FREQUENCY ESTIMATION OF ENDOMETRIAL CANCER ASSOCIATED WITH MICROSATELLITE INSTABILITY AND MISMATCH REPAIR GENE DEFECTS

By

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A Thesis submitted in Conformity with the Requirements for the Degree of Masters of Science Graduate Department of Laboratory Medicine and Pathobiology University of Toronto October 1999
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By Anna L. Millar, Masters of Science Thesis
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ABSTRACT

Endometrial cancer (EC) is the most common gynecological malignancy in North America. While most EC is sporadic, it represents the most common extracolonic cancer in the hereditary nonpolyposis colorectal cancer syndrome (HNPCC). Mutations in mismatch repair genes (MMR), of which hMLH1 and hMSH2 are the most commonly mutated, are known to cause HNPCC, resulting in microsatellite instability. However, the proportion of EC associated with microsatellite instability and mismatch repair gene defects remains unknown. We found 18% of women with double primary cancers of the colorectum and endometrium, to harbour germline hMLH1 and hMSH2 mutations. As well, 21% of apparently sporadic endometrial cancers, diagnosed in women less than 50 years of age, showed microsatellite instability and of those, 30% lack expression of hMSH2. Therefore, women diagnosed with multiple primary cancers of the colorectum and endometrium or diagnosed with EC before 55 years of age, are prime candidates for diagnosis of HNPCC.
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ABBREVIATIONS

ADP  adenosine diphosphate
APC  adenomatous polyposis coli gene
ATP  adenosine triphosphate
BAX  apoptosis regulator BCL-X
CHK1  checkpoint kinase 1
CI  confidence interval
CRC  colorectal cancer
DAM  DNA adenine methylation
DNA  deoxyribonucleic acid
EC  endometrial cancer
EDTA  ethylene diamine tetra-acetic acid
FAP  familial adenomatous polyposis
GTBP  G/T binding protein
H&E  haematoxylin and eosin
hMLH1  human mutL homolog I
hMSH2  human mutS homolog II
hMSH3  human mutS homolog III
hMSH6  human mutS homolog VI
HNPCC  hereditary nonpolyposis colorectal cancer
hPMS1  human DNA mismatch repair protein I homolog (Schizosaccharomyces pombe)
hPMS2  human DNA mismatch repair protein II homolog (Schizosaccharomyces pombe)
IGFIIIR  insulin-like growth factor II receptor
K-RAS  Kirsten murine sarcoma virus
LOH  loss of heterozygosity
MMR  mismatch repair
MSI  microsatellite instability
NF1  neurofibromatosis type 1
OCR  Ontario Cancer Registry
PAGE  polyacrylamide gel electrophoresis
PCR  polymerase chain reaction
PTEN  phosphatase and tensin homolog deleted in chromosome 10
Rb  retinoblastoma
RER  replication error
RNA  ribonucleic acid
SSCP  single strand conformation polymorphism
TBS  tris borate saline
TGF-βRII  transforming growth factor-beta type II receptor
VHL  von-Hippel-Lindau disease
CHAPTER 1: INTRODUCTION

Part 1: ENDO METRIAL TUMORIGENESIS

1.1.1 Endometrial Cancer

1.1.1.1 General Features

Endometrial cancer (EC) is the most common invasive gynecological malignancy in North America. With an estimated 34,000 new cases reported each year, EC represents 48% of all female genital cancers and approximately 7% of all cancers in the female population (Silverberg, 1987). Despite this high frequency, EC is associated with a low rate of mortality. This is partly due to the fact that EC is usually diagnosed in older women between the ages of 55 and 65, with 75-80% of all EC patients being post-menopausal (Creasman et al, 1985; DiSaia et al, 1985). It is therefore often accompanied by abnormal (post-menopausal) bleeding, making it readily detectable, thus allowing for treatment at an early stage. Prognosis depends largely on clinical stage, histologic grade and tumour type at diagnosis. Approximately 80% of women diagnosed with EC in North America have clinical stage I well-differentiated to moderately well-differentiated lesions (Silverberg, 1987). Treatments of stage I EC with surgical removal alone or in conjunction with radiation therapy results in a 5-year survival rate of 80-90% (Malkasian et al, 1977; Salazar et al, 1977; Underwood et al, 1977). This survival rate diminishes rapidly for tumours diagnosed at more advanced stages and decreased differentiation. A survival rate of 63% is seen in stage II EC (Grigsby et al, 1985) and to 25% in stage III EC (Kottmeier et al, 1977), as well as poorer differentiation and/or more aggressive histological subtypes (Gore et al, 1966; Kurman et al, 1976).
1.1.2.2 Pathology

Grossly, EC may appear as a localized polypoid tumour or as a diffuse tumour involving the entire surface of the endometrium. Diffuse tumours are often accompanied by extensive hemorrhage and necrosis. Spread of EC is usually a result of direct myometrial invasion, with eventual metastasis through the peritoneum. Other means of metastasis include tubal transport to adjacent organs, as well as vascular and lymphatic flow to other organs such as lung, bone and liver. Staging of endometrial adenocarcinoma, using the FIGO criteria (Table 1), describes the extent to which the tumour has spread.

FIGO stage I tumours are sub-classified according to grade, based on histologic architecture and nuclear atypia. Histologic grading of EC is determined using the following guidelines: grade 1, well-differentiated adenocarcinoma with clearly recognizable gland patterns (less than 5% solid); grade 2, differentiated adenocarcinoma with some solid areas (5-50% solid); grade 3, predominantly solid or undifferentiated carcinoma (greater than 50% solid). Prognosis is closely linked to the stage, degree of differentiation and cell type. Most endometrial tumours are adenocarcinomas, demonstrating well-differentiated gland patterning, lined by malignant stratified columnar epithelium (Silverberg, 1987). Papillary serous carcinoma and clear cell carcinoma are rare histologic EC types and have been shown to behave aggressively (Gore et al, 1966; Kurman et al, 1976). They are therefore classified as poorly differentiated, regardless of their degree of differentiation.
Table 1. FIGO Classification of Endometrial Cancer

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
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<tbody>
<tr>
<td>Stage I</td>
<td>The carcinoma is confined to the corpus</td>
</tr>
<tr>
<td>1a</td>
<td>The length of the uterine cavity is 8 cm or less</td>
</tr>
<tr>
<td>2a</td>
<td>The length of the uterine cavity is 8 cm or more</td>
</tr>
<tr>
<td></td>
<td>Stage I cases should be sub-grouped according to histological grade of</td>
</tr>
<tr>
<td></td>
<td>adenocarcinoma:</td>
</tr>
<tr>
<td></td>
<td>G1 – highly differentiated</td>
</tr>
<tr>
<td></td>
<td>G2 – moderately differentiated</td>
</tr>
<tr>
<td></td>
<td>G3 – poorly differentiated</td>
</tr>
<tr>
<td>Stage II</td>
<td>The carcinoma involves the corpus and the cervix</td>
</tr>
<tr>
<td>Stage III</td>
<td>The carcinoma extends outside the corpus but not the true pelvis</td>
</tr>
<tr>
<td>Stage II</td>
<td>The carcinoma involves the bladder or rectum or extends outside the</td>
</tr>
<tr>
<td></td>
<td>corpus but not the true pelvis</td>
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</tbody>
</table>
Adenocarcinoma can be divided into two types, those which arise in association with estrogen stimulation and a history of endometrial hyperplasia, known as *endometrioid*, and those which arise irrespective of these factors. Endometrioid tumours are perhaps the best understood and are shown to occur at an earlier age, display distinct glandular patterning (cribriform) and have better prognosis. Cell proliferation in these estrogen dependant tumours is stimulated by estrogen and inhibited by progestin (Hsueh et al, 1975). Factors which increase the risk for EC development include late menopause (Pike et al, 1987), nulliparity (Kvale et al, 1988), estrogen replacement therapy (IARC, 1987) and obesity (Henderson et al, 1983; Swanson et al, 1993). Thus, factors increasing the risk of EC suggest a link with increased exposure to unopposed estrogens (Key and Pike, 1988).

1.1.2 Molecular Biology of Cancer

Cancer cells escape normal growth control mechanisms as a consequence of constitutive activation of cellular proto-oncogenes, which are normally tightly controlled growth-signaling genes, loss of tumour suppressor function or loss of genetic repair gene function. Tumour suppressor gene inactivation can be the result of chromosome instability (Hartwell et al, 1992; Cahill et al, 1998) or hypermutability (Ionov et al, 1993; Thibodeau et al, 1993). Inactivation of DNA mismatch repair genes and their role in tumour hypermutability will be discussed in more detail later in Chapter 1, Part 2, as it represents a distinct genetic pathway, which may evolve in conjunction with the tumour suppressor inactivating mutations.

An additional pathway that has been shown to contribute to cancer progression is the epigenetic or methylation pathway, which generates patterns of DNA methylation that
govern the overall genomic gene expression (Chuang et al, 1996). DNA methylation organizes the genome of higher order eukaryotes into transcriptionally active and inactive zones. The principal site of methylation are the “CpG islands” or small CpG dinucleotides, which are associated with transcription start sites of about 40,000 human genes (Antequera and Bird, 1993). Cancer cells show altered patterns of DNA methylation (Laird and Jaenisch, 1996) resulting usually in an overall decrease in methylation (Gama-Sosa et al, 1983). Normally methylated CpG sites can be hypermethylated, causing an epigenetic equivalent to the genetic mutation of important tumour suppressors (Laird and Jaenisch, 1996) or repair pathway genes (Simpkins et al, 1999) in sporadic cancers. For example, hypermethylation of the mismatch repair protein hMLH1 results in a sporadic mismatch repair phenotype similar to (familial) HNPCC.

Proto-oncogenes are activated by the mutation of a single allele, which acts dominantly, causing an uncontrolled increase in growth signaling, leading to transformation and tumour progression. While proto-oncogenes accelerate cellular proliferation, tumour suppressor genes have an important role in maintaining control of cellular proliferation rate by inhibiting cell cycle machinery. Tumour suppressor mutations are recessive at the cellular level because they require the inactivation of both gene copies before the suppressor function is lost and the tumourigenic phenotype manifests (Knudson, 1985). Tumour suppressor inactivation results from genetic instability or hypermutability (Nowell et al, 1976). This first manifests in chromosomal alterations such as aneuploidy and loss of chromosome material. This mechanism results in the loss of heterozygosity (LOH), a common phenotype which is involved in a large
proportion of tumour suppressor inactivation, hence it is termed the “suppressor pathway” (Thibodeau et al, 1993; Cahill et al, 1998).

Many of the gene products of proto-oncogenes and tumour suppressor genes are involved in complex signal transduction pathways which control cell differentiation, sense DNA damage, initiate repair mechanisms, control cell cycle entry and exit, and programmed cell death (apoptosis). Most tumours have mutations in multiple oncogenes and tumour suppressors which vary depending on the tissue site and primary tumour origin, suggesting that cells use multiple parallel pathways to control cell growth, differentiation, DNA damage control and death. Variations in mutation frequencies of key oncogenes and tumour suppressors amongst different tumour sites indicate tissue specificity of certain signaling pathways.

Cancer development has perhaps been best described in the multi-step model or suppressor model of colorectal cancer, which hypothesizes that tumourigenesis is a slow and progressive process, resulting from an accumulation of several function-altering mutations in tumour suppressor genes and proto-oncogenes (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996). In colorectal cancer, mutations in tumour suppressor genes such as APC (adenomatous polyposis coli) and p53, and in the K-RAS proto-oncogene, have been shown to be important contributors for tumour progression in colorectal cancer (Figure 1) (Vogelstein et al, 1988).

Studies of the rare familial colorectal cancer syndrome, familial adenomatous polyposis (FAP) led to the discovery of the APC (adenomatous polyposis coli) gene. Located on chromosome 5q21, APC encodes a 2843 amino acid protein, which binds to β-catenin to inhibit transcriptional activity via the WNT signaling pathway (Rubinfeld et
al, 1993; Su et al, 1993). Somatic APC mutations are found in the majority of sporadic colorectal tumours (Miyoshi et al, 1992; Powell et al, 1992) and have been shown to be an early event in tumourigenesis (Jen et al, 1994). FAP patients carry a germline APC mutation. Loss of the wild-type allele or LOH leads to the development of thousands of polyps. Although these polyps are usually benign, the high number virtually guarantees the development of cancer in these patients. For this reason, APC is believed to act as the “gatekeeper” of colonic epithelial proliferation because mutations in this gene result in uncontrolled proliferation and the initiation of tumourigenesis with the formation of polyposis, the precursor to colorectal carcinoma (Kinzler and Vogelstein, 1996). Somatic APC mutations are found in the majority of sporadic colorectal tumours (Miyoshi et al, 1992; Powell et al, 1992) and has been shown to be an early event in tumourigenesis (Jen et al, 1994).

