for this study was obtained from recent studies including two main series of families from a population-based registry 1) 81 HNPCC families (Nyström-Lahti et al. 1996, Holmberg et al. 1998), 2) 65 classical FAP families (Moisio et al. 2002) that were screened for predisposing mutations by conventional DNA- and RNA-based mutation detection methods. Families without a detected cancer-predisposing mutation were considered as an "MMR/APC mutation negative panel" and consisted of 26 Finnish MMR gene (MLH1, MSH2, MSH6, PMS1 and PMS2) mutation negative HNPCC families (study I and II) and 29 Finnish APC mutation negative FAP families (study IV) whose background of cancer predisposition remained unknown. Four Swedish MMR gene mutation negative HNPCC families were also included in study I.

In addition, 12 Uruguayan HNPCC or putative HNPCC families were included in conventional and advanced mutation detection screens (III) for comparison of the epidemiology of HNPCC in Uruguayan and Finnish populations. Uruguay provides a useful reference for Finland since the size of the population is similar, the majority of the Uruguayan population originates from Southern Europe, and well-organized colon cancer registries offer an excellent possibility for high standard scientific research.

Pedigree information and clinical data concerning Finnish HNPCC and FAP families were obtained from the Hereditary Colon Cancer Registry of Finland maintained by Dr. Jukka-Pekka Mecklin and Dr. Heikki Järvinen. Pedigree information about Swedish HNPCC families (I) was provided by Dr. Annika Lindblom and about Uruguayan families (III) by Dr. Henry T. Lynch. All human specimens were collected after informed consent was given on a voluntary basis according to the guidelines of the institutional review boards. Patients with identified mutations were included in genetic counseling programs.

### SAMPLE PREPARATION (I–IV)

Total cellular DNA was extracted from cultured lymphocytes or lymphoblasts (Lahiri and Nurnberger 1991). Total cellular RNA was extracted by the guanidium thiocyanate method (Chomczynski and Sacchi 1987). Two different kinds of cDNAs were reverse transcribed from total cellular RNA (RT-PCR). For hexamer cDNA preparation (I, II, IV), 1 ug of template RNA was incubated at 42°C for 60 min and at 95°C for 5 min in a 20µl reaction with

standard 5x amplification buffer with 1.5 mM $MgCl_2$ (Promega), 10 mM dNTPs (Finnzymes), random hexamers (Promega), RNase ribonuclease inhibitor (Promega) and 200 U of M-MLV reverse transcriptase. Oligo-dT cDNA was prepared with oligo-dT and SuperScript II according to the manufacturer's instructions (SuperScriptTM First-Strand Synthesis System for RT-PCR, Invitrogen).

Normal and tumor DNA was extracted from paraffin-embedded tissue sections (Isola et al. 1994) and microscope slides of sliced (4-5μm) tissue material were prepared for immunohistochemical analyses.

## MUTATION DETECTION BY CONVENTIONAL METHODS

### Denaturing Gradient Gel Electrophoresis, DGGE (I, III)

DGGE was used to screen polymorphisms (http://www.insight-group.org) and mutations in 26 Finnish and 12 Uruguayan HNPCC families in MLH1 exons 2 (T66T), 8 (I219V, I219L), 12 (S406N), 17 (L653L) and 19 (del TTC at nucleotide 2268 +33 in the 3' untranslated region of MLH1) and MSH2 exons 2 (R96H, K110R, K113K), 6 (G322D), 11 (L556L, K579K, S585S) and 13 (G713G, Q718Q) to verify the presence of two allelic copies of the respective gene/exon and to allow allelic discrimination in mRNA expression studies. The sequences for PCR primers are given in Wu et al. (1997). MSH6 exons 1 (A36A, G39E, R62R), 2 (P92P), and 3 (D180D) were screened for polymorphisms by exon-specific sequencing (Chadwick et al. 2001).

DGGE samples (250 ng genomic DNA) and a healthy control to show the exact size of each exonic product and one negative control including water instead of gDNA were amplified with AmpliTaq® DNA polymerase (Applied Biosystems) by the following protocol (working concentrations in parentheses: 1 μl dNTP mix (concentration of each nucleotide 10mM, Finnzymes), 1 µl both forward and reverse DGGE primers (25 pmol/µl), 5 µl GeneAmp® 10X PCR Buffer (Applied Biosystems), 0.2 µl AmpliTaq DNA polymerase (250 U) and 36.8 µl $H_20$ to bring the total volume to 50 µl.

The PCR program consisted of the following steps: an initial incubation at +94°C for 1 min, followed by 35 additional cycles, each with 1 min denaturation at +94°C, 1 min annealing at various temperatures, and 2 min elongation at +72°C, followed by a final extension for 3 min at +72°C. To increase the amount of heteroduplex formation the samples were denatured for 10 min at +96°C and renatured for 60 min at +52°C. Then the PCR products were ethanol-

precipitated and diluted in 15 µl neutral DGGE-loading buffer (2 g sucrose, 100 µl 1M Tris-HCl, pH 7.8, 20 µl 0.5M EDTA pH 8.0, 10 mg BPB added up to 10 ml with MilliQ water).

The equipment, details of preparing and running gradient gels, and detailed discussion of how to prepare samples and determine the melting behavior and optimal electrophoresis times are described by Myer (Myers et al. 1985a, Myers et al. 1985b).

The 15-µl aliquots of amplified exons were separated by size by electrophoresis in 0.75-1 mm thick 6.5% polyacrylamide (PAA) gels (acrylamide:bisacrylamide 37.5:1, BioRad) containing a denaturing gradient of 5-70% urea-formamide (UF), which ranges from below to above the calculated melting gradients of the DNA fragments in a PAA gel. Samples were run at 110V until the lower loading bands front came out of the gel and the upper loading bands front had passed the middle point of the gel. The separation pattern was visualized on a UV transilluminator after ethidium bromide staining (500 ng/ul) for 30 min and documented.

### APC Promoter Analysis (IV)

The APC promoter region (U02509, http://www.ncbi.nlm.nih.gov) was screened for alterations in FAP families belonging to subgroup II: unbalanced mRNA expression (IV). Samples were amplified with primers 5-gctagcatagctttctggtaac-3' and 5'-cagtgacaccctggcgggctg-3' and purified PCR products were sequenced using an automated sequencer ABI 3730.

### Protein Truncation Test, PTT (IV)

In this study PTT was used to screen for truncating mutations in FAP families that had entered the study after 2002 and thus were not included in previous mutation screens by Moisio et al. (2002). For PTT analysis, the entire coding region of the APC gene was divided into 5 (1A, 1B, 2, 3 and 4) fragments. Exons 1-14 were amplified in two overlapping cDNA fragments: 1A encompassing exons 1-9 and 1B encompassing exons 8-14. Exon 15 was covered with fragments 2-4. RT-PCR primers for fragments 1A and 1B are described by Soravia et al. (1998) and primers for gDNA amplification of fragments 2-4 are described by Powell et al. (1993). PTT was performed using the TNT coupled reticulocyte lysate system (Promega Corporation) according to the manufacturer's instructions. Translation products (4 µl) were run in precast 4-20% TG gels (Novex). The aberrant protein bands were sequenced from DNA samples. Mutations identified in cDNA fragments 1A and 1B were further verified in genomic DNA.

