THE ROLE OF *MSH6* MUTATIONS IN NORTH AMERICAN PATIENTS RECEIVING CLINICAL GENETIC TESTING FOR HEREDITARY NONPOLYPOSIS COLORECTAL CANCER

by

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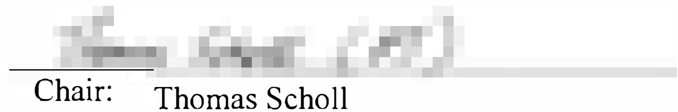

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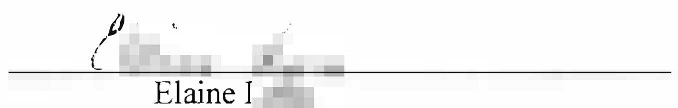

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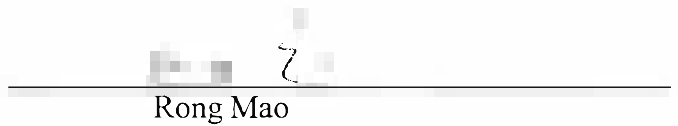

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ABSTRACT

Hereditary nonpolyposis colon cancer (HNPCC) is caused by mutations in a group of genes, *MLH1*, *MSH2*, *MSH6*, *MSH3*, *PMS1* and *PMS2*, which function in the mismatch repair (MMR) pathway. *MSH6* mutations account for approximately 11% of disease causing mutations in HNPCC families, but only a few of these families fulfill the Amsterdam/Bethesda criteria for patient selection for clinical genetic testing. It is difficult to determine the prevalence of *MSH6* mutations in the HNPCC patient population, since reported mutation rates vary greatly among studies in accordance with selection criteria. The majority of the genetic defects in MMR genes result from point mutations, but genomic rearrangements also account for a significant portion, which might escape detection by using conventional diagnostic techniques.

To understand the role of *MSH6* mutations in North American patients receiving HNPCC clinical genetic testing, a consecutive set of 406 samples were made anonymous and screened for mutations in *MSH6*, *MLH1* and *MSH2*. The spectrum of point mutations versus rearrangements across these genes is also determined by augmenting DNA sequencing with MLPA and Southern blot analysis. From using MLPA probe kit P008 *MSH6/PMS2* of MRC-Holland, the deletion of the entire *PMS2* gene was identified in a patient with colorectal cancer.

Sixty-four deleterious mutations and 38 genetic variants of uncertain clinical significance (VUS) in *MLH1* and *MSH2* were identified by DNA sequencing in the

original set of samples. From this set, 279 specimens were subjected to *MSH6* mutation testing by DNA sequencing for the entire coding region and consensus splice sequences adjacent to the exons. Six protein truncating mutations and 8 missense VUS were detected in *MSH6*. *MLH1* and *MSH2* mutations accounted for 89% of total mutations detected in HNPCC in this sample set. Of these mutations, 25% were rearrangements in *MLH1* and *MSH2* detected by Southern blot analysis. *MSH6* mutations accounted for 11% of total mutations detected in HNPCC in these samples. There were no rearrangement mutations detected in *MSH6* by MLPA analysis. Four of the 6 *MSH6* mutations were identified in samples from patients affected with endometrial cancer.

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CHAPTER I

INTRODUCTION

Hereditary nonpolyposis colon cancer (HNPCC) is an autosomal dominant condition characterized primarily by increased risk of colon cancer, and cancer of other tissues including the endometrium, ovary, stomach, small intestine, hepatobiliary tract, upper urinary tract, brain, and skin. The average age of diagnosis is the early to mid 40s, but tumors can emerge in individuals in their early 20s (Baudhuin 2004). HNPCC is one of two prevalent hereditary cancer syndromes, the other being familial adenomatous polyposis (FAP), which is caused by mutations in the APC gene. Patients affected with HNPCC do not present with large numbers of colonic polyps (sometimes many hundreds or even thousands), which occur in FAP.

Individuals with HNPCC carry an 80% lifetime risk for developing colon cancer