Additional mutations in other genes such as K-RAS and p53 are common in developing colonic tumours. K-RAS mutations are very common (73%) in early tumour lesions, including pre-cancerous aberrant crypt foci (Pretlow et al, 1993) and are implicated in the progression of early to intermediate adenomas (Vogelstein et al, 1988). Somatic p53 mutations are found in greater than 80% of colorectal cancers (Baker et al, 1990) and are predominantly associated with late stage tumour progression from adenoma to carcinoma (Kinzler and Vogelstein, 1996).
Figure 1. Multi-step tumour suppressor pathway of tumourigenesis in the colorectum
While \textit{K-RAS} and \textit{p53} are shown to contribute to the further progression of the colorectal neoplasm (Vogelstein et al, 1988; Shibata et al, 1993), mutations in \textit{K-RAS} and \textit{p53} without a preceding \textit{APC} mutation are hypothesized not to develop into colorectal cancer (Garber et al, 1991; Jen et al, 1994). These studies demonstrate that the order in which these mutations accumulate, as well as the spectrum of mutations, are important determinates in the outcome of tumourigenesis.

Colorectal tumourigenesis serves as a prototype model for cancer development in general. It is likely that different gatekeeper genes exist and contribute unique functions to distinct tissue sites. For example, the \textit{NF1} gene has been suggested to play a gatekeeper role in Schwann cells and the \textit{Rb} and \textit{VHL} genes have been linked to retinal and renal cells, respectively (reviewed in Knudson, 1993).

1.1.3 Biological Basis of Sporadic Endometrial Cancer

Relatively little is known about the genetic events that underlie endometrial tumourigenesis. The theory of progression from endometrial hyperplasia to adenocarcinoma was first proposed in 1949 (Hertig and Sommers, 1949). Recent studies confirm these observations and show endometrial tumourigenesis to be a multi-step process involving the accumulation of genetic alterations in important growth regulating and cell cycle control genes, similar to that described and characterized in colorectal cancer. EC progresses in a stepwise fashion involving a series of well-defined histological changes, e.g. normal endometrial tissue, hyperplasia, followed by atypical hyperplasia, then endometrial adenocarcinoma and ultimately metastatic disease.

Similar to the multi-step model elucidated in colorectal cancer, EC appears to follow a stepwise accumulation pattern of somatic mutations in tumour suppressor genes
and proto-oncogenes (Figure 2). The specific genes involved in endometrial
tumourigenesis are not necessarily the same as those described for colorectal cancer. For
example, APC may only act as a gatekeeper in colorectal tumourigenesis. Although it is
ubiquitously expressed, its role may be redundant in other tissues.

A recently discovered PTEN (phosphatase and tensin homolog deleted in
chromosome ten) tumour suppressor gene, located within chromosomal region 10q23,
may be a candidate gatekeeper or tumour initiator in EC. Several lines of evidence
support this hypothesis: 1) inactivating somatic PTEN mutations are found in up to 50% of
endometrial neoplasms (Risinger et al, 1997; Tashiro et al, 1997); 2) somatic PTEN
mutations are found at equal frequencies in all stages of EC as well as complex
hyperplasias, which suggests it to be an early event for EC specifically, (Tashiro et al, 1997; Levine et al, 1998) despite the fact that PTEN is predominantly mutated in
advanced glial (Liu et al, 1997; Rasheed et al, 1997; Wang et al, 1997; Bostrom et al,
1998) and prostate tumours (Cairns et al, 1997; Suzuki et al, 1998); and 3) a
heterozygous PTEN -/+ mouse model strain has been shown to develop multiple cancers
including early EC (Podsypanina et al, 1999).
Figure 2. Multi-step tumour suppressor pathway of tumourigenesis in the endometrium
K-RAS and p53 are shown to play similar roles in multi-step endometrial
tumourigenesis as seen in colorectal cancer. However the significance of these mutations
in EC is not yet certain. K-RAS mutations have been shown in 14-15% of EC and are less
common in endometrioid EC (Varras et al, 1996; Semczuk et al, 1998), but no
correlations have been found between K-RAS and any other clinicopathological features.
This suggests that K-RAS mutations may occur randomly in endometrial tumourigenesis.
Similarly, p53 mutations are more common in non-endometrioid (15%) EC, showing an
inverse relationship with estrogen and progesterone receptor status (Geisler et al, 1999;
Niwa et al, 1999).

Most cases of EC are sporadic, resulting from the multistep tumour suppressor
pathway as depicted earlier; however, endometrial adenocarcinoma presents as the most
common extracolonic cancer in HNPCC (hereditary nonpolyposis colorectal cancer)
patients. Unlike sporadic cancers, tumourigenesis in HNPCC patients arises as a result of
genetic instability due to loss of function of mismatch repair genes, also known as the
mutator pathway. This will be discussed in further detail in sections 1.2.4 and 1.2.5 of
this chapter.
Part 2: ENDOMETRIAL CANCER ASSOCIATED WITH HNPCC

1.2.1 General Features of HNPCC

Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominant cancer susceptibility syndrome with high penetrance (80-85%) caused by germline mutations in DNA mismatch repair genes (Lynch et al, 1993; Marra et al, 1995; Jass et al, 1998). HNPCC is characterized by the early onset of right-sided colorectal cancer (CRC) as well as extracolonic cancers of the endometrial, gastric, hepatobiliary and urinary tract. HNPCC patients carry a high risk of metachronous CRC's (Mecklin et al, 1986; Lynch et al, 1988; Vasen et al, 1989) as well as an increased cumulative risk (40%) of developing new CRC's 10 years after initial diagnosis (Mecklin et al, 1986; Lynch et al, 1988). Endometrial neoplasms are the most common cancer presentation after CRC and like CRC, occur at an early age (Mecklin et al, 1991). Women carriers of HNPCC have a 20-63% risk of developing EC compared to 3% for the general population (Watson et al 1994; Aarnio et al, 1995). HNPCC represents an estimated 1-6% of the total burden of CRC (Aaltonen et al, 1994; Lynch et al, 1996). The exact contribution of HNPCC to the total incidence of EC remains unknown.

1.2.2 Clinical Presentation

Clinical detection is based on the presentation of multiple CRC's and early onset of CRC (<50 years) in large kindreds. These criteria, known as the Amsterdam criteria, are as follows: 1) At least three relatives should have histologically verified colorectal cancer: one of them should be a first degree relative of the other two. 2) At least two successive generations should be affected. 3) In one of the relatives colorectal cancer
should be diagnosed under 50 years of age. 4) Familial adenomatous polyposis should be excluded as a diagnosis (Vogelstein and Kinzler, 1996).

While these criteria are useful in identifying HNPCC families and reflect the presence of underlying germline \textit{hMLH1} and \textit{hMSH2} mutations (Liu et al, 1996; Weber et al, 1997), they may be too stringent for smaller families. Chance clustering, environmental impact on penetrance and phenocopies obscure accurate estimates of HNPCC frequency. In addition, the Amsterdam criteria do not account for extracolonic cancers, of which EC is the most common, and may also lead to misdiagnosis of EC due to HNPCC. In fact, in some HNPCC families, endometrial carcinoma may even occur as the only or primary presentation of the HNPCC syndrome.

Newer criteria guidelines, such as the Bethesda criteria, which include HNPCC-related extracolonic cancers as well as histopathologic features for clinical diagnosis (Boland et al, 1998) have been proposed in order to address these possible limitations.

1.2.3 Molecular Basis of HNPCC

1.2.3.1 Mismatch Repair Genes

In the last five years, it has been shown that defects in mismatch repair (MMR) genes form the molecular basis of HNPCC. To date, mutations of six MMR genes have been implicated in HNPCC, including \textit{hMSH2}, \textit{hMLH1}, \textit{hPMS1} and \textit{hPMS2}, \textit{hMSH3} and \textit{hMSH6} (also known as \textit{GTBP}). Of these, germline mutations in \textit{hMSH2} and \textit{hMLH1} account for ~70% of all HNPCC families and over 90% of the reported mutations to date (Fishel et al, 1993; Leach et al, 1993; Bronner et al, 1994; Nicolaides et al, 1994; Papadopoulos et al, 1994; Palombo et al, 1995). Mutations in \textit{hPMS1}, \textit{hPMS2} and \textit{hMSH6} are only found in rare families. Therefore, mutations in \textit{hMLH1} and \textit{hMSH2} are
the most likely MMR gene mutations to identify HNPCC kindreds. However, recent studies have shown that hMSH6 mutations may play an important role in extracolonic cancers such as endometrial and gastric cancer (Edelmann et al, 1997; Imai, 98; Wijnen et al. 99).

Mutations in hMLH1 and hMSH2 are distributed throughout the entire length of both genes. Virtually all of the reported mutations are point mutations. In hMSH2, these are mostly insertion and/or deletions, and in hMLH1 they are mostly single-base substitutions. Overall, deletions have been seen to be more common than insertions (69% vs. 31%) and transitions more common than transversions (61% vs. 39%). Most mutations in hMSH2 are of frameshift (60%) or nonsense type (23%), whereas mutations in hMLH1 are predominantly frameshift (40%) and missense (31%) type (Peltomaki and Vasen, 1997). In general, most mutations in hMLH1 and hMSH2 are unique. Recurrent mutations are shared by two or more kindreds. Mutations affecting multiple kindreds with different ethnic or geographic origin include a splice donor site mutation of hMSH2 exon 5 (Figure 3) which accounts for 12% of British kindreds (Froggart et al, 1995) and a 3-base pair deletion at hMLH1 codon 616 (Figure 4). A genomic deletion of hMLH1 exon 16 affecting the highest number of kindreds (25) is specific to the Finnish population.

1.2.3.2 hMSH2

hMSH2 is a homologue of the bacterial MutS and yeast MSH proteins. hMSH2 is a 2802 bp gene containing 16 exons and maps to the human chromosome 2p22-21 (Figure 3). The encoded protein spans 934 amino acids, with the most conserved region located between amino acids 573-764 of the human protein being 85% identical to the yeast homologue. The most highly conserved region of hMSH2 encompasses a helix-
turn-helix domain spanning approximately 150 amino acids, forming an adenine nucleotide and magnesium-binding motif. This site has 100% identity amongst MutS homologues and is hypothesized to play a critical role in ATPase function as mutation of a conserved Lysine residue creates a dominant mutator phenotype (Discussed further in 1.2.4) (Fishel, 1998).

1.2.3.3 hMLH1

hMLH1 is a homologue of the bacterial MutL and yeast MLH proteins. hMLH1 is a 2268 bp gene containing 19 exons and maps to the human chromosome 3p21 (Figure 4). The encoded protein spans 756 amino acids, with the most conserved region located between amino acids 1-295 of the human protein. HMLH1 contains a region important for the interaction with hPMS2 at the carboxy terminus, between amino acid residues 506-675. Mutations in this region result in a dramatic decrease in binding affinity (>80%), leading to decreased mismatch repair function (Guerrette et al, 1999).
Figure 3. Schematic diagram of hMSH2 including common kindred mutations.
Figure 4. Schematic diagram of hMLH1 including common kindred mutations
1.2.4 Mismatch Repair

Much of our understanding of mismatch repair is based on studies of bacterial and yeast systems, which are highly conserved in human cells. The most extensively studied MMR system is the DNA adenine methylation (DAM)-instructed pathway in *Escherichia coli* (Modrich, 1989). The bacterial gene products of *mutH*, *mutL*, *mutS* and *mutU (uvrD)* repair DNA mismatches with a long-patch (~2kb) excision repair reaction. The newly replicated DNA strand is identified and specifically targeted for repair in this system by means of transient undermethylation of the adenine nucleotide within the GATC DAM sequence.