**Heteroduplex Analysis of APC (IV)**

Exon specific amplification products of APC exons 1-14 were incubated at 95°C for three minutes and allowed to cool until they reached a temperature of 37°C for heteroduplex formation. The samples were loaded onto 0.5% MDE gels (AT Biochem, Malvern, USA), prepared according to the manufacturer's instructions, and run at 12 mA and 9 W for 11-15 hours at room temperature. The bands were visualized by silver staining. PCR products showing aberrant heteroduplex patterns were sequenced from genomic DNA.

**MUTATION DETECTION BY ADVANCED METHODS**

**Single Nucleotide Primer Extension, SNuPE (I, III, IV)**

Primer extension has been used to map the 5' termini of mRNAs, detect splice variants of mRNAs, map precursors and processing intermediates of mRNA, and measure the abundance of particular target mRNAs (Boorstein and Craig 1989). In this study, a non-radioactive modification of SNuPE was used to quantify the relative expression of mRNA transcripts in patients heterozygous for a coding polymorphism. The extension reaction of SNuPE is based on the incorporation of a single ddNTP that is selected to allow differential extension of a labeled primer annealed close to the polymorphic site (Figure 4, a).
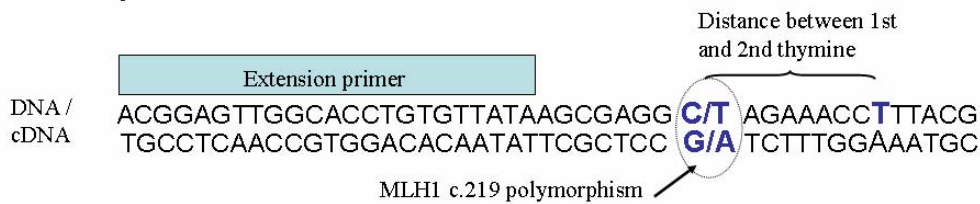
Parallel gDNA and cDNA samples from the same individuals were PCR-amplified and purified by incubating 10 µl PCR product, 1 µl Exonuclease I (10 U/µl) and 1 µl SAP (Shrimp alkaline phosphatase, 2 U/µl) together at +37°C for 15 min and 15 min at 80°C to inactivate the SAP and Exonuclease. The Exonuclease I removes residual single-stranded primers and any extraneous single-stranded DNA produced by the PCR. SAP removes the remaining dNTPs from the PCR mixture, which could interfere with the primer extension reaction. This purification method will not remove double stranded DNA elements, such as primer-dimers or non-specific amplicons, and those must be removed prior to the extension reaction by additional purification by a spin column method, such as the QIAquick PCR Purification Kit Protocol (Qiagen).

The sequences of primers used for amplification and extension reactions are given in I: Table 2 and IV: methods. The total primer extension reaction volume was 20 µl including 2 µl of purified PCR product (gDNA or cDNA), 2 µl of ddXTP (the nucleotide to be labeled), 2 µl each of other dNTPs, 0.2 µl of extension primer (20 pmol/µl), 7.6 µl of $H_2O$, 0.2 µl Thermo Sequenase™ DNA Polymerase (Amersham Pharmacia Biotech) and 2 µl of Thermo Sequenase Reaction Buffer (concentrated, 1x 1ml 260 mM Tris-HCl, pH 9.5, 65 mM $MgCl_2$).
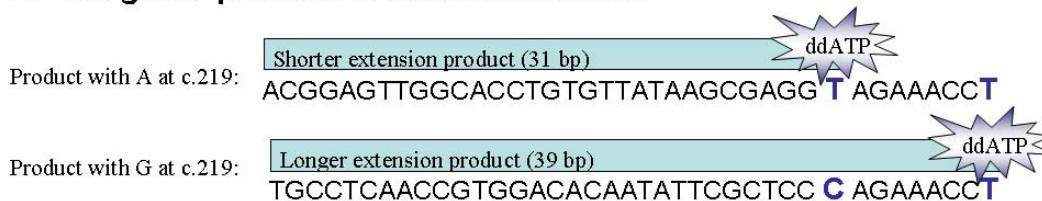
Extension was performed by PCR with the first denaturation for 2 min at +95°C, and subsequently 25 cycles consisting of 20s denaturation at +95°C, 20 s annealing at a primer-specific temperature, and 40 s elongation at +70°C, after which the final extension was done for 10 min at +70°C.

The products of the primer extension reaction (Figure 4b) were electrophoresed through 9% LongRanger Gel in an ABI 377 sequencer and analyzed by the Genotyper program (I) or by Genemapper (IV). The allelic cDNA ratio (peak area from the shorter extension product divided by peak area from the longer extension product) was normalized by the corresponding ratio obtained from genomic DNA (Figure 4c).

## A: Principle of SNuPE

Distance between 1st and 2nd thymine

DNA / cDNA

| Extension primer |
ACGGAGTTGGCACCTGTGTTATAAGCGAGG **C/T** AGAAACC**T**TTACG
TGCCTCAACCGTGGACACAATATTCGCTCC **G/A** TCTTTGG**A**AATGC

MLH1 c.219 polymorphism

## B: Elongation products of extension reaction

Product with A at c.219:
| Shorter extension product (31 bp) | ddATP
ACGGAGTTGGCACCTGTGTTATAAGCGAGG **T** AGAAACC**T**

Product with G at c.219:
| Longer extension product (39 bp) | ddATP
TGCCTCAACCGTGGACACAATATTCGCTCC **C** AGAAACC**T**

## C: Examples of SNuPE results

A/G heterozygote: Balanced mRNA expression

A/G heterozygote: Unbalanced mRNA expression

Nucleotide at c.219 (in extension product)  A   G       A   G

Peaks from genomic DNA

Peaks from cDNA (mRNA)

31 bp fragment from allele A    39 bp fragment from allele G    Decrease of 50% of allele A mRNA expression
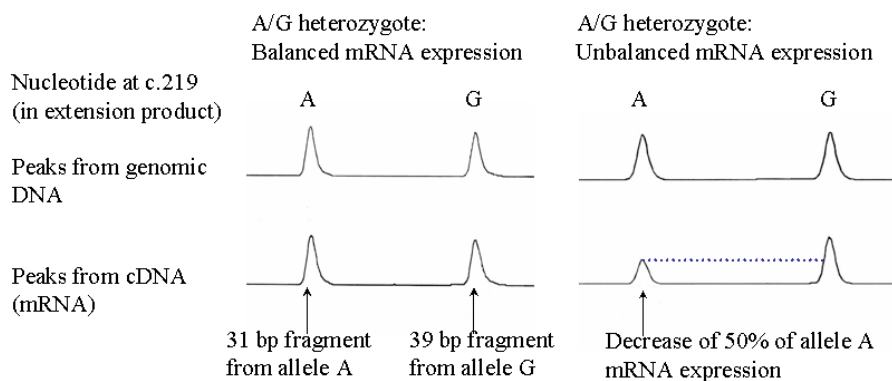
**Figure 4**: Major steps of the single nucleotide primer extension (SNuPE) protocol, using the MLH1 exon 8 A/G polymorphism as an example.

**cDNA Cloning of MLH1 and MSH2 (I, II)**

Full-length oligo-dT transcribed MLH1 (2.3 kb) and MSH2 (2.8 kb) cDNA was amplified (primers from Nomura et al. 2000) using the Expand Long Template PCR system (Roche, Basel Switzerland). The amplification products were cloned into pCRII-TOPO vector taking advantage of the TOPO TA cloning system (Invitrogen) to separate the cDNA products of mutant and healthy alleles. The resulting plasmids were used to transform XL1-Blue (Stratagene, La Jolla, CA) cells, and recombinant clones were identified based on white color on Xgal-containing plates. Among positive colonies, all available or at least 25 separate colonies were selected for DNA isolation and subsequent analysis.