Single base pair mismatches created by polymerase misincorporation and larger mismatches due to strand slippage, causing insertions and deletions, are repaired by mismatch MutHLS pathway (Figure 5) homologue repair proteins, which work as a multimeric complex. During mismatch repair, replication errors are recognized by a MSH2/GTBP (MSH6) heterodimer or MSH2/MSH3 heterodimer (Modrich and Lahue, 1996, Palombo et al, 1996). MLH1 and PMS2 are then recruited and the mismatched strand is excised and the correct sequence is resynthesized.
Figure 5. MutHLS homolog protein mismatch repair pathway
Bidirectional Mismatch Repair

In studies of the molecular mechanism mismatch repair involving hMSH2-hMSH6 mismatch recognition and ATP-induced dissociation, it has been recently proposed that the intrinsic ATPase activity of hMSH2 and hMSH6 functions as a molecular switch, which signals the timing and assembly of the mismatch machinery (Gradia et al, 1997). In this model (Figure 6), the hMSH2-hMSH6 complex remains in an ADP-bound (active) form in the absence of DNA. Upon recognition and binding of a DNA mismatch, ADP→ATP is exchanged by hMSH2-hMSH6 and the complex dissociates (inactive – ATP-bound) from the DNA strand to be recycled back to an ADP-bound form (Gradia et al, 1997). More recent studies indicate that this hMSH2-hMSH6 molecular switch forms a sliding clamp, which is capable of binding and diffusing along the DNA backbone, such that, multiple ATP-bound sliding clamps can diffuse bidirectionally, trigger the activation of the repair machinery (Gradia et al, 1999). A similar model has been proposed for hMSH2-hMSH3, which can bind alternately to DNA mismatches. However, mismatch binding was found to be necessary but not sufficient alone to cause ADP→ATP exchange (Wilson et al, 1999).
Figure 6. Sliding clamp model of bidirectional mismatch repair
1.2.5 Microsatellite Instability

Repetitive DNA sequences constitute the vast majority (>90%) of the human genome. Microsatellites are small (about 100-300 bp) tracts consisting of tandem repeats of mono-, di-, tri- and tetra-nucleotide sequences that exhibit variation in the number of repeats block of microsatellite (Weber and May, 1989). The (CA)n repeats are found in all eukaryotes and represent one of the most abundant types of interspersed repetitive DNA in the human genome. These repetitive sequences in the genome are estimated to be between 35,000-130,000 (Sun et al, 1984; Litt and Luty, 1989; Weber and May, 1989). Microsatellites are abundant and evenly distributed throughout the genome. While alterations at these sites may be benign if they occur in non-coding regions, they create a recognizable phenotype known as microsatellite instability (MSI) indicating a defect in mismatch repair (MMR) function. Microsatellites markers are excellent indicators of MMR since they are easily genotyped using PCR-based methods, due to their small size. Virtually all HNPCC tumours show this MSI phenotype, whereas only 10-15% of sporadic colorectal tumours, are MSI positive.

HNPCC individuals carry one mutant germline copy of a MMR gene and one wild-type allele. Similar to tumour suppressor gene inactivation, loss of the wild-type allele by somatic mutation results in the loss of MMR gene activity. MMR genes recognize and repair polymerase slippage errors created during DNA replication. Loss of this important activity leads to an accumulation of replication errors and genetic instability, known as the mutator phenotype (Aaltonen et al, 1993; Thibodeau et al, 1993). This condition leads to an increase in the rate of somatic mutations in other cancer causing genes including the important growth regulatory genes, such as proapoptotic
genes BAX (Yamamoto, et al, 1998) and CHK1 (Bertoni, et al, 1999), as well as growth factor receptors IGFIIR (Souza et al, 1996) and TGF-β type II receptor (Markowitz, et al, 1996), which contain mononucleotide repeats. Secondary mutations are also common in other MMR genes, which contain repeat sequences, such as hMSH3 and GTBP. This may enhance the progression of tumourigenesis, hypothesized to require multiple mutations (Loeb et al, 1994).

1.2.6 Mutator vs. Suppressor Phenotype

Studies in colorectal cancer have revealed two distinct pathways of tumourigenesis. The classic tumour suppressor pathway model involves mutations, seen as LOH, in the APC tumour suppressor gene, P53 and K-RAS proto-oncogene. In the mutator phenotype, mutations in the nearly diploid tumours are as a result of defects in the mismatch repair pathway which lead to alterations in short repeat tracts in the coding regions of genes involved in carcinogenesis. This pathway and the gene mutations involved have been described in colorectal cancer predominantly. Mutations arising in the mutator pathway, are often called “secondary”, as they are the result of primary mutations in hMLH1 and hMSH2 (in over 90% of reported cases). This includes other MMR genes (i.e. hMSH6, hMSH3), as well as the important growth regulating and proapoptotic genes mentioned in the previous section.

Mutations arising form this pathway contribute to tumourigenesis in a stepwise fashion (Figure 7), similar to that seen in the suppressor pathway, but accelerated due to
Figure 7. Mutator pathway in endometrial tumourigenesis
hypermutability of these tumours (Loeb et al, 1991; Shibata et al, 1996). The
accumulation of inactivating somatic mutations of growth regulating genes such as
*IGFIIIR* (Souza et al, 1996) and *TGF-β* type II receptor (Markowitz et al, 1996),
contribute to tumour progression. Mutations of the *BAX* gene give the selective advantage
of resistance to programmed cell death and in tumour cells is likely to play a role in
malignant transformation (Yamamoto, et al, 1998). *BAX* mutations are seen in 50% of
MSI colorectal tumours and may account for low levels *p53* mutations (Rampino et al,
1997).

Differences seen in tumour phenotype, location and prognosis are linked to the
genetic pathways involved. Tumours located distally tend to be infiltrating, with more
frequent recurrence and have an unfavourable prognosis (Bottger et al, 1993; Tang et al,
1995). Also, aneuploid tumours associated with the suppressor pathway, are usually
located in the distal colorectum have poor prognosis (Meling et al, 1993; Moerkerk et al,
1994; Breivik et al, 1994, 1997; Yamashita et al, 1995; Changchien et al, 1997; Borresen-
Dale et al, 1998). Conversely, MSI have been associated with polypoid tumors located
proximal to the splenic flexure and better prognosis (Lothe et al, 1993; Thibodeau et al,
1993; Breivik et al, 1997).

The specific genetic events underlying tumourigenesis in EC are still being
investigated. As in colon cancer, mutations in *hMLH1* and *hMSH2* account for most of
the reported germline MMR gene mutations in EC patients (Millar et al, 1999; Simpkins
et al, 1999). Germline alterations in *hMSH2* have been shown to be more common in
families presenting with extracolonic cancers (Vasen et al, 1991; Millar et al, 1999). MSI
is seen in 17-23% of EC (Risinger et al, 1993; Burks et al, 1994; Duggan et al, 1994;
Many of the secondary genes targeted in the mutator pathway EC are similar to those mutated in colorectal cancer with the exception of TGFβRII, which is rarely mutated in EC (Myeroff et al., 1995; Schwartz et al., 1999) and the PTEN tumour suppressor, which has been suggested to play a possible "gatekeeper" role in EC. Mutations in PTEN are found 75-85% of MSI+ tumours and only 30-35% of MSI- tumours (Tashiro et al, 1997). However, the basis of this relationship remains unclear.

1.2.7 MSI-H vs. MSI-L

New evidence supports the distinction between MSI-High (MSI-H) and MSI-Low (MSI-L) categories within the mutator phenotype for colorectal cancer (Jass et al, 1999). It is likely that this will be true for EC as well however this has not been yet well characterized for EC. MSI-H tumours show allelic alterations at 40% or more of the microsatellites analyzed compared to less than 40% for MSI-L tumours. A standardized panel of five microsatellite markers for MSI analysis of colorectal cancer is recommended (Boland et al, 1998) and includes mononucleotides BAT25 and BAT26 and dinucleotides D2S123, D5S345 and D17S250. Dinucleotide markers D2S123 and D5S346 are sensitive for detection of both MSI-H and MSI-L cancers. Whereas BAT26 has been shown to predict MSI-H cancer. Germline mutations in hMLH1 and hMSH2 are not common in patients with MSI-L tumours. These patients may be candidates for other MMR gene mutations such as GTBP (Akiyama et al, 1997, Miyaki et al, 1997) or hMSH3, which is shown to repair some replication errors when introduced into an hMSH3 deficient endometrial cancer cell line (Risinger et al, 1996).

Apart from a mild mutator phenotype, MSI-L colon tumours tend to have more in common with MSI-stable (MSS) tumours. As in MSS tumours, MSI-L tumours
infrequently show mutations in growth related genes such as BAX, IGFIIR and TGF-β type II receptor and have similar frequencies of LOH. However, MSI-L tumours have a higher frequency of K-RAS mutations and reduced BCL-2 expression than MSS tumours (Jass et al, 1999).

1.2.8 Hypermethylation of the hMLH1 Promoter Region

MSI is a hallmark of HNPCC, occurring in virtually all HNPCC tumours. On the other hand, 15% of apparently sporadic colorectal tumours and 17-23% of apparently sporadic endometrial tumours are MSI (Aaltonen et al, 1993; Thibodeau et al, 1993; Simpkins et al, 1999). There exists a strong correlation between MSI and MLH1/MSH2 protein expression in cancers of the colorectum (Thibodeau et al, 1996; Dietmaier et al, 1997). Yet, MMR gene mutations have only been shown in one third of sporadic tumours (Peltomaki and de la Chapelle, 1997), suggesting the role for epigenetic alterations in MMR protein expression. Recent studies have determined that hypermethylation of the hMLH1 promoter region accounts for most of these sporadic colorectal (Kane et al, 1997) and (>70%) MSI positive cases of sporadic EC (Gurin et al, 1999; Simpkins et al, 1998). Hypermethylation has not been shown for hMSH2 protein expression, therefore these are likely to represent germline mutations. Moreover, germline hMSH2 mutations are shown to be more common in HNPCC families with increased frequency of cancers such as EC (Vasen et al, 1996; Millar et al, 1999).

1.2.9 β-catenin Mutations in EC

β-catenin is a multifunctional protein involved in two independent processes. Acting as a cell-cell adhesion regulator when coupled with cadherin (Hulsken et al, 1994)
and as a member of the Wnt signal pathway (Gumbiner et al, 1995). Phosphorylation of β-catenin by GSK (glycogen synthase kinase)-3β of serine/threonine residues is required for APC binding, which inhibits transcription activity through the Tcf-Lef pathway. Either APC mutation or stabilizing β-catenin mutation through phosphorylation of the serine/threonine residues, encoded in exon 3, will result in β-catenin stabilization and increased transcriptional activity through the Wnt signaling pathway.

APC mutations are shown in about 85% of colorectal tumours (Kinzler and Vogelstein, 1996; Korinek et al, 1997; Rubinfeld et al, 1997) and now, β-catenin mutations are being shown in a large proportion (up to 50%) of those tumours which have intact APC expression (Ilyas et al, 1997; Morin et al, 1997; Iwao et al, 1998; Sparks et al, 1998). Although the underlying mechanism is not understood, β-catenin mutations have also been associated with MSI+ tumours (Sparks et al, 1998; Kitaeva et al, 1997; Muller et al, 1998) in colorectal cancer. However, β-catenin mutations are seen equally in MSI+ and MSS tumours in EC (Mirabelli-Primdahl et al, 1999). While codon 41 and 45 in exon 3 appear to be mutational targets in colorectal cancer, codons 33 and 37 have been shown to be preferentially mutated in EC (Mirabelli-Primdahl et al, 1999; Kobayashi et al, 1999).
CHAPTER 2: RATIONALE & HYPOTHESIS

HNPCC is an inherited cancer predisposition syndrome, characterized by the early onset of colorectal cancer as well as an increased frequency of extracolonic cancers. After colorectal cancer, endometrial cancer is the most common cancer associated with HNPCC. However, the actual proportion of the overall incidence of EC, which is attributed to HNPCC, is uncertain and relative risk estimates for developing EC among HNPCC patients vary over a wide range. More accurate estimates of the contribution of HNPCC to the general population incidence of EC and the relative risk of EC in HNPCC patients, will be useful for developing future screening and counseling strategies for patients and other at risk family members.

In general, hereditary predisposition to cancer manifests in several ways, including two or more tumours arising in a single individual, the presence of many cancers in a family, and cancer diagnosis at an early age.

Therefore, we hypothesized that: 1) double primary cancer diagnosis of the two principal HNPCC associated cancer sites, the colorectum and endometrium, indicates the likelihood of an HNPCC diagnosis. 2) The presentation of EC before 50 years of age, suggests an underlying genetic predisposition and a possible diagnosis of HNPCC in at least a subset of these patients.
CHAPTER 3: STUDY OBJECTIVES

Despite the advances made in our understanding of the molecular basis of HNPCC, little is known about the contribution to the overall incidence of endometrial cancer (EC). Using germline molecular screening, microsatellite instability analysis and immunohistochemistry to detect molecular phenotypic changes found in HNPCC tumour DNA, it should be possible to more accurately determine the proportion of EC cases due to HNPCC. 

The overall aim of this study is to examine the incidence of EC associated with HNPCC in a series of (A) women diagnosed with endometrial and colorectal cancer, and a series of (B) young women diagnosed with EC (<50 years of age).

Specific objectives are:

(A) 1) To determine the frequency of germline hMLH1 and hMSH2 mutations in a series of women diagnosed with double primary (colon and endometrial) cancers.
   2) To determine the frequency of MSI+ tumours amongst a series of women with double primary (colon and endometrial) cancers.
   3) To perform immunohistochemical analysis of hMLH1 and hMSH2 protein expression for those cases which are MSI+.
   4) To evaluate any significant correlations between family history, mutation status, tumour microsatellite instability and hMLH1 and hMSH2 protein expression in double primary cancer patients.

(B) 1) To determine the frequency of MSI+ tumours in a population series of young women diagnosed with EC before 50 years.
2) To investigate the association of the expression of hMLH1 and hMSH2 proteins by immunohistochemical analysis with those cases which are MSI+. 
CHAPTER 4: hMLH1 AND hMSH2 MUTATIONS CONTRIBUTE TO DOUBLE PRIMARY CANCERS OF THE COLORECTUM AND ENDOMETRIUM

The contents of this chapter have recently been published in the May 1999 issue of Human Molecular Genetics. (see Appendix A)

4.1 AIM OF STUDY

In this study, we have examined a cohort of women with two most common HNPCC related cancers (colorectal and endometrial) for mismatch repair gene defects in hMLH1 and hMSH2 and the mutator pathway, in an effort to estimate the contribution of HNPCC to the development of double primary cancers at these tissue sites.

4.2 MATERIALS AND METHODS

This study has been approved by the Human Ethics Committee at the University of Toronto.

4.2.1 Accrual of Cases

This work was carried out in collaboration with Dr. S. Narod and Dr. T Pal at the Centre for Research in Women’s Health. Using the databases of the Ontario Cancer Care Registry at Cancer Care Ontario and the Princess Margaret Hospital tumour registry, we have identified a series of 109 double primary cancer patients. These study subjects were women diagnosed with cancer of the colorectum and endometrium by 70 years of age between 1971-1996. Of the total number of patients identified, 65 were living and of those we were able to contact 55. Family histories were obtained from 46 of these patients and blood samples were donated by 40 of these patients for mutation analysis. Where possible, we collected archival endometrial and/or colon tissues for microsatellite
instability (MSI) analysis. We have successfully obtained tumour tissue from 23 of the 40 patients included in the germline mutation analysis.

4.2.2 MMR Mutation Analysis

Germline mutations in hMSH2 and hMLH1 were screened using PCR-SSCP (polymerase chain reaction -single stranded conformational polymorphism) which is a sensitive method (sensitivity shown to be 70% or greater) of rapidly screening specific gene fragments (<300 bp) for conformational changes caused by genetic alterations as small as a single base change (Orita et al, 1989, Sheffield et al, 1993). Conditions for PCR have been optimized for the 16 exons of hMSH2 and the 19 exons of hMLH1 using flanking primers for the individual exonic regions. Exonic regions of hMSH2 and hMLH1 were amplified independently with random incorporated αP³³. An aliquot of PCR product was heat denatured to separate the double stranded DNA into single stranded DNA products, which can then assume their individual conformation under non-denaturing conditions. These changes become visible band patterns as the products migrate differently according to their conformational structure, with electrophoresis. Putative mutations detected by SSCP were confirmed and further characterized by sequencing.

PCR conditions for amplification of the exonic regions of hMLH1 and hMSH2 were as follows: 1.0-3.5μl MgCl₂, 0.25-1.0μl of 100ng/μl primer and 1.2μCi (αP³³) (Dupont-NEN Research Products, Boston, MA) in a total reaction volume of 25μl. PCR reaction was initiated at 94°C for 5 min, followed by 35, 1 min cycles of 94°C denaturing, with different annealing temperatures (52-57°C) and extension at 72°C. DNA products were denatured at 95°C for 5 min and 6μl PCR containing 50% loading dye,
was electrophoresed through a 5% non-denaturing polyacrylamide gel (29:1 acrylamide:bisacrylamide) in 0.5x TBE buffer (0.045 M Tris (pH 8.3), 0.04 M borate and 0.001 M EDTA) at 4°C for 24 hours at 7W. Gels were then dried and exposed to Kodak Biomax MR autoradiograph film.

4.2.3 Verification and Microdissection

The cases, for which tumour tissue samples were available, were sectioned and haematoxylin and eosin (H&E) stained for histologic review and the diagnosis of endometrial adenocarcinoma verified under the supervision of Dr. Mark Redston, a staff pathologist at Mount Sinai Hospital. Unstained 10μ slide sections were cut from the archival tissue and microdissected according to marked regions on the H&E stained slide sections to obtain enriched samples of normal and tumour cells (>70% cellularity) for DNA extraction and molecular analysis (Figure 8).

Separate areas of normal and tumour cells were scraped manually and digested overnight in 60-100μl of digestion buffer containing 10mM Tris-HCl (pH 8.3), 100mM KCl, 2.5mM MgCl2, and 0.45% Tween 20 (Amersham, Oakville, Ontario), mixed thoroughly with a 2.5 mg/ml concentration of proteinase K (Boehringer Mannheim, Laval, PQ). The samples were then overlaid with mineral oil and allowed to digest overnight at 65°C. Samples were heat denatured at 94°C for 5 min to inactivate any remaining proteinase K and stored at 4°C.
Figure 8. H&E slide with normal and tumour cells marked for microdissection
4.2.4 MSI Analysis

DNA extracted from the normal and tumour archival tissues was analyzed for microsatellite instability status (MSI+/-). The following (CA)n (D2S123, D5S346, D17S250) and (A)n (BAT26, BAT25) microsatellites were used for analysis. Alterations found in 40% or more (≥2 of 5) satellites were determined MSI positive (MSI+).

PCR conditions for amplification of the 5 microsatellite markers were as follows: 1.5μl MgCl2, 1.0μl of 100ng/μl primer and 1.2μCi (γP33) (Dupont-NEN Research Products, Boston, MA) in a total reaction volume of 25μl. PCR reaction was initiated at 94°C for 5 min, followed by 35, 1 min cycles of 94°C denaturing, with different annealing temperatures (55-60°C) and extension at 72°C. DNA products were denatured at 95°C for 5 min and 6μl PCR containing 8μl of 6x loading dye, were electrophoresed through a 6% denaturing DNA PAGE polyacrylamide gel with 0.42g/ml urea in 1x TBE buffer (0.045 M Tris (pH 8.3), 0.04 M borate and 0.001 M EDTA) at room temperature for approximately 2 hours at 80W. Gels were then dried and exposed to Kodak Biomax MR autoradiograph film.

4.2.5 Immunohistochemistry

Paraffin-embedded tissue blocks were sectioned (6μ) then deparaffinized and rehydrated using xylene and ethanol. The slides were pretreated by microwave antigen retrieval (10 mM citrate buffer, pH 6.0). Endogenous peroxidase and non-specific binding were blocked with 3% aqueous hydrogen peroxide and 20% Protein blocker (Signet Laboratories, Inc) in TBS. Sections were incubated overnight at room
temperature with mouse monoclonal antibodies against (a) hMLH1 protein (G168-728, PharMingen) at a 1:50 dilution and (b) hMSH2 protein (FEI11, Oncogene Science) at a 1:100 dilution. Antibodies were detected with the avidin-biotin method, using diaminobenzidine as the chromagen and counterstained with hemotoxylin. Cancers were considered to demonstrate inactivation of HMLH1 or hMSH2 when there was complete absence of detectable nuclear staining of neoplastic cells. Intact nuclear staining of adjacent non-neoplastic epithelium, stromal cells, or lymphocytes served as an internal control and was required for adequate evaluation. Dr. Victoria A. Marcus assessed all the cases independent of any knowledge of family history, microsatellite instability information, or germline mutation status.

4.2.6 Statistical Analysis

The observed number of cases (O) in the first degree relatives was determined by review of family pedigrees. The expected number of cases (E) for each cancer was calculated from the product of the person years and the Ontario provincial cancer rate for each age range. Patients were considered to be at risk of cancer from birth until death or age in 1996. The expected number of cases was calculated for each of the age ranges selected. The relative risk of cancer in the first degree relatives of probands was estimated by comparing observed to expected figures. The confidence intervals (CI) were calculated assuming a Poisson distribution, CI=O_L/E-O_U/E, where O_L and O_U are the lower and upper bounds respectively.
4.3 RESULTS

4.3.1 Germline Mutation Analysis and Association with Family history among double primary probands

Of the 40 cases examined, 6 were found to carry \textit{hMSH2} mutations and one had an \textit{hMLH1} mutation (17.5\%). All of the mutations were novel and truncating in nature. Mutation positive probands had stronger family histories and an earlier age of diagnosis (Table 2). Five of these 7 patients with MMR gene mutations had strong family histories (71\%), according to familial HNPCC criteria developed in our previous study (Table 3), and 3 of these 7 families met the Amsterdam criteria (Fig. 4.1-4.7). This compares with 7 of 33 cases (21\%) having strong cancer family histories out of 33 of the mutation negative cases (odds ratio = 9.3, \(P=0.02\)).

As well, 6 of the 7 patients with MMR mutations had been diagnosed with both colorectal and EC by the age of 55. The other case was diagnosed with colorectal cancer at age 48, but EC at age 65. Of the 33 mutation negative probands, 8 were diagnosed with both of these cancers by age 55. Overall, mutations were found for 6 of 14 (43\%) women who had both cancers before the age of 55 years (odds ratio=18.8, \(P=0.004\)). No difference was seen between the average age of diagnosis in the living cases of our study and the deceased cases (with pedigrees) which were excluded from mutational analysis.

4.3.2 MSI Analysis

Tumour blocks were available for 23/40 of the total cases included in this study for mutation analysis. Only colorectal tissue was available for 4 cases and only endometrial tumour was available for 4 cases. Both colorectal and endometrial tissue was available for 15 cases.
Table 2. Summary of mutation analysis of women with double primary cancers of the colorectum and endometrium

<table>
<thead>
<tr>
<th>Case #</th>
<th>Gene</th>
<th>Exon</th>
<th>Codon</th>
<th>Nucleotide Change</th>
<th>Mutation Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>hMSH2</td>
<td>8</td>
<td>452</td>
<td>A→T at 1355</td>
<td>Creates novel splice site</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>→ 30 bp del</td>
</tr>
<tr>
<td>2</td>
<td>hMSH2</td>
<td>15</td>
<td>878</td>
<td>Del AG at 2633</td>
<td>Frameshift</td>
</tr>
<tr>
<td>3</td>
<td>hMSH2</td>
<td>15</td>
<td>878</td>
<td>Del AG at 2633</td>
<td>Frameshift</td>
</tr>
<tr>
<td>4</td>
<td>hMSH2</td>
<td>12</td>
<td>SD of exon 12</td>
<td>Del 11 bases at 2005 + 2</td>
<td>Splice defect</td>
</tr>
<tr>
<td>5</td>
<td>hMSH2</td>
<td>1</td>
<td>45</td>
<td>Del 29 bases at 134</td>
<td>Frameshift</td>
</tr>
<tr>
<td>6</td>
<td>hMLH1</td>
<td>12</td>
<td>397</td>
<td>Del T at 1190</td>
<td>Frameshift</td>
</tr>
<tr>
<td>7</td>
<td>hMSH2</td>
<td>7</td>
<td>389</td>
<td>C→T at 1165</td>
<td>Arg-stop</td>
</tr>
</tbody>
</table>
Table 3. Study criteria used to classify double primary cancer families