**MSI Analysis (I, III)**

Most of the tumors included in this study was analyzed with the complete Bethesda panel (BAT25, BAT26, D5S346, D2S123, and D17S250), but all of the tumors were analysed with at least the BAT25 and BAT26 markers. Tumors were classified according to the number of unstable markers: none or one unstable marker was considered as microsatellite stable (MSS, MSI-negative) while two or more unstable markers were considered as microsatellite unstable (MSI-positive).

**MLH1 Promoter Methylation Analysis (I)**

Methylation status of four HpaII sites in the MLH1 promoter region (CCGG at -567, -527, -347, and 341) relative to the A of the initiation codon of MLH1) was studied by taking advantage of methylation sensitive restriction enzyme HpaII that is unable to restrict methylated DNA. Results were obtained by comparing the methylation status of normal colonic DNA and tumor DNA from the same individuals. Primers and protocols were as described by Kuismanen et al. (1999).

**MLH1 and MSH2 Haplotype and Genetic Linkage Analyses (I)**

A possible MLH1-linked haplotype was sought using a marker panel (ptel - D3S1266 - D3S3727 - D3S1611 (MHL1) - D3S1277 - D3S1298 - D3S1289 - cen) that spreads over a 23 cM region around MLH1. An MSH2-linked haplotype was sought by a marker panel (ptel-D2S2259-D2S391-(MSH2/MSH6)-D2S123-D2S337-D2S2368-cen) that covers 15 cM region around MSH2. Primer sequences for microsatellite markers can be found at http://www.gbd.org.

**MLH1 and MSH2 Promoter Mutation Analysis (I)**

To detect possible mutations in the promoter regions of MLH1 and MSH2, we sequenced 929 bp gDNA upstream of the MLH1 initiation codon and 457 bp of gDNA upstream of the MSH2 initiating codon. Primers for two overlapping fragments of MLH1 and one fragment of MSH2 promoter regions are listed in I: methods.

**Multiplex Ligation-Dependent Probe Amplification, MLPA (III, IV)**

The MLPA test is designed to detect large genomic rearrangements of specific genes (MRC-Holland, Amsterdam, The Netherlands). The MLPA method is based on sequence-specific probe hybridization to genomic DNA, followed by PCR amplification of the hybridized probe (with one amplification primer fluorescently labeled), and semiquantitative analysis of the PCR products. Target-specific products are identified according to their differential length (Figure 5).

The MLPA Salsa P043 kit (III, Figure 5) was used to screen DNA samples for large genomic rearrangements of APC according to the manufacturer's instructions. This includes probes for each coding exon of APC, three probes for the APC promoter region, and 11 probes for other human genes located on different chromosomes.

MLPA Salsa P003 and Salsa P008 (IV) kits were used to analyze DNA samples of HNPCC families for large genomic rearrangements of MMR genes according the protocols provided by MRC-Holland. The Salsa P003 kit is designed to detect deletions/duplications of one or more exons of the MLH1 and MSH2 genes and contains probes for the 19 exons of the MLH1 gene and for the 16 exons of the MSH2 gene. In addition, 7 probes for other human genes located on different chromosomes are included as controls.

The Salsa P008 kit provides a simple method to determine the copy number of many exons of PMS2, MLH3, MUTYH, MSH3 and MSH6 genes and includes probes for 7 of the 10 MSH6 exons, 11 of the 15 PMS2 exons, MSH2 exon 1, two probes for the TACSTD1 gene, three probes for MLH1 exon 1. In addition, four probes are present for the MUTYH gene, five probes are present for MLH3 and MSH3 exons, and one probe for APC.

FAM-labeled PCR products were analyzed on an ABI 3730 capillary sequencer (Applied Biosystems) using Genescan-ROX 500 size standards (Applied Biosystems). Fragment analysis was performed using Genemapper software. A deletion was suspected when the peak area was reduced more than 40-50% compared to normal controls in at least two independently repeated MLPA analyses and was confirmed by the observation of the

involvement of contiguous exons (whole-gene deletions) or the demonstration of a deletion-specific fragment in cDNA (exon 4 deletion).
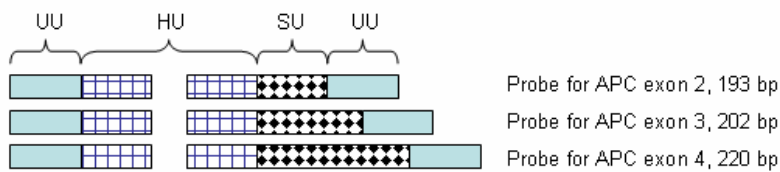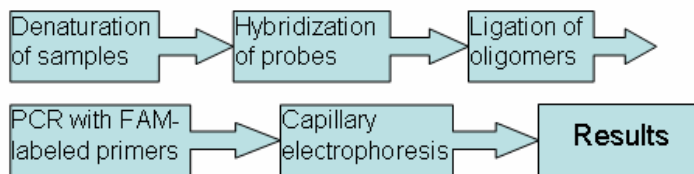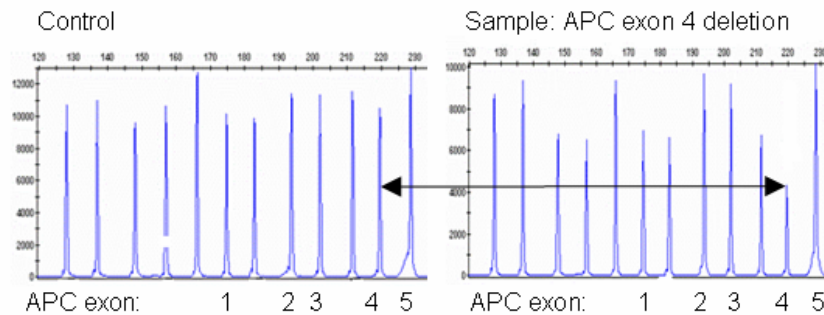


**Figure 5:** Principles, flowchart and examples of results of multiplex ligation-dependent probe amplification (MLPA), using a few reactions of Salsa P043 kit for APC as an example.

**APC Haplotype Analysis (IV)**

Eight polymorphic microsatellite markers or single nucleotide polymorphisms around and within APC, respectively, were analyzed for haplotype construction with the following marker panel: cen - D5S409 - D5S1965 - APC promoter a/g polymorphism, rs 2019720 (Sieber et al. 2002) - APC intron 7 a/t polymorphism, rs 1914 (Sieber et al. 2002) - APC exon 11 C/T polymorphism (nt.1458) - APC exon 15 A/G polymorphism (nt. 5037)] (Sieber et al. 2002) - D5S346 - MCC (Peltomäki et al. 1992) - tel. Primer sequences for microsatellite markers are listed at http://www.gdb.org.