<table>
<thead>
<tr>
<th>1) Familial</th>
<th>1) Amsterdam criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1) at least three relatives should have histologically verified colorectal cancer: one of them should be a first degree-relative of the other two</td>
</tr>
<tr>
<td></td>
<td>2) at least two successive generations should be affected</td>
</tr>
<tr>
<td></td>
<td>3) In one of the relatives colorectal cancer should be diagnosed under 50 years of age</td>
</tr>
<tr>
<td></td>
<td>4) familial adenomatous polyposis should be excluded</td>
</tr>
<tr>
<td></td>
<td>or</td>
</tr>
<tr>
<td></td>
<td>2) two or more first-degree relatives of proband with: colorectal cancer or endometrial cancer diagnosed at &lt;55 years of age; kidney, urethral, esophageal, stomach, small intestinal, pancreatic or ovarian cancer diagnosed at any age</td>
</tr>
<tr>
<td></td>
<td>or</td>
</tr>
<tr>
<td></td>
<td>3) two or more first-degree relatives of proband with at least one colorectal cancer diagnosed at &lt;55 years of age with another diagnosed at any age</td>
</tr>
<tr>
<td></td>
<td>or</td>
</tr>
<tr>
<td></td>
<td>4) two or more first-degree relatives of proband with at least one endometrial cancer diagnosed at &lt;55 years of age with another diagnosed at any age</td>
</tr>
<tr>
<td></td>
<td>or</td>
</tr>
<tr>
<td></td>
<td>5) two or more first-degree relatives of proband with colorectal cancer or endometrial cancer diagnosed at &lt;55 years of age with another colorectal cancer or endometrial cancer diagnosed at any age</td>
</tr>
</tbody>
</table>

| 3) Possibly familial | 1) one first-degree relative of proband with: colorectal or endometrial cancer diagnosed at <55 years of age; kidney, urethral, esophageal, stomach, small intestinal, pancreatic or ovarian cancer diagnosed at any age |
|                      | or |
|                      | 2) two or more first-degree relatives of proband with colorectal or endometrial cancer diagnosed at >55 years of age |

| 3) Non-familial      | none of the above |
Table 4. Summary of clinical features for mutation positive vs mutation negative probands

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>Mutation Positive* (n=7)</th>
<th>No Mutation (n=33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ave. Age of Diagnosis of Colorectal Cancer</td>
<td>44.9 yrs</td>
<td>54.8 yrs</td>
</tr>
<tr>
<td>Ave. Age of Diagnosis of Endometrial Cancer</td>
<td>49.7 yrs</td>
<td>56.8 yrs</td>
</tr>
<tr>
<td>Number of Probands meeting the Amsterdam Criteria</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Familial**</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Possibly Familial</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Non- familial</td>
<td>1</td>
<td>16</td>
</tr>
</tbody>
</table>

* mutation positive = *hMLH1* or *hMSH2* mutation identified
** Familial includes pedigrees that meet Amsterdam Criteria
Table 5. Relative risks of cancers in first-degree relatives of mutation positive vs mutation negative probands.

<table>
<thead>
<tr>
<th>Cancer Site</th>
<th>Age at Diagnosis</th>
<th>Mutation positive (n=7)</th>
<th>Mutation negative (n=33)</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>RR</td>
<td>CI</td>
</tr>
<tr>
<td>Any cancer</td>
<td>&lt;55</td>
<td>5.4</td>
<td>2.9-9.3</td>
</tr>
<tr>
<td></td>
<td>≥55</td>
<td>1.3</td>
<td>0.5-2.7</td>
</tr>
<tr>
<td></td>
<td>Any age</td>
<td>2.7</td>
<td>1.6-4.1</td>
</tr>
<tr>
<td>Colon</td>
<td>&lt;55</td>
<td>24.5</td>
<td>7.9-57.2</td>
</tr>
<tr>
<td></td>
<td>≥55</td>
<td>3.8</td>
<td>0.8-11.1</td>
</tr>
<tr>
<td></td>
<td>Any age</td>
<td>8.1</td>
<td>3.5-15.9</td>
</tr>
<tr>
<td>Endometrial</td>
<td>&lt;55</td>
<td>62.8</td>
<td>12.6-183.4</td>
</tr>
<tr>
<td></td>
<td>≥55</td>
<td>8.3</td>
<td>0.1-46.3</td>
</tr>
<tr>
<td></td>
<td>Any age</td>
<td>23.8</td>
<td>6.4-61.0</td>
</tr>
</tbody>
</table>
Table 6. MSI Analysis compared with Immunohistochemistry

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>Case #</th>
<th>Mutation Status</th>
<th>MSI Status</th>
<th>IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amsterdam</td>
<td>3</td>
<td>hMSH2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>hMSH2</td>
<td>+</td>
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</tr>
<tr>
<td></td>
<td>33</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Strongly Familial</td>
<td>4</td>
<td>hMSH2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>-</td>
<td>+</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>-</td>
<td>+</td>
<td>- MSH2</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Possibly Familial</td>
<td>9</td>
<td>-</td>
<td>+</td>
<td>- MLH1</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>-</td>
<td>+</td>
<td>WT</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>-</td>
<td>+</td>
<td>- MLH1</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Non-familial</td>
<td>10</td>
<td>-</td>
<td>+</td>
<td>- MLH1</td>
</tr>
<tr>
<td></td>
<td>29</td>
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<td>+</td>
<td>- MLH1</td>
</tr>
<tr>
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<td>14</td>
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<td></td>
<td>27</td>
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<td>8</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Figure 9. Pedigrees for mutation positive cases #1-7. #2 is an HNPCC/Muir-Torre kindred. #8 is a mutation negative family that met Amsterdam criteria. Blood samples were obtained from probands for each family and analyzed for germline *hMSH2* and *hMLH1* mutations. Probands are indicated by an arrow (→). Affected and unaffected members are shown as solid and open symbols respectively. Cancer site(s) and age of diagnosis are indicated. Bl, bladder; bn, brain; bo, bowel; br, breast; co, colorectum; en, endometrium; es, esophagus; lu, lung; ov, ovary; pa, pancreas; pr, prostate; psu, primary site unspecified; st, stomach; th, throat; ur, ureter; wt, Wilm’s tumour.
All of the patients with MMR mutations exhibited MSI (MSI+). Of the mutation negative probands, 7 were MSI+. The one patient, whose pedigree met Amsterdam criteria but did not carry a MMR mutation, was MSI-. Of the 12 patients with weak (non-familial) cancer family histories, 10 were MSI- (Table 4.4).

4.3.3 Immunohistochemical Analysis

Immunohistochemical analysis was performed for the 7 MSI+ cases for which no germline mutations of hMLH1 or hMSH2 were found. Lack of hMLH1 antibody staining was seen in 4 cases, one case showed abnormal expression of hMSH2 and normal expression of both proteins was seen in the 2 remaining cases.

A proportion of the cases lacking hMLH1 expression may harbour mutations missed during our germline mutation screening. However, these are more likely to be the result of epigenetic alterations, specifically hMLH1 promoter hypermethylation, which is shown to account for a significant proportion of sporadic MSI+ tumours of the colorectum and endometrium (Kane et al, 1997, Simpkins et al, 1999). The abnormal hMSH2 antibody stained case is more likely to represent such a mutation.

The 4 cases with no observed abnormal expression of hMLH1 or hMSH2 may carry mutations in other MMR genes or as yet undefined genes contributing to the MSI phenotype.

4.3.4 Relative Risk Estimates

Cancer risk estimates for first-degree relatives of double primary probands are presented in Table 6. A marked increase in relative risk is seen for cancer of the colorectum and endometrium in relatives of probands carrying hMSH2 or hMLH1 mutations. This increased risk in relatives is diminished after the age of 55.
The relative risk of colorectal cancer and EC remains high for the mutation-negative patients, specifically those diagnosed before the age of 55 years. This increased risk is not seen in patients diagnosed over the age of 55 years.

Risks were similar for relatives of the 40 women who consented to genetic testing, compared to the total 46 women for whom pedigrees were available and consent for genetic testing was requested.

### 4.4 CONCLUSION

Based on our study we conclude that a diagnosis of double primary cancers of the colorectum and endometrium in a patient is a strong indicator of HNPCC, particularly for those diagnosed with both cancers before 55 years of age. Physicians are advised to consider the possibility of HNPCC when a patient presents with both colorectal and ECs before the age of 55 years or has a strong family history of HNPCC associated cancers and to perform a comprehensive genetic evaluation.
CHAPTER 5: A POPULATION STUDY OF MICROSATELLITE INSTABILITY IN TUMOURS OF YOUNG ENDOMETRIAL CANCER

5.1 AIM OF STUDY

The overall aim of this population based study is to determine the frequency of microsatellite instability in tumours of patients diagnosed with endometrial adenocarcinoma before 50 years age and then to determine what proportion of these are due to the mismatch repair deficiency of *hMLH1* and *hMSH2*.

5.2 MATERIALS AND METHODS

This study was approved by the Human Ethics Committee at the University of Toronto.

5.2.1 Accrual of Cases

In collaboration with the Ontario Cancer Registry (OCR) at Cancer Care Ontario, under the direction of Dr. Eric Hollowaty and Darlene Dale, we have used the OCR’s computerized database to identify all the young cases (≤50 yrs) of primary endometrial adenocarcinoma diagnosed within a five-year period (1989-1993). The OCR database is based on passive retrieval of all diagnosed cancers, reported by Ontario hospitals. In our study, the search parameters included ages 0-49 years, diagnosis years 1989-1993 and cancer site being the endometrium (OCR site code 182), to collect all reported cancer from hospitals located within the geographical area of Central-Eastern Ontario. 40 hospitals and 2 diagnostic medical labs were included in this study (see table). Copies of all the pathological reports, including hospital names, diagnosis dates and procedures, have been obtained. Archival tissue samples have been requested from the hospitals.
specified in the reports and collected at Mount Sinai Hospital with assigned study numbers to protect patient anonymity.

5.2.2 Verification and Microdissection

The pathology reports of all the identified cases were reviewed and the diagnosis endometrial adenocarcinoma verified under the supervision of Dr. Mark Redston. Slides from the archival tissue were then microdissected to obtain enriched samples of normal and tumour cells (>70% cellularity) for DNA extraction and molecular analysis (for a more detailed description see 4.2.2).

5.2.3 Microsatellite Instability Analysis.

Microsatellite instability analysis will be performed according to the protocol used in the double primary series (see chapter 4, section 4.2.4). DNA extracted from the normal and tumour archival tissues was analyzed for microsatellite instability status (MSI+/-). The following dinucleotide (D2S123, D5S346, D17S250) and mononucleotide (BAT 26, BAT25) microsatellites will be used for analysis (Figure 12). Alterations found in 30% or more satellites will be determined MSI positive (MSI+). MSI+ cases are further categorized as MSI-HHH (very high) which refers to tumours unstable at 4 or more microsatellites and MSI-H tumours, which are unstable at 2 or 3 microsatellites. As well MSI- cases are subdivided into MSS (microsatellite stable) and MSI-L (low) which includes tumours that show instability at only one microsatellite.

5.2.4 Immunohistochemistry

Immunohistochemical analysis of hMLH1 and hMSH2 protein expression was performed for all of the MSI+ (MSI-H and MSI-HHH) and MSI-L cases as well as a
subset of MSS cases. The methods used are identical to those described earlier in chapter 4. For a detailed review, refer to section 4.2.5. Immunohistochemistry for this study, was reviewed by Dr. Robert Travis Ogilvie, under the supervision of Dr. Mark Redston (Figures 10 and 11).
Figure 10. Low (X100, top) and high (X200, bottom) power views of immunohistochemical hMSH2 (left) and hMLH1 (right) staining of tumour sections from one patient, demonstrating a lack of hMSH2 protein expression (A and C). Nuclear staining of expressed hMLH1 protein is visible (D), whereas no nuclear staining of hMSH2 is seen (C) in tumour cells. Normal expression of hMSH2 and hMLH1 can be seen in the nuclei of normal epithelium and stromal cells (A and B).
Figure 11. Low (X100, top) and high (X200, bottom) power views of immunohistochemical hMSH2 (left) and hMLH1 (right) staining of tumour sections from one patient, demonstrating a lack of hMLH1 protein expression (B and D). Nuclear staining of expressed hMSH2 protein is visible (C), whereas no nuclear staining of hMLH1 is seen (D) in tumour cells. Normal expression of hMSH2 and hMLH1 can be seen in the nuclei of normal epithelium and stromal cells (A and B).
5.3 RESULTS

5.3.1 Accrual Results

In total, Cancer Care Ontario identified 256 women diagnosed with adenocarcinoma of the endometrium. After obtaining and carefully reviewing the pathology reports of all the cases in this study, 36 cases were noted not have a primary diagnosis of endometrial adenocarcinoma and were excluded from the study. These miscoded cases included women with endometrial tumours originating from another primary cancer site, such as the cervix or ovary, and endometrial pathology other than adenocarcinoma, such as complex hyperplasia or leiomyomas. After further review of cut slide sections, 28 cases were excluded based on misdiagnosis or because there was insufficient tissue for molecular analysis. Misdiagnosed cases were often complex hyperplasias, especially common in diagnosis based on curetting alone, leiomyomas, or secondary endometrial cancers arising from a primary cancer in a different tissue site. Nineteen cases were identified for which only curetting samples were available. Matched hysterectomy tissue was located for 8 cases, offering good matched normal tissue for MSI analysis. Adequate normal and tumour tissue was available in 2 curettings. Six cases have only tumour tissue for MSI analysis. An additional 8 cases were unavailable for molecular analysis.