**Analysis of APC for LOH in Tumor Tissue (IV)**

Paired tumor and normal DNA samples were examined for LOH in the APC region by SNuPE (as described above). Polymorphisms of APC exon 11 and microsatellite markers closely linked to APC, D5S1965 (200 kb upstream of APC) and D5S346 (less than 100 kb downstream of APC), were investigated. The marker-specific fluorescent amplification products were run on an automated sequencer and analyzed by Genemapper. The ratios of allelic peak areas in tumor DNA relative to normal DNA were interpreted to suggest LOH if below 0.60 or above 1.67 (indicating that one allele had decreased 40% or more), and putative LOH if between 0.60 and 0.80, or between 1.25 and 1.67 (indicating a decrease of 21-39% for one allele).

**AXIN2 Mutation Screen (IV)**

Total 29 APC mutation negative FAP/AFAP families were sequenced for AXIN2 alterations. Primers and conditions for exon specific sequencing of AXIN2 are described at http://helsinki.fi/science/dentgen (Lammi et al. 2004).

**Statistical Methods (I, IV)**

T-tests for independent samples or Fisher's exact test (two-tailed) were used to evaluate the statistical significance of differences between known MMR gene mutation positive HNPCC families and subgroups MMR gene mutation negative subgroups 1 and 2 (I) and between FAP subgroups (IV).

## RESULTS

**Genetic Basis of MMR Gene Mutation Negative HNPCC Families (I, II)**

Our expression-based strategy included the study of the major MMR genes (MLH1, MSH2, and MSH6) for protein expression and the examination of relative allelic mRNA expression of MLH1 and MSH6. The status of MSH2 mRNA expression could not be clarified due to homozygosity for the coding polymorphisms tested. This strategy linked 42% (11/26) of earlier mutation negative Finnish HNPCC families to an MMR defect based cancer predisposition. Evidence of a hidden mutation in MLH1 was obtained in 23% (6/26) of families, whereas 15% (4/26) and 4% (1/26) of the families were associated with hidden MSH2 and MSH6 mutations, respectively (I: Table 4).

Further studies (RT-PCR combined with cDNA cloning of MLH1 and MSH2, splicing analyses and sequencing) unraveled two predisposing mutations (Table 6): R100X of MLH1 (in F73) and deletion of AGAA in MLH1, codon 70 (in F95). Both of these mutations were associated with multiple exon skipping in the mutation containing allele. An increase of exon skipping with R100X was statistically significant and, furthermore, it was observed in two family members in F73, indicating that the aberrant splicing cosegregated with the cancer phenotype. In addition, the A allele (MLH1, exon 8, codon 219) of two affected family members of F73 containing a R100X point mutation showed an expression reduction of similar magnitude for MLH1 mRNA with no protein expression evident in immunohistochemistry.

Four Swedish HNPCC families were screened for unbalanced mRNA expression of MLH1 and MSH6 according to the defective gene pinpointed by immunohistochemical analysis. Two of three families with no MLH1 protein expression showed unbalanced allelic mRNA expression (I: Table 4). Moreover, a predisposing mutation in at least one family (genomic deletion of MLH1 exon 11 in family S4) was identified by quantitative multiplex PCTR (Di Fiore et al. 2004, Table 6).

**Frequency and Genetic Basis of Uruguayan HNPCC Families (III)**

A retrospective analysis of unselected CRC cases (n = 461) included 12 patients (2.6%) who fulfilled the ACI or ACII requirements for HNPCC, 26 (5.6%) were considered to represent the population at increased risk (PIR) of cancer, and 423 (91.8%) were patients with sporadic cancers and did not fulfill the criteria for either of these two groups. Families fulfilling the ACI or ACII were screened for point mutations in major MMR genes by combination of

conventional and advanced mutation detection methods (see details in III) The genetic basis of colorectal cancer predisposition could be specified at the nucleotide level in two Uruguayan families (Table 6). In one (U4082), two affected individuals had a germline mutation of MLH1, del A at nucleotide 663, codon 221, that resulted in the truncation of the protein at codon 228. In the other family (U8161) a nonsense mutation of MSH2, R406X, was observed in a proband. Other family members were not available for mutation detection. In this cohort of Uruguayan HNPCC families, no unbalanced mRNA expression or large genomic rearrangements existed.

**Table 6:** Summary of HNPCC families with evidence of "hidden mutations". (MSI = microsatellite instability, del = deletion, nt. = nucleotide)

| Finnish family ID | Mutation | Expression based evidence (IHC/mRNA expression) | Other evidence |
|---|---|---|---|
| F36 | * | MLH1 - / Unb. expr. of MLH1 | MSI |
| F44 | | MLH1 - / | |
| F48 | | MLH1 - / | MSI |
| F53 | | MLH1 - / | MSI |
| F57 | | MLH1 - / | MSI |
| F65 | MSH2: Q593X | MSH2&6 - / | MSI |
| F73 | MLH1: R100X | MLH1 - / Unb. expr. of MLH1 | MSI, splicing |
| F70 | | MSH2&6 - / | MSI |
| F71 | ** | MSH2&6 - / | MSI |
| F81 | | MSH2&6 - / | MSI |
| F88 | | MSH2&6 - / | MSI |
| F84 | | MSH6 - / | MSI |
| F95 | MLH1: del AGAA at nt. 210 | MLH1 - / | MSI |

| Swedish family ID | Mutation | Expression based evidence (IHC/mRNA expression) | Other evidence |
|---|---|---|---|
| S4 | MLH1 ex11 del | MLH1 - / Unb. expr. of MLH1 | MSI |
| S237 | | MLH1 - / | MSI |
| S346 | | MLH1 - / Unb. expr. of MLH1 | MSI |
| S11 | | MSH2&6 - / | MSI |

| Uruguayan family ID | Mutation | Expression based evidence (IHC/mRNA expression) | Other evidence |
|---|---|---|---|
| U4082 | MLH1: del A at nt. 663 | | |
| U8161 | MSH2: R406X | | |

* MLPA evidence of exonic deletion of MLH1 (unpublished data)

** MLPA evidence of exonic deletion of MSH2 (unpublished data)

**Clinical Characteristics in Finnish and Uruguayan HNPCC Families (I, III)**

Finnish HNPCC families with expression-based evidence of MMR involvement (I: group 1) resemble those with previously detected mutations in one of the major MMR genes by similar age of onset (45.8 y. vs. 45.2 y., respectively), and an equal number of affected family members within a family (6 vs. 8, respectively). In addition, the age of onset of these groups closely resembles Uruguayan HNPCC families (45 y.).

Finnish HNPCC families with no evidence of MMR gene involvement (I: group 2) have a significantly higher age of onset (53.7 y.) than Finnish mutation positive families (P < 0.0001 by two-tailed T-test) However, the age of onset in group 2 is markedly higher than in Uruguayan patients with increased cancer risk or sporadic CRCs, suggesting that it represents a genetically and clinically distinct category of familial colorectal cancer.

**Genetic Basis of "APC Mutation Negative" FAP/AFAP Families (IV)**

Previously, Moisio et al. (2002) screened 65 FAP families, among which 28% (18/65) showed no APC germline mutations by conventional methods (Table 5): PTT, heteroduplex analysis, and exon-specific sequencing of cDNA. We added another cohort of 11 Finnish FAP/AFAP families that were screened for mutations by the same methods without identification a predisposing mutation. These 29 families were screened for "hidden" mutations in APC by more advanced mutation detection methods (Table 5) – MLPA, SNuPE of APC mRNA, and LOH analysis of tumor tissue using markers from the APC region.

The genetic basis of previously APC mutation negative FAP/AFAP families could be determined in a total of 38% (14/29) of families (summarized in IV: Table 2). In 10% (3/29) MLPA detected a large genomic deletion of an entire APC allele and in one (4%) a deletion of APC exon 4 was found. In seven families no sequence alteration in the APC gene was detected, but a constitutionally unbalanced expression of APC mRNA combined with frequent APC-LOH in tumor tissue strongly suggests a mutation that was not detected by the methods applied. Finally, in three (10%) of the families a predisposing mutation was detected in genes other than APC. One family harbored a germline mutation in AXIN2 (Lammi et al. 2004), whereas in two families a biallelic germline mutation of MYH explained the predisposition to cancer (Alhopuro et al. 2005).

Unfortunately, in 52% of the FAP/AFAP families (15/29) the role of APC in tumor predisposition could neither be confirmed nor excluded and AXIN2 and MYH studies did not reveal any mutations. However, in some of the families (FAP57 and FAP74) a shared disease haplotype (Table 7) was observed, and in 60% (9/15) of families from which tumor samples

were available showed strict or putative LOH in the APC region (IV: Tables 2 and 3), possibly serving as the second hit needed for tumor development.

**Table 7:** Haplotypes across the APC region from centromere to telomere based on allele sharing within a family.

| | | APC | | | | | |
|---|---|---|---|---|---|---|---|
| **Family: Individual** | D5S1965 | promoter | intron 7 | exon 11 | exon 15 | D5S346 | MCC |
| 57:1 | 185 | A | T | A | G | 121 | 75 |
| | 181 | G | A | G | A | 111 | 75 |
| 57:2 | 185 | A | T | A | G | 121 | 75 |
| | 185 | G | T | G | G | 111 | 75 |
| 57:3 | 185 | A | T | A | G | 121 | 75 |
| | 185 | G | A | G | A | 111 | 75 |
| 57:4 | 185 | A | T | A | G | 121 | 75 |
| | 183 | G | A | G | A | 111 | 75 |
| 57:5 | 185 | A | T | A | A | 105 | 75 |
| | 185 | G | A | G | A | 111 | 89 |
| 74:1 | 185 | A | T | A | G | 111 | 75 |
| | 183 | A | T | A | A | 119 | 89 |
| 74:2 | 185 | A | T | A | G | 111 | 75 |
| | 191 | A | T | A | A | 119 | 75 |

*Group III* spans all rows.

In box = shared haplotype within family;

## Genotype-Phenotype Correlations in FAP/AFAP Families (IV)

FAP families with large genomic deletions displayed similar clinical features as those with truncating APC mutations detected by conventional methods (Table 8 and IV: Table 1). These features included profuse polyposis (over 100 polyps/patient), early onset and excess of extracolonic manifestations. A milder phenotype, AFAP, was observed in a family with a deletion of APC exon 4 with a remarkably higher mean age (69 y.) at polyposis than in those with the whole APC gene deleted (27.3 y.). Families with unbalanced mRNA expression of APC alleles showed classical polyposis with a significantly higher (P = 0.04) mean age of onset and less frequent extracolonic manifestations than FAP families with detectable genomic mutations in APC. Families with no direct evidence of APC involvement or families with MYH or AXIN2 mutations often showed attenuated or atypical FAP. The polyp count in

MYH-associated polyposis (MAP) was low and no clear evidence of dominant inheritance existed. Dental agenesis was specifically linked with AXIN2-associated polyposis.

**Table 8**: Comparison of clinical features between genetically discrete groups of Finnish FAP and AFAP families.

| | Proportion of families with classical FAP (>100 polyps in at least 1 patient) | Number of families with | | | | Mean age at polyposis diagnosis (years), in probands | Proportion of families with extracolonic manifestations |
|---|---|---|---|---|---|---|---|
| | | multiple patients | | vs. | single patient | | |
| | | dominant | resessive | | | | |
| **Group I** | 4/4 (100%) | 1 | 0 | vs. | 3 | 37.8 | 4/4 (100%) |
| **Group II** | 7/7 (100%) | 4 | 0 | vs. | 3 | 47.3 | 3/7 (43%) |
| **Group III** | 12/15 (80%) | 5 | 0 | vs. | 10 | 45.2 | 7/15 (47%) |
| **Group IV (AXIN2)** | 0/1 (0%) | 1 | 0 | vs. | 0 | 56 | 1/1 (100%) |
| **(MYH)** | 0/2 (0%) | 0 | 1 | vs. | 1 | 48.5 | 2/2 (100%) |
| **APC mutation-positive FAP (Moisio et al. 2002)** | 47/47 (100%) | 37 (79%) | 0 | vs. | 10 (21%) | 35.6 | 42/47 (89%) |

**Unbalanced mRNA Expression in MMR Genes and APC Gene (I, IV)**

Studies of MMR gene mutation positive HNPCC families (6 different MLH1 mutations, 1 MSH6 mutation) indicated that all studied frameshift mutations were associated with unbalanced allelic mRNA expression, whereas neither missense nor in-frame mutations resulted in imbalance in allelic expression. Importantly, only mRNA of the mutation containing MMR gene showed with unbalanced expression while the remaining MMR genes displayed balanced mRNA expression. Notably, the allele linked with the HNPCC phenotype was the allele showing decreased mRNA expression, showing that the expression alteration was a characteristic of the mutation residing in the allele. These observations imply that the cancer predisposition in the majority of HNPCC families with expression-based evidence of MMR gene involvement (Table 6) may eventually be explained by mutations of the

frameshift or nonsense type that, for some reason, escaped detection by the present methods used.

For comparison, all nine FAP families with eight different truncating APC mutations showed balanced mRNA expression of APC; the same was true for MMR gene mutation negative HNPCC families without MSI and healthy controls. These data suggest that unbalanced expression is associated with frameshift mutations of MMR genes but not of APC, suggesting probable gene-specific differences for degradation by nonsense-mediated mRNA decay. The constitutionally unbalanced mRNA expression of APC that was not associated with any detectable premature nonsense codons is likely to have a different genetic basis. While the genomic cause is unknown at present, structural changes that lie deep in introns or far upstream or downstream of APC remain a possibility.

# DISCUSSION

## Frequency of HNPCC in Different Populations

The incidence of hereditary nonpolyposis colorectal cancer (HNPCC) is not exactly known and the published estimations from different populations range from 0.3-13% (Stephenson et al. 1991, Evans et al. 1997). The studies investigating the frequency of HNPCC vary in methodological design, inclusion and exclusion criteria, and the mutation detection methods applied, which may partly affect the observed estimates. The use of Amsterdam criteria I, and later, Amsterdam criteria II has unified these studies and estimates. The proportion (2.6%) of HNPCC families fulfilling the ACI and ACII among Uruguayan CRC patients were in accordance with studies representing populations from different parts of Europe, in which the frequency of HNPCC among all CRC cases varied from 0.3-5.5% (Westlake et al. 1991, Mecklin 1987, respectively and III: Table 4). Notably, even within the same population estimates differ, for example depending on i) the study design, from 0.5-2.6% in Italy (de Leon et al. 1999, Cornaggia et al. 2000), or ii) the geographic origin of patients, from 0.7-5.5% in Finland (Mecklin 1987, Mecklin et al. 1995).