5.3.2 MSI Results

The remaining 184 verified cases of adenocarcinoma were included in the study and analyzed for MSI status. Of these, 167 cases amplified for all loci (5/5), 13 amplified for 4 loci (4/5) and 4 cases amplified for 3 loci (3/5). Of the total 184 cases, 39 were MSI+ (21.2%), including 16 MSI-H (16/39) and 23 MSI-HHH (23/39) cases. Of the
Table 7. Distribution of endometrial tumour samples according to hospital of origin in South-Central Ontario.

<table>
<thead>
<tr>
<th>Hospital of Origin</th>
<th>No. TOC</th>
<th>No. TEC</th>
<th>No. TSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oakville-Traf</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Tor E Gen</td>
<td>15</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Etob Gen</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Queensway Gen</td>
<td>9</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>NY Branson</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>NYork Gen</td>
<td>14</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Sunnybrook</td>
<td>11</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>York Finch Gen</td>
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<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Scarboro Cent</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Scarboro Gen</td>
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<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Central Hosp</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Doctors</td>
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<td>3</td>
<td>5</td>
</tr>
<tr>
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<td>Toronto Gen</td>
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<td>Wellesley</td>
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<td>Women's</td>
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<td>Kitch Waterloo</td>
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<td>Stouffville</td>
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<td>1</td>
<td>1</td>
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<tr>
<td>Toronto Hosp</td>
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<td>3</td>
</tr>
<tr>
<td>Hillside Lab</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>256</strong></td>
<td><strong>72</strong></td>
<td><strong>184</strong></td>
</tr>
</tbody>
</table>
Figure 12. Normal (N) and matched tumour (T) showing examples of allelic alteration (*) versus stable alleles for a panel of 5 microsatellites.
MSI- cases, 135 were MSS, 7 were MSI-L and 15 did not amplify for all the microsatellite loci after 3 attempts. These are considered MSI- or MSS if 4 of the 5 markers were negative (9 cases), MSI-L if 1 marker was altered (2 cases) and MSI-H if 2 or more markers were altered (4 cases).

More than 1 marker did not amplify for 4 cases. Of these cases, 3 showed stability for the 4 marker loci which amplified, however it is not certain if these are true MSS cases or possibly MSI-L. These MSI- cases are therefore left as query and require further classification. The remaining cases showed alterations in 2 markers and were classified as MSI-H.

**Individual Marker Sensitivity**

Only marker information, of cases for which amplification was successful for all (5/5) loci, was included in the specific marker sensitivity analysis. Therefore, 4 MSI-H cases and 2 MSI-L cases were excluded as some markers did not amplify for these cases and 2 currettings (1 MSI-H and 1 MSI-L) were also excluded from analysis because only BAT 25 and BAT 26 were available for these cases.

One or more of the mononucleotides markers were altered for most of the MSI+ cases. This was true for both MSI-H and MSI-HHH cases. Only 2 MSI-H cases were negative for both mononucleotide markers. Both BAT25 and BAT26 were altered in 21 of the 23 MSI-HHH cases (Table 8).
Table 8. Results of Individual microsatellite marker sensitivity analysis for a panel of 5 microsatellite loci.

<table>
<thead>
<tr>
<th>Marker</th>
<th>MSI-HHH (n=23)</th>
<th>MSI-H (n=11)</th>
<th>MSI-L (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Locus Positive</td>
<td>Locus Negative</td>
<td>Locus Positive</td>
</tr>
<tr>
<td>BAT25</td>
<td>22</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>BAT26</td>
<td>21</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>D2S123</td>
<td>22</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>D5S346</td>
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<td>4</td>
<td>2</td>
</tr>
<tr>
<td>D17S250</td>
<td>21</td>
<td>2</td>
<td>8</td>
</tr>
</tbody>
</table>
Immunohistochemistry Results

Immunohistochemical analysis of MLH1 and MSH2 protein expression was performed for 37/39 MSI+, 6/7 MSI-L and 9 MSS cases. Normal expression was seen in 40.5% of the MSI+ cases, abnormal expression of MLH1 was seen in 29.7% of MSI+ cases and abnormal MSH2 expression was seen in the remaining 29.7% of MSI+ cases. 90.9% of abnormal MSH2 expression was seen in MSI-HHH cases. All of the MSI-L and MSS cases showed normal expression of both MLH1 and MSH2. The results of immunohistochemistry were uninformative for 12 cases (Table 9).
<table>
<thead>
<tr>
<th>6</th>
<th>9</th>
<th>1</th>
<th>5</th>
<th>9</th>
<th>01</th>
<th>9</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>WT</td>
<td>+ve</td>
<td>+ve</td>
<td>MSH2</td>
<td>WT</td>
<td>MSH2</td>
<td>WT</td>
</tr>
<tr>
<td>MSS</td>
<td>MSL-T</td>
<td>MSL-H</td>
<td>MSL-HHH</td>
<td></td>
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5.4 DISCUSSION

Of the 256 cases identified by the OCR at Cancer Care Ontario, 49 cases were not primary endometrial adenocarcinomas and were excluded from our study. Fifteen cases were verified as adenocarcinomas, but unfortunately, could not be analyzed because there was insufficient tissue for molecular analysis. Few cases were indeed misdiagnosed. We found that it was fairly common for curettings to be reported for diagnostic purposes as well-differentiated adenocarcinoma or suspicious of adenocarcinoma, however, for molecular analysis, they were determined to be complex hyperplasias and were not included in our molecular analysis. An additional 8 cases could not be located or were unavailable for molecular analysis. Therefore the total number of true endometrial adenocarcinoma cases with verified diagnosis and identified for analysis was 199 and of these we were able obtain tumour tissue for molecular analysis for 184, which is a high (92%) accrual rate.

Of the 184 endometrial cancer cases analyzed for MSI status in our study, 21% (37/184) were MSI+ (alterations in ≥40% of microsatellite loci). This is within the reported range of MSI + tumour frequencies for endometrial cancer (Risinger et al, 1993; Burks et al, 1994; Duggan et al, 1994; Peiffer et al, 1994). MSI+ tumours were further classified into groups of tumours, which show alterations at ≥40% but < 80% of microsatellite loci (2-3/5 loci), termed MSI-H, and those which show alterations at ≥ 80% of microsatellite loci (4-5/5 loci), termed MSI-HHH.

Immunohistochemical analysis was performed in order to determine the proportion of MSI+ cases that were due to genetic defects in MMR proteins, specifically MLH1 and MSH2. Abnormal expression of MLH1 protein and MSH2 protein equally
with each representing 28% (11/39) of the total MSI+ cases, the remaining 43.5% of cases showed normal wild-type expression of both proteins.

The patients included in our study were diagnosed with EC at an early age, and are therefore more likely to be representative of a hereditary genetic MMR deficiency. Here, we have found that roughly one third of our MSI+ cases lack hMSH2 expression and as hypermethylation is not shown to affect its expression, it is likely that these represent germline mutations. The mutation detection rate based on hMSH2 alone is 29%. A proportion of the MSI+ tumours lacking hMLH1 expression are likely due to hypermethylation, which cannot be determined without conducting hypermethylation analysis. In comparison to a similar study of apparently sporadic EC (not based on young onset cancers), a smaller proportion of our cases, show abnormal hMLH1 expression in our study than reported by Simpkins et al (1999). This highlights the distribution differences of hMLH1 and hMSH2 mutations in young EC, suggesting a predominance of hMSH2 mutations in the genetic contribution of young EC.

Interestingly, upon further classification, we observed a large proportion of MSI-HHH cases (23/39) within our total MSI+ cases as compared to the proportion of MSI-H cases (16/39). Within this subset of very high MSI cases (MSI-HHH), we observed a high frequency of cases showing abnormal hMSH2 (10/11) expression. The distribution of tumours lacking MLH1 expression, in contrast, was roughly even between the 2 groups.

Again, this strongly indicates the important hereditary contribution of hMSH2, to the genetic instability of endometrial tumours.

Notably, there remains a large proportion of both MSI-H and MSI-HHH for which no abnormal expression of MLH1 or MSH2 was found. This supports the
possibility of other candidates, such as GTBP or other, as yet unidentified genes, which may contribute heavily to the microsatellite phenotype.

Although several studies to date have described microsatellite markers that are likely to be highly sensitive and specific for CRC, very few studies have commented specifically on similar markers for EC. Our study represents one of the largest collections of population based EC analysis for MSI status. We observed that similar to CRC, both BAT25 and BAT26 are sensitive markers in our series.

Specific allelic sensitivity analysis of each of the microsatellite markers used for MSI analysis demonstrated high sensitivity for both of the mononucleotides, BAT25 and BAT26 (A)n repeats, as well as the D17S250 (CA)n repeat. BAT25 and BAT26 showed similar allelic sensitivity and were both altered in 21 of 23 MSI-HHH tumours, therefore over 91% of MSI+ EC could have been identified as based on analysis of the mononucleotides alone. This is useful for designing future strategies for MSI analysis of cases for which only tumour tissue is available, such as is often the case for endometrial curettings. Twenty-six (26/199) curettings were first identified for our study. Only 2 of the total number of MSI+ cases were negative for both BAT25 and BAT26.
CHAPTER 6: SUMMARY AND CONCLUSIONS

Endometrial cancer (EC) is the most common extracolonic malignancy in HNPCC yet the accurate proportion of EC associated with this hereditary cancer predisposition syndrome and the inherited risk of EC amongst HNPCC patients remains unclear. Lifetime risk estimates for developing EC may vary from 20-63% in women carriers of MMR gene mutations and may even surpass the risk for colorectal cancer (Dunlop et al, 1997). These women tend to present with EC about 15 years earlier than the general population (Watson et al, 1994) at an average age of 45 years and have a predisposition to colorectal cancer as well as EC as it is the most frequent cancer amongst its associated extracolonic cancers. In some small or atypical HNPCC families, there may be a predominance of HNPCC related extracolonic cancers for which current clinical diagnostic criteria may not detect, and past studies based on the Amsterdam criteria, would not have included. Two such families are documented in our study of women with double primary cancers of the colorectum and endometrium. One family has a very small pedigree making family history uninformative for clinical diagnosis of HNPCC (Figure 9-6). The second family has a strong history of HNPCC-related extracolonic cancers but there is no direct transmission of colorectal cancer and would therefore not be diagnosed using the Amsterdam criteria (Figure 9-4). These factors create an under representation of the contribution of HNPCC to the overall incidence of EC.

EC carries a high morbidity rate (most common gynecological cancer), but with early detection, it often has good long-term prognosis. This is highly dependent on early detection, as stage I, well-differentiated adenocarcinomas that are diagnosed and treated quickly, have a 90% 5-year survival rate. This underscores the importance of developing
rigorous screening and diagnostic strategies as early detection is determinate of the overall prognosis. Yet the contribution of HNPCC within the general incidence of EC is uncertain and some women may carry and underestimate risk for EC development. The focus of this thesis is to more accurately determine the frequency of HNPCC-related EC in populations of women that are likely candidates for this hereditary cancer predisposition. These include, patients with double primary cancers of the colorectum and endometrium and women diagnosed with EC before 50 years of age.

The first study, examining the frequency of germline \textit{hMLH1} and \textit{hMSH2} mutations in women with double primary cancers of the two most common cancers in the HNPCC syndrome, was based on a cohort of women for whom we had family history knowledge. These patients were accrued through hospital-based registries and represent patients that one might typically expect to see in a genetic counseling clinic. The results of this study will be useful in designing counseling and clinical diagnostic strategies based on the patient family history. We determined that a high rate of HNPCC germline mutations exists (18\%) amongst women with double primary cancers of the colorectum and endometrium. This is particularly high for women diagnosed with both cancers before 55 years of age. It is important to note that the relative risk of colorectal and endometrial cancers is elevated for the first-degree relatives of these patients who carry an \textit{hMLH1} or \textit{hMSH2} mutation. Thus, highlighting the importance of screening and detection for the individual as well as their family members.