Although the estimates of the frequency of HNPCC among all CRC cases are similar in the Uruguayan population and previously published studies of non-southern American populations, the frequency of molecularly confirmed (mutation positive) HNPCC shows greater variation. In general, among HNPCC families meeting ACI, a predisposing mutation is detected in approximately 60% (Lindor et al. 2005), whereas among families not meeting these criteria (ACII and putative HNPCC families), less than 30% show mutations by routine diagnostic methods (Nyström-Lahti et al. 1996, Wijnen et al. 1997). Despite comprehensive methodologies to detect point mutations, large genomic rearrangements and unbalanced mRNA expression, a predisposing mutation in the major MMR genes (MLH1, MSH2 and MSH6) was detected in only 25% (3/12) of the cases from Uruguay. This figure is at the lower border of results from other studies using similar inclusion criteria that detected mutations in 25-65% of ACI and ACII families (Weber et al. 1997, Scott et al. 2001). Differences in mutation detection rates may be due to 1) interpopulation/intrapopulation differences in the incidence of MMR gene mutations, 2) influence of modifier genes, 3) role of novel susceptibility genes, 4) environmental carcinogens, or 5) study design (de la Chapelle 2005, III). It is worthwhile to note that the Uruguayan CRC panel consisted of a hospital-based series and therefore the observed frequencies may or may not reflect the whole Uruguayan population.

For CRC families preselected based on MSI and immunohistochemical analyses of MMR protein expression, mutation detection rates up to 70% have been reported (Lynch and de la Chapelle 1999). Unfortunately, tumor samples were unavailable from Uruguayan CRC patients preventing the use of this preselection strategy and also limiting our ability to clarify the role of MMR genes in cancer predisposition in these patients. In addition, had tumor samples been available the use of less stringent inclusion criteria (Bethesda guidelines) would have been possible, which would have allowed us to include some of the present PIR families in the molecular analysis, possibly resulting in increased number of mutations. The proportion of PIR cases (5.6%) is similar to those of populations from the UK and Italy (Evans et al. 1997, de Leon et al. 1999). This may reflect a genetic profile of the Uruguayan population which originates from southern Europe (Spain, Italy, French, and Portugal) (Sans 1992).

**Detection of Distinct Genetic Subgroups among Hereditary Colorectal Cancers**

The detection of mutation carriers at high risk of cancer is important for the implementation of surveillance programs to decrease the morbidity and mortality in hereditary colorectal cancer syndromes. To date, more than 400 mutations predisposing to HNPCC and more than 800 to FAP have been described. Around 30% of HNPCC and FAP families and around 90% of AFAP families remain mutation negative by conventional methods, emphasizing the need for more sophisticated mutation detection methods. We used a comprehensive approach combining methods to detect possible expression changes of mRNA and/or protein with a recently designed method, MLPA, to detect large genomic rearrangements in genomic DNA.

By our strategy, HNPCC families were divided into two genetically different categories. The first group (42%, 11/26) showed evidence of an MMR-gene linked basis of cancer predisposition, whereas in the second group of (58%, 15/26) MMR-gene involvement was unlikely. A similar strategy applied for mutation negative FAP and AFAP divided this originally single group into four genetically different groups: 1) families with large genomic deletions of APC (14%, 4/29), 2) families with unbalanced mRNA expression of APC (24%, 7/29), 3) families without defined molecular etiology of predisposition (52%, 15/29), and 4) families with predisposing mutations in genes other than APC (AXIN2 in 4%, 1/29; MYH in 7%, 2/29).

In many suspected HNPCC families, immunohistochemical analysis of tumor tissue reveals an abnormal expression of one of the major MMR genes (MLH1, MSH2, and MSH6) which in most of cases is associated with a high level of MSI (Wahlberg et al. 2002). Therefore, the combination of MSI and IHC analyses have turned out to be valuable for preselection of HNPCC families with MMR defects (Thibodeau et al. 1996, Salahshor et al. 2001, Wahlberg et al. 2002). Although in our study all tumors with MSI displayed a lack of expression of one

of the major MMR genes, occasional tumors with MSI-H show normal expression of MLH1, MSH2, and MSH6. In a proportion of such cases, selective loss of the PMS2 protein may be observed (Gill et al. 2005). The phenotype of early age of onset, right sided tumors and no outstanding family history may guide a researcher to look for selective PMS2 loss in the case of MSI and normal expression of major MMR genes (Truninger et al. 2005, Gill et al. 2005).

Unfortunately, MSI and IHC from the tumor tissue are unable to distinguish hereditary alterations from acquired changes. In contrast, our mRNA expression based SNuPE assay performed on leukocytes can provide evidence of a constitutional defect in MMR genes. In SNuPE, homozygosity for coding polymorphisms is an important limitation. To circumvent this, several coding polymorphisms of MLH1, MSH2 and MSH6 should first be tested and tests developed for those with adequate numbers of informative cases.

Besides linking putative HNPCC families to an MMR defect, the combination of MSI, IHC, and SNuPE can also be used to gather cumulative evidence against MMR gene involvement. Recently, a distinct clinical phenotype has been reported for families that fulfill ACI, but have no evidence of an MMR defect. These share a lower incidence of colorectal cancer within a family (Lindor et al. 2005, I), higher age of onset (Müeller-Koch et al. 2005, I), and less frequent extracolonic tumors (Müeller-Koch et al. 2005), especially EC in females (I). This suggests the existence of a clinically and molecularly separate category of inherited colorectal cancers recently named familial colorectal cancer type X (Lindor et al. 2005). Estimates for the frequency of familial colorectal cancer type X range from 39-76% among MMR gene mutation negative HNPCC families (Lindor et al. 2005, Woods et al. 2005, Müeller-Koch et al. 2005).

Different clinical features have been reported for apparent FAP families with no defined molecular etiology by routine mutation detection methods. In Heinimann et al. (2001) and our previous study (Moisio et al. 2002) mutation negative families were associated with a milder phenotype, whereas Bisgaard et al. (2004) observed a more severe phenotype for these families. The differences may be explained by different types or locations of undetected APC mutations, the possible involvement of susceptibility genes other than APC or the influence of modifier genes. In our investigation, three of the 15 FAP families with no defined molecular etiology (52%, 15/29) seemed to display AFAP/atypical FAP phenotype (FAP78, FAP100, FAP111), whereas the remaining families in this group fulfilled the inclusion criteria of classical polyposis. The group is probably genetically heterogeneous. Shared haplotypes in the APC region are observed for FAP57 and FAP74 (Table 7), and 55% (11/20) of the tumors displayed strict or putative LOH in the APC region. Therefore, a germline mutation of APC

may exist in at least some cases whereas (an) other susceptibility gene(s) may be involved in the remaining families.

Recently, biallelic mutations of the human Mut Y homolog, MYH, encoding a DNA glycosylase (Al-Tassan et al. 2002), was associated with a phenotypic subtype of FAP, MAP. The clinical features of this syndrome include variable polyp count (below and above 100) and autosomal recessive inheritance (Sieber et al. 2003). The frequency of germline mutations of MYH was studied in collaboration with us (Alhopuro et al. 2005), and in 7% (2/29) of the apparent APC mutation negative FAP families showed biallelic MYH mutations. MYH mutation screening should be considered if 1) classical or attenuated FAP is present, 2) pathogenic APC mutations are absent, and 3) family history suggests an autosomal recessive mode of inheritance.

During our investigation of FAP families, a mutation in the Wnt-signaling regulator AXIN2 was identified in a family (FAP113) with severe dental anomalies (Lammi et al. 2004). Subsequently, the remaining mutation negative FAP families were screened for AXIN2 mutations, with no observed apparently pathogenic alterations. The role of other Wnt-signaling members should therefore be considered as possible candidates for cancer predisposition. AXIN2 mutation screening should be considered if 1) classical or attenuated FAP is present, 2) pathogenic APC/MYH mutations are absent, and 3) severe dental agenesis is observed.

**Significance of Genomic Deletions in HNPCC and FAP Predisposition**

Until recently, the true frequency of mid-size genomic rearrangements in MMR genes and APC was unknown. Several methods are available for detecting deletions and insertions of a few base pairs or very large, cytogenetically visible deletions [e.g. fluorescence in situ hybridization (FISH) and its applications (Cetin et al. 2005)]. However, most methods used for mutation detection do not detect deletions of a few kilobases, such as deletions of single exons or deletions of the whole gene. Recently two methods, multiplex amplification subsequent with probe hybridization (MAPH) and MLPA, were designed to allow easy, reliable and low-cost detection of deletions in 40 target sequences at once (Sellner and Taylor 2004).

The failure to detect germline mutations in mutation negative HNPCC families may in part be caused by methodological difficulties in detecting mutations, such as the presence of mid-sized genomic rearrangements not detected by routine mutation detection techniques (DGGE SSCP, PTT, and DNA sequencing). Previous studies of HNPCC families have detected genomic rearrangements of MLH1 and MSH2 in 5-27% of HNPCC probands (Wang et al.

2003, Wagner et al. 2003). The frequencies vary between populations and individual MMR genes (Table 9). Genomic rearrangements seem more frequent in MSH2 than in MLH1 (Wijnen et al. 1998, Charbonnier et al. 2002, Nakagawa et al. 2003, Wang et al. 2003, Wagner et al. 2003, Bunyan et al. 2004, van der Klift et al. 2005, Table 9), whereas very few genomic rearrangements of MSH6 and PMS2 are reported to date (van der Klift et al. 2005). The frequencies of genomic rearrangements are in accordance with our unpublished results of a partial deletion of MSH2 in one HNPCC family (1/26, 4%) and a partial deletion of MLH1 in another HNPCC family (1/26, 4%) (I, Table 6). Most reported genomic rearrangements lie within repetitive sequences, mostly within Alu repeats (van der Klift et al. 2005).

**Table 9**: Frequencies of mid-size genomic rearrangements in MMR genes in HNPCC.

| | Renkonen et al. 2003 (I) and unpublished data | Bunyan et al. 2004 | van der klift et al. 2005 |
|---|---|---|---|
| **Detection method** | MLPA | MLPA | Southern blot |
| **Population** | Finland | UK | Germany, USA, Italy, Australia |
| **Frequency of MMR deletions among mutation negative HNPCC probands** | | | |
| total | 2/26 (7.7%) | 7/122 (5.7%) | 68/439 (15.5%) |
| MLH1 | 1/26 (3.8%) | none | 14/439 (3.2%) |
| MSH2 | 1/26 (3.8%) | 7/122 (5.7%) | 48/439 (10.9%) |
| MSH6 | none | not studied | 2/439 (0.5%) |
| PMS2 | not studied | not studied | 4/439 (0.9%) |

A variety of techniques for the detection of deletions (real-time quantative multiplex PCR, semi-quantative PCR, RT-PCR, MLPA) have been applied to FAP and AFAP cohorts without truncating APC mutations (Sieber et al. 2002, Venesio et al. 2003, Bunyan et al. 2004, Michils et al. 2005, IV). According to recent studies, the overall APC deletion frequency in FAP and AFAP together varies from 3-14% (Table 10). Furthermore, the frequency of APC deletions in FAP exhibiting more than 100 adenomas is even higher, varying from 12-33% (Sieber et al. 2002, De Rosa et al. 1999). The phenotype of APC deletion carriers seems similar to FAP families with truncating mutations (Sieber et al. 2002, Michils et al. 2005, IV) in that both groups showed profuse polyposis, early age of onset and frequent extracolonic manifestations (Table 8, IV). Our results are in line with the observation that large deletions

are very rare, maybe even absent in AFAP (Table 10). Interfamilial phenotypic variability, a property of FAP (Crabtree et al. 2002), was also observed in APC deletion carriers. For example, the Finnish family FAP5 displayed many extracolonic manifestations (desmoids, duodenal adenomas, fundic gland polyps, osteomas, and epidermal cysts) whereas in FAP91 only fundic gland polyps were detected (IV: Table 1). Large deletions of the APC region are frequently associated with mental retardation, dysmorphic features, or extracolonic manifestations, such as CHRPE (Raedle et al. 2001, De Rosa et al. 1999).

**Table 10**: Occurence of mid-size genomic rearrangements in FAP and AFAP families.

|  | Renkonen et al. 2005 (IV) | Michils et al. 2005 | Sieber et al. 2002 | Bunyan et al. 2004 |
|---|---|---|---|---|
| **Population** | Finland | Belgia | UK 82.7%, D 5.4%, S 5.4%, P | UK |
| **Proportion of mutation negative FAP/AFAP** | | | | |
| total | 29/95 (31%) | 55/85 (65%) | Not reported | 24/74 (32%) |
| FAP | 23/29 (79%) | 27/55 (49%) | 60 | not reported |
| AFAP | 6/29 (21%) | 28/55 (51%) | 143 | not reported |
| **Frequency of APC deletions in mutation negative FAP and AFAP** | | | | |
| total | 4/29 (14%) | 4/55 (7%) | 7/203 (3%) | 6/24 (6%) |
| FAP | 4/23 (17%) | 4/27 (15%) | 7/60 (12%) | not reported |
| AFAP | 0/6 (0%) | 0/28 (0%) | 0/60 (0%) | not reported |
| **Frequency of deletions of whole APC in FAP** | 3/23 (13%) | 3/27 (11%) | 6/60 (10%) | not reported |
| **Frequency of deletion of single exon of APC in FAP** | 1/23 (4%) | 1/27 (3.7%) | 1/60 (2%) | not reported |

UK= the United Kingdom, D= Denmark, S=Switzerland, P=Portugal, I=Italy

**Unbalanced mRNA Expression in HNPCC and FAP Predisposition**

A challenging problem in the diagnosis of human mutations is that alterations in one allele may occasionally be masked by the normal sequence of the wild type allele, making allele-specific approaches obligatory. Conversion technology (Yan et al. 2000), as well as SNuPE, aims to circumvent this problem by analyzing the paternal and maternal alleles separately.

The genomic copies are physically separated by converting the human chromosome complement to a haploid state through fusion to a rodent cell line. For the measurement of relative mRNA levels, conversion technology can be combined with digital single nucleotide polymorphism analysis. In SNuPE, single nucleotide polymorphisms are used to distinguish paternal and maternal alleles of gDNA or mRNA fragments and their allelic expression can be monitored by a simple semiquantitative fluorescent primer extension. Subsequent cloning of full-length RT-PCR products allows the physical separation for cDNA sequencing.

By SNuPE, 8% (2/26) of Finnish HNPCC families displayed unbalanced mRNA expression of MLH1 and, by allelic separation and subsequent cDNA cloning and sequencing, a predisposing mutation of MLH1 (R100X) was detected in one of them (I). Among two Swedish families with unbalanced mRNA expression of MLH1, one showed an out-of-frame deletion of MLH1 exon 11 by quantative multiplex PCR of short fluorescent fragments (Di Fiore et al. 2004). This finding was consistent with the unbalanced MLH1 mRNA expression of MLH1 in this family. Unbalanced mRNA expression was absent in Uruguayan HNPCC patients, reflecting a possible population-based differences in their genetic background (III).