The second population-based study is representative of a random North American population, as it includes a large sample of EC patients identified from both rural and urban settings from 42 different hospitals (Table 7). The results of this study are of great
importance for estimating the overall frequency of mismatch repair deficiency in endometrial tumours and the contribution of MMR genes, specifically \textit{hMLH1} and \textit{hMSH2} alterations, in these tumours. Because our understanding of the genetic basis of EC tumourigenesis and the role of mismatch repair deficiency and its associated MMR genes remains uncertain, compared to colorectal cancer, much of the resulting knowledge gained about the sensitivities of individual markers and the contribution of the most commonly reported MMR genes in HNPCC related colorectal cancer, will be very valuable for future EC studies.

Based on the results of this study, we estimate that approximately 22\% of apparently sporadic young EC tumours are MSI+. Of these ~29\% are due to \textit{hMSH2} mutation and 29\% may involve \textit{hMLH1} mutation. Further, we have found that this rate of mutation increases dramatically for \textit{hMSH2} in women whose tumours show alterations at 80\% or more of the microsatellites tested. Interestingly, a large proportion of these women have not been shown to have alterations in \textit{hMLH1} or \textit{hMSH2}, suggesting a possible role for other MMR genes, such as \textit{hMSH6}, which has been recently shown to play an important role in endometrial cancer (Wijnen et al, 1999) or even other genes to be determined.

The clinical implications drawn from these studies are clear. Women diagnosed with multiple primary cancers of the colorectum and endometrium or diagnosed with EC before 55 years of age, are prime candidates for diagnosis of HNPCC. Genetic testing in the form of MSI analysis of tumours followed by immunohistochemical analysis of \textit{hMLH1} and \textit{hMSH2} protein expression would be advised. Patients exhibiting defective
mismatch repair should then have germline mutation testing for \textit{hMLH1} and/or \textit{hMSH2} and follow up genetic counseling.
CHAPTER 7: REFERENCES


hMSH2-hMSH6 forms a hydrolysis-independent sliding clamp on mismatched DNA.


Risinger JI, Hayes AK, Berchuck A, Barrett JC. PTEN/MMAC1 mutations in endometrial cancers. *Cancer Res* 1997 Nov 1; 57(21):4736-8


Title Page

Submission category: article

Title: Mismatch repair gene defects contribute to the genetic basis of double primary cancers of the colorectum and endometrium.

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Abstract

Hereditary nonpolyposis colorectal cancer (HNPCC) is a dominantly inherited cancer syndrome caused by germline defects of mismatch repair (MMR) genes. Endometrial cancer is the most common extracolonic neoplasm in HNPCC and is the primary clinical manifestation of the syndrome in some families. The cumulative incidence of endometrial cancer among HNPCC mutation carriers is high, estimated to be from 22 to 43%.

We hypothesized that women with double primary cancers of the colorectum and endometrium are likely to be members of HNPCC families. In order to determine how frequently HNPCC manifests in the context of double primary cancers, we examined alterations of two MMR genes, hMSH2 and hMLH1, in 40 unrelated women affected with double primary cancers. These cases were identified using hospital-based and population-based cancer registries in Ontario, Canada. MMR gene mutations were screened by single-strand conformation polymorphism analysis and confirmed by direct sequencing. Eighteen percent (7/40) were found to harbor mutations of one of the two MMR genes. Analysis of colorectal and/or endometrial tumors of mutation negative probands found microsatellite instability in 7/20 cases. Six of seven mutation positive probands had strong family histories suggestive of HNPCC. First-degree relatives of mutation positive probands had a very high relative risk of colorectal cancer (RR= 8.1, CI 3.5-15.9) and endometrial cancer (RR= 23.8, CI 6.4-61.0). The relative risk of mutation negative cases was 2.8 (CI 1.7-4.5) for colorectal cancer and 5.4 (CI 2.0-11.7) for endometrial cancer. We recommend that all double primary patients with cancers at these sites should have a genetic evaluation, including molecular analysis for HNPCC where appropriate.
Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominant cancer susceptibility syndrome frequently caused by germline mutations in DNA mismatch repair (MMR) genes\(^1\). HNPCC is characterized by an early onset of colorectal cancer (CRC) as well as cancers of the endometrium, stomach, ovary, pancreas and the hepatobiliary and urinary tracts. CRC is the most common cancer in the syndrome (~63% of all HNPCC tumors) and endometrial neoplasms are the most common extracolonic cancers\(^2\). As with CRC, endometrial cancers occur at an early age in HNPCC\(^3\). Women who carry HNPCC mutations have a 22-43% lifetime risk of developing endometrial cancer compared to 3% for the general population\(^4,5,6\).

Recent advances in our understanding of the molecular basis of HNPCC should lead to a more accurate diagnosis of the syndrome. Defects in mismatch repair (MMR) genes have been shown to cause HNPCC (including \(h\)\(MSH2\), \(h\)\(MLH1\), \(h\)\(PMS2\), and \(h\)\(MSH6\) (\(GTBP\))). To date, germline mutations in \(h\)\(MSH2\) and \(h\)\(MLH1\) account for ~90% of all reported MMR gene mutations, \(h\)\(PMS2\) and \(h\)\(MSH6\) account for the remainder\(^7\). However, the overall representation of \(h\)\(MSH2\) and \(h\)\(MLH1\) mutations in HNPCC families is ~50%, with equal contributions of each\(^8,9,10\). The proteins encoded by MMR genes recognize and repair DNA errors created during replication. Loss of MMR gene activity leads to an accumulation of replication errors and genetic instability, also known as the mutator phenotype\(^11\). Particularly sensitive sites for polymerase slippage errors are regions of short tandem repeat sequences, which are called microsatellites. While alterations at these sites may be benign if they occur in the non-coding regions, they create a phenotype known as microsatellite instability (MSI) indicating a defect in MMR.
function. Over 90% of HNPCC tumors show this MSI phenotype, whereas only 10-15% of sporadic CRC tumors, are MSI positive\textsuperscript{12,13,14,15}. MMR mutations lead to an increase in the rate of somatic mutations in specific cancer-related genes including the important growth regulatory genes, \textit{BAX}\textsuperscript{16}, \textit{IGFII}R\textsuperscript{17} and \textit{TGF-β type II} receptor\textsuperscript{18}. This may enhance the progression of tumorigenesis, which is hypothesized to require multiple mutations\textsuperscript{19}.

Diagnosis of HNPCC is based on the presentation of multiple CRC’s in several generations and the early onset of CRC (<50 years). These criteria, known as the Amsterdam criteria, are useful in identifying HNPCC in large families\textsuperscript{20} and reflect the presence of underlying germline \textit{hMLH1} and \textit{hMSH2} mutations\textsuperscript{21,22}. However, these criteria may be too stringent for smaller families, or when complete pedigree information is not available. In addition, the Amsterdam criteria do not account for extracolonic cancers. Since endometrial cancer is the second most common cancer associated with HNPCC, strict adherence to the Amsterdam criteria may also lead to a missed diagnosis of HNPCC. Newer guidelines, such as the Bethesda criteria\textsuperscript{23}, have been proposed in order to address these possible limitations. These criteria include HNPCC-related extracolonic cancers as well as histopathologic features of colorectal tumors for clinical diagnosis.

Hereditary predisposition to cancer often manifests in several ways, including two or more tumors arising in a single individual, the presence of many cancers in a family, and cancer diagnosis at an early age. Therefore, double primary cancers of the two principal HNPCC associated sites, the colorectum and endometrium, suggests the possibility of a diagnosis of HNPCC.
The overall aim of our study was to estimate the proportion of double primary cancers of the colorectum and endometrium, which are due to HNPCC. To accomplish this we have obtained family histories on a series of women with double primary cancers of these sites, identified through hospital and population-based registries. The results of our previous pedigree analysis demonstrated an elevated relative risk for developing cancer at these sites in first-degree relatives of double primary probands when compared to that for first-degree relatives of probands with cancer at a single site, in particular for women affected at a young age\textsuperscript{24}.

Our current objective is to extend these earlier clinical observations. In the present study, we have conducted molecular genetic analysis of \textit{hMLH1} and \textit{hMSH2} genes and microsatellite instability in this series for 40 unrelated patients affected with double primary cancers of the colorectum and endometrium.
**Materials and Methods**

**Patient Accrual**

The population-based Ontario Cancer Registry (OCR) at Cancer Care Ontario and the hospital-based tumor registry at Princess Margaret Hospital (Toronto) contain information on patients diagnosed with cancer of all sites. Identification and ascertainment of patients for this study was based primarily on these registries. Study subjects were women diagnosed with both colorectal and endometrial cancer by the age of 70 years during the period 1971-1996.

In total, 109 women were identified and confirmed. Eighty-four patients were identified by the OCR and 51 were identified through Princess Margaret Hospital. Twenty-six patients were recorded in both provincial and hospital registries. This includes 44 deceased and 65 living cases. We were able to contact 53 of the living patients. Of these, 46 agreed to provide their family histories and 40 agreed to give blood samples for molecular testing. Pedigree information was available for 10 of the deceased women. Tumor specimens were obtained for 23 of the 40 probands for whom germline mutation analysis was performed. Both endometrial and colorectal tissue was available for 15 of these cases and at least one tissue was available for the remaining 8 cases (4 endometrium, 4 colorectum).

Interviews were conducted with the 46 living probands and with the first-degree relatives of the 10 deceased probands. The interviews were conducted in person or by telephone. The family history included current age or age of death, age of diagnosis of cancer, and site of cancer in all first-degree relatives of the proband. Pathology
confirmation was obtained for 39 of the 67 reported cases of cancer (58%) in first-degree relatives. The cancer diagnosis was taken as reported by the first-degree relatives when pathological confirmation was not available. The diagnosis of cancer reported by first-degree relatives is very likely to be accurate.

Clinical data, family histories and blood samples were obtained according to the protocol approved by the University of Toronto Human Ethics Committee. Families were classified as Amsterdam criteria, strongly familial, possibly familial and non-familial (Table 1) as previously described. Genetic counseling was offered to all probands and their families before and after molecular testing.

**Mutation Analysis**

Single-stranded conformation polymorphism (SSCP) analysis was used to detect putative germline hMSH2 and hMLH1 mutations in 37 of the 40 probands. Three remaining patients overlapped with the Mount Sinai Hospital Familial Gastrointestinal Cancer Registry and had been assessed and reported independently using protein truncation test assay (PTT) according to methods described elsewhere. Patient samples showing aberrant electrophoretic patterns were further analyzed by sequencing to confirm and characterize putative mutations.

Sixteen exons of hMSH2 and 19 exons of hMLH1 were amplified individually using intronic primer sequences. Genomic DNA was extracted from blood lymphocytes using the NH4Cl-TRIS/salt precipitation method and 100-200 ng DNA was amplified in a PCR reaction volume of 15 µl with 1 µCi of [α-33P]dCTP, 1.0-2.0 mM MgCl2 and 50 ng each of the forward and reverse primer. PCR conditions included one
minute cycling at 95°C, 52-62°C and 72°C for 35 cycles. An equal volume of denaturing formamide dye was added and the products were heat denatured at 95°C for 5 min. followed by rapid cooling on ice. The denatured products were analyzed by electrophoresis through a 5% polyacrylamide gel containing 10% glycerol at 4°C (7-10 W) for 17 to 20 hours. The samples showing unique electrophoretic patterns were reamplified from genomic DNA in an independent PCR and sequenced using the Thermosequenase kit (Amersham Pharmacia Biotech, Inc., Cleveland, Ohio, USA).

MSI Analysis

DNA for MSI analysis was obtained from paraffin-embedded blocks of colorectal and endometrial tissues. All blocks were examined by a GI pathologist (CS or MR) to identify areas enriched in normal and tumor cell populations (>70% cellularity) for microdissection. Samples were deparaffinized at 95°C for 10 min., cooled to room temperature and incubated at 65°C for 12 hours with a digestion buffer (100µl) containing proteinase-K (20 mg/µl), 10mM Tris-HCL (pH 8.0), 100 mM KCL, 2.5 mM MgCl₂ and 0.45% Tween 20. Samples were then heat denatured and stored at -20°C.

Matched normal and tumor DNA was analyzed using 5 microsatellite loci including 3 dinucleotide repeats (D2S123, D5S346, D17S250) and 2 polyA repeats BAT25 and BAT26. MSI was defined by the presence of altered/additional alleles in the PCR amplified product of tumor DNA as compared to matched normal DNA samples. Tumors were designated microsatellite instability positive (MSI+) if alterations were observed in at least 40% loci (≥2 of 5) analyzed.
**Statistical Analysis**

The observed number of cases (O) in the first-degree relatives of the probands was determined by review of family pedigrees. The expected number of cases (E) for each cancer was calculated from the product of the person years and the Ontario provincial cancer rate for each age range\(^{30}\). Patients were considered to be at risk of cancer until death or 1996. The expected number of cases was calculated for each of the age ranges selected. The relative risk of cancer in the first-degree relatives of probands was estimated by comparing observed to expected numbers. The confidence intervals (CI) were calculated assuming a Poisson distribution, \(CI = O_U/E - O_L/E\), where \(O_L\) and \(O_U\) are the lower and upper bounds respectively\(^{31}\).
Results

Of the 40 cases examined, 7 were found to carry mutations in *hMLH1* or *hMSH2* (17.5%) (Table 2). The majority of these (6 of 7) were *hMSH2* mutations and all resulted in a truncated protein. One missense mutation was detected (DP# 1, Table 2), containing a germline A→T substitution at nt.# 1358 in exon 8 of *hMSH2*, which creates a novel splice site consensus sequence. By reverse transcriptase (RT)-PCR and PTT analysis, this mutation was shown to cause a 30 bp deletion spanning codons 453 to 462, leading to a truncated protein.

Overall, mutation positive probands had stronger family histories and an earlier age of diagnosis (Table 3). Five of these 7 patients with *hMLH1/hMSH2* gene mutations had strong family histories (71%) and 3 of these 7 families met the Amsterdam criteria (Fig. 1-1 to 1-7). This compares with 7 of 33 cases (21%) having strong cancer family histories among the 33 mutation negative cases (odds ratio = 9.3, P=0.02).

As well, 6 of the 7 patients with known germline mutations had been diagnosed with both colorectal and endometrial cancer by the age of 55. The other case was diagnosed with colorectal cancer at age 48, but endometrial cancer at age 65. Of the 33 mutation negative probands, 8 were diagnosed with both of these cancers by age 55. Overall, mutations were found for 6 of 14 (43%) women who had both cancers before the age of 55 years (odds ratio=18.8, P=0.004). No difference was seen between the average age of diagnosis in the living cases of our study and the deceased cases (with pedigrees) which were excluded from mutation analysis.

Tissue was available from 3 of the 7 mutation positive probands, and all 3 were MSI+ (Fig. 1-3, 1-4,1-5). Of the 20 mutation negative probands analyzed, 7 had MSI+
tumors. Strong family histories were seen in 2 of these patients, 3 were possibly familial and 2 were non-familial. Among 11 non-familial probands analyzed, 9 were MSI- (Table 4). Finally, one proband, whose pedigree met the Amsterdam criteria, did not carry an hMLH1 or hMSH2 mutation and had an MSI- tumor (Fig. 1-8).

Cancer risk estimates for first-degree relatives of double primary probands are presented in Table 5. A marked increase in relative risk is seen for cancer of the colorectum and endometrium in relatives of probands carrying hMSH2 or hMLH1 mutations. This increased risk in relatives is diminished after the age of 55. The relative risk of colorectal and endometrial cancer remains high for the mutation negative patients, specifically those diagnosed before the age of 55 years (Table 5). This increased risk is not seen in patients diagnosed over the age of 55 years.

Risks were similar for relatives of the 40 women who consented to genetic testing, compared to the total 46 women for whom pedigrees were available and consent for genetic testing was requested (Table 6).
Discussion

The development of multiple primary cancers in the same individual is uncommon and suggestive of genetic predisposition to cancer. Relatives of women with double primary cancers of the colorectum and endometrium are at an increased risk for these cancers, compared to relatives of patients with cancer at only one of these sites. This association is particularly strong for patients diagnosed at an early age. In our study, mutations were found in 6 of 14 patients when both cancers were diagnosed by age 55.

The majority of women found to carry MMR mutations in our study had strong family histories. Three of the four families in our study that met the Amsterdam criteria, had germline *hMLH1/hMSH2* mutations and as expected, had MSI+ tumors. No mutation was found in the fourth family and MSI analysis was negative. However, we have identified four additional patients with MMR gene mutations based on the presence of double primary cancer, who would not have been identified using the Amsterdam criteria alone. It is possible, that by using patient ascertainment based on colorectal and endometrial double primary cancers, we have selected for HNPCC kindreds with a strong history of extracolonic cancers (Fig. 1-2,1-4). This may also contribute to the high proportion of *hMSH2* mutations (6/7) observed in our series of patients, as *hMSH2* mutation carriers have been shown to have an increased risk for extracolonic cancers. Wijnen et al (1998) recently showed that the presence of both colorectal cancer (with an early age of diagnosis) and endometrial cancer, in a patient from a family meeting the Amsterdam criteria, is a strong predictive factor for germline *hMSH2* and *hMLH1* mutations. In their study, the mutation detection rate increased from 45%, for a kindred
meeting the Amsterdam criteria, to 90% if the kindred also included one member with both colorectal and endometrial cancer\textsuperscript{34}.

First-degree relatives of the double primary cancer patients in our study have an increased relative risk for colorectal and endometrial cancer. We found this risk to be greater for first-degree relatives of the women with MMR mutations, compared to those for whom no mutation is found. However, it is important to note that the risk for relatives of patients for whom no mutations were found remains significantly elevated. This observation is in accordance with our MSI analysis findings, which show a high rate of MSI+ (7/20) tumors in our mutation negative families. These families, particularly those with strong family histories of cancer, may contain mutations in other MMR genes, such as, \textit{hMSH6}, \textit{hPMS2} or as yet unidentified genes. Some families with weaker histories of cancer may be sporadic, having somatic \textit{hMLH1} or \textit{hMSH2} mutations. Therefore, it is likely that the actual proportion of double primary endometrial and colorectal cancers due to HNPCC is higher than that observed in the present study. A similar observation has been recently reported by Simpkins and colleagues (1998), who found 12/15 apparently sporadic MSI+ endometrial cancers were due to \textit{hMLH1} promoter hypermethylation\textsuperscript{35}.

The exclusion of deceased cases from our study raises the possibility of bias because HNPCC related cancers may have a better prognosis compared to sporadic cancer patients\textsuperscript{36}. However, there was no significant difference seen between the average age of diagnosis of the living cases and the deceased cases for which pedigrees were available (data not shown).

Based on our study we conclude that a diagnosis of double primary cancers of the colorectum and endometrium in a patient is a strong indicator of HNPCC, particularly for
those diagnosed with both cancers before 55 years of age. Physicians are advised to consider the possibility of HNPCC when a patient presents with both colorectal and endometrial cancers before the age of 55 years or has a strong family history of HNPCC associated cancers and to perform a comprehensive genetic evaluation.
Acknowledgments

We are grateful to the patients who agreed to participate in our study and the Cancer Registry at Cancer Care Ontario for making this study possible. We thank Ms. Margot Mitchell-Lehman for her excellent efforts in the initial ascertainment and recruitment of patients. This work was supported in part by NCIC grant 8034 (S.G., B.B.) and University of Toronto Open Fellowship (to A.M.).
References


Table 1

1) Familial
   a) Amsterdam criteria
      1) at least three relatives should have histologically
         verified colorectal cancer: one of them should be a
         first degree-relative of the other two
      2) at least two successive generations should be
         affected
      3) In one of the relatives colorectal cancer should be
         diagnosed under 50 years of age
      4) familial adenomatous polyposis should be
         excluded
   b) Strongly familial
      1) two or more first-degree relatives of proband
         with: colorectal cancer or endometrial cancer
         diagnosed at <55 years of age; kidney, ureteral,
         esophageal, stomach, small intestinal, pancreatic or
         ovarian cancer diagnosed at any age
         or
      2) two or more first-degree relatives of proband
         with at least one colorectal cancer diagnosed at <55
         years of age with another diagnosed at any age
         or
      3) two or more first-degree relatives of proband
         with at least one endometrial cancer diagnosed at
         <55 years of age with another diagnosed at any age
         or
      4) two or more first-degree relatives of proband
         with colorectal cancer or endometrial cancer
         diagnosed at <55 years of age with another
         colorectal cancer or endometrial cancer diagnosed
         at any age

2) Possibly familial
   1) one first-degree relative of proband with:
      colorectal or endometrial cancer diagnosed at <55
      years of age; kidney, ureteral, esophageal, stomach,
      small intestinal, pancreatic or ovarian cancer
      diagnosed at any age
      or
   2) two or more first-degree relatives of proband
      with colorectal or endometrial cancer diagnosed at
      <55 years of age

3) Non-familial
   none of the above
Table 2 – summary of mutational analysis

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<td>397</td>
<td>Del T at 1190</td>
<td>Frameshift</td>
</tr>
<tr>
<td>DP7</td>
<td>Non- familial</td>
<td>hMSH2</td>
<td>7</td>
<td>389</td>
<td>C→T at 1165</td>
<td>Arg-stop</td>
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</table>
Table 3 – summary of clinical features for mutation positive vs mutation negative probands

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>Mutation Positive (n=7)</th>
<th>Mutation Negative (n=33)</th>
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</thead>
<tbody>
<tr>
<td>Age of Diagnosis of Colorectal Cancer</td>
<td>44.9 yrs</td>
<td>54.8 yrs</td>
</tr>
<tr>
<td>Age of Diagnosis of Endometrial Cancer</td>
<td>49.7 yrs</td>
<td>56.8 yrs</td>
</tr>
<tr>
<td>Amsterdam Criteria</td>
<td>3</td>
<td>1</td>
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<tr>
<td>Strongly Familial</td>
<td>2</td>
<td>6</td>
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<tr>
<td>Possibly Familial</td>
<td>1</td>
<td>10</td>
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<tr>
<td>Non-familial</td>
<td>1</td>
<td>16</td>
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Table 4 - summary of microsatellite instability status vs. clinical features for mutation positive and mutation negative probands (n=23)

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Case #</th>
<th>Mut+/−</th>
<th>MSI+/−</th>
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<tbody>
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<td>Amsterdam Criteria</td>
<td>DP3</td>
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<td>+</td>
</tr>
<tr>
<td>DP5</td>
<td>hMSH2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>DP33</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>Strongly Familial</td>
<td>DP4</td>
<td>hMSH2</td>
<td>+</td>
</tr>
<tr>
<td>DP26</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>DP31</td>
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<td>+</td>
<td></td>
</tr>
<tr>
<td>DP36</td>
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</tr>
<tr>
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<td>DP13</td>
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<td>DP35</td>
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<td>+</td>
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</tr>
<tr>
<td>DP17</td>
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</tr>
<tr>
<td>DP16</td>
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<tr>
<td>Non-familial</td>
<td>DP10</td>
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<td>DP29</td>
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<td>DP32</td>
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<td>DP34</td>
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<tr>
<td>Cancer Site</td>
<td>Age of Diagnosis</td>
<td>Mutation positive (n=7)</td>
<td>Mutation negative (n=33)</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------</td>
<td>-------------------------</td>
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</tr>
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<td>CI</td>
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<tr>
<td>Any cancer</td>
<td>&lt;55</td>
<td>5.4</td>
<td>2.9-9.3</td>
</tr>
<tr>
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<td>≥55</td>
<td>1.3</td>
<td>0.5-2.7</td>
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<tr>
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<td>1.6-4.1</td>
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<td>≥55</td>
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<td>12.6-183.4</td>
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<td>0.1-46.3</td>
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<td>6.4-61.0</td>
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Table 6—relative risks for genetically tested vs total living probands

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<tr>
<th>Cancer Site</th>
<th>Age of Diagnosis</th>
<th>Genetically tested probands (n=40)</th>
<th>Total living probands in study (n=46)</th>
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<tbody>
<tr>
<td></td>
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<td>RR</td>
<td>CI</td>
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<td>1.0-1.7</td>
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<tr>
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<td>5.9-19.1</td>
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<tr>
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<td>0.6-9.1</td>
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<td>Any age</td>
<td>7.8</td>
<td>3.7-14.4</td>
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</tbody>
</table>
Figure 1:

Pedigrees for mutation positive cases #1-7. #2 is an HNPCC/Muir-Torre kindred. #8 is a mutation negative family that met Amsterdam criteria. Blood samples were obtained from probands for each family and analyzed for germline hMSH2 and hMLH1 mutations. Probands are indicated by an arrow (→). Affected and unaffected members are shown as solid and open symbols respectively. Cancer site(s) and age of diagnosis are indicated. Bl, bladder; bn, brain; bo, bowel; br, breast; co, colorectum; en, endometrium; es, esophagus; lu, lung; ov, ovary; pa, pancreas; pr, prostate; psu, primary site unspecified; st, stomach; th, throat; ur, ureter; wt, Wilm's tumor.