In addition to HNPCC, unbalanced mRNA expression may occur in FAP (Yan et al. 2002), and even modest decreases in transcripts of approximately 50% of the normal (APC) allele can lead to a severe disease. To our knowledge, our study is the first to address the occurrence of unbalanced APC mRNA expression in a larger panel of FAP patients. We observed that 24% (7/29) of APC mutation negative families with classical FAP showed a decrease of APC mRNA expression of one allele from 32% (partial silencing) to 100% (complete silencing of one allele). Furthermore, our studies revealed that constitutionally decreased expression of one allele was accompanied by the selective loss of the other (wild-type) allele in tumor tissue. This supported the idea that haploinsufficiency of the APC protein contributed to the development of cancer in these families.

In our investigations, unbalanced mRNA expression was associated with all truncating mutations studied in the MMR genes (6 MLH1 and one MSH6) (I, II), but none of eight different APC truncating mutations from FAP patients (IV). In particular, constitutionally unbalanced mRNA expression was a characteristic of the subgroup of seven FAP families with no truncating mutations in APC (IV). The possible mechanisms of unbalanced mRNA expression were discussed above (see results).

**Aberrant Splicing – a Genetic Marker for Undetected Mutation**

In addition to unbalanced mRNA expression, aberrant splicing of multiple exons unrelated to the mutation site, as observed for the MLH1 nonsense mutation R100X (II), may serve as a

genetic marker for mutation-containing mRNA transcripts. Subsequent sequencing of cDNAs displaying such a splicing phenomenon may lead to the detection of a predisposing mutation. To our knowledge, aberrant splicing of this kind in MMR genes has not been reported before and seems to be a very rare phenomenon as we found only one other disease, maple syrup urine disease, with a splicing defect resembling that which we describe (Fisher et al. 1993). While the reasons for aberrant splicing in both cases are unknown, the phenomenon may depend on the mutation site and type or altered secondary structure of mutated transcripts. According to our investigations, it is not merely a consequence of the type of the mutation, since among six different nonsense or frameshift mutations leading to premature termination, only R100X was associated with aberrant splicing.

**Genetic Basis of Families that Remained Mutation Negative Even by Advanced Methods**

In our experience, the expression based strategy we used for mutation negative HNPCC (MSI, IHC, and SNuPE), combined with the detection of large genomic rearrangements by MLPA, are valuable additions to routine mutation diagnostics in hereditary cancer syndromes, in order to gather more evidence either for or against the role of MMR genes in cancer predisposition. In the case of FAP, SNuPE and MLPA analysis should be combined with the screening of MYH and AXIN2 for inherited defects. After these steps, the remaining "truly" MMR and APC gene mutation negative cohorts of HNPCC and FAP/AFAP families offer highly valuable material for future searches for new colon cancer susceptibility genes.

The genetically undefined group of putative HNPCC families may include families from proposed novel colorectal cancer syndromes, such as hereditary colorectal cancer type X (HCCX), which are thought to arise from genetic defects not involving MMR. Whereas the HCCX phenotype resembles that of HNPCC, closer examination suggests that it forms a molecularly and clinically separate entity (Lindor et al. 2005, Müeller-Koch et al. 2005).

Studies of familial clusters of colorectal cancers with early age of onset of adenomas have suggested a new putative locus for CRC. A high density genome wide screen of microsatellite markers of an Ashkenazi HMPS family showed linkage to a region in 15q13-14 (Jaeger et al. 2003). Haplotype analysis subsequently merged this locus together with a locus 15q14-22 of inherited susceptibility to colorectal adenomas and carcinomas (CRAC1) in another Ashkenazi family (Tomlinson et al. 1999). This HMPS/CRAC1 locus is further supported by the observation of LOH of the wild-type allele in tumor samples (Jaeger et al. 2003) and by the narrow area of LOH previously reported by the same group (Tomlinson et al. 1999). The region between D15S1031 and D15S118 contains some good candidate genes, a few with known function, as well as various predicted genes yet to be tested by a candidate gene strategy.

Another study linked familial colorectal neoplasia (Wiesner et al. 2003) to a region of 9q22.2-31.2 and was followed by the discovery of an even more defined locus 9q22.32-31.1 in a Swedish family with a likely inherited colorectal cancer predisposition (Skoglund et al. 2006). The area of 7.9 cM between markers D9S280 and D9S277 contains several good candidate genes, such as TGFßRI, PTCH, and XPA, which may have a role in colorectal cancer predisposition.

Mutation negative FAP/AFAP families may involve many genetic and clinical subgroups of inherited colorectal polyposis even after the exclusion of hidden APC, MYH and AXIN2 mutations. Several potential candidate genes that are frequently mutated in sporadic gastrointestinal tumors are known (Becker et al. 1994, Samowitz et al. 1999). These include ß-catenin (CTNNB1) and CDH1, both suggested to participate in the development of colorectal cancer. However, the roles of ß-catenin and CDH1 in hereditary cancer have probably been overestimated, since, to date, no constitutional alterations have been observed (Venesio et al. 2003). Unfortunately, a genome wide linkage analysis strategy similar to that used for HNPCC and HNPCC-like phenotypes, HCCX or HMPS is not possible in our APC mutation negative polyposis families, since the families are often small and are likely to have a heterogenous genetic background.

Despite methodological improvements and intensive research, a significant fraction of HNPCC and FAP families are likely to remain molecularly unexplained. It is important to understand that a negative test result does not rule out inherited predisposition to polyposis and colorectal cancer. These patients and their family members should be offered cancer surveillance, as was done in the pretesting era (Church et al. 2001).

## CONCLUSIONS AND FUTURE PROSPECTS

The identification of inherited defects predisposing to the hereditary cancer syndromes, HNPCC and FAP, is clinically important since it enables targeted inclusion of at-risk family members in preventive screening programs.

A majority of HNPCC-linked MLH1, MSH2, and MSH6 mutations are associated with marked under-representation of the mutant mRNA transcript, which can be utilized as a diagnostic tool.

The unbalanced mRNA expression of APC may provide a genetic marker for FAP predisposition in FAP families were APC mutations has not been found.

Aberrant splicing may pinpoint mutation carrying alleles of MLH1.

Among clinically verified HNPCC families with no detectable germline MMR gene mutations by conventional methods, 42% were associated with a "hidden" mutation in one of the three major MMR genes, leaving the remaining 58% of families MMR gene mutation negative.

Recognition of these "hidden" mutations in known susceptibility genes is essential to provide truly mutation negative cohorts for future searches for new susceptibility genes.

Advanced mutation detection techniques, such as MLPA and methods based on discrimination of paternal and maternal alleles, allow the detection of "hidden" mutations, such as large genomic rearrangements and mutations that are masked by the wild type allele.

We detected large genomic deletions in 14% of apparently APC mutation negative FAP families and 8% of apparently MMR gene mutation negative HNPCC families.

The true incidence of "hidden" mutations in HNPCC and FAP remains unknown and should be clarified by studying larger series of apparently mutation negative HNPCC and FAP families.

Truly mutation negative cohorts are highly valuable for future searches for new genes for colorectal cancer predisposition. The methods may include a combination of positional and functional cloning and a candidate gene strategy.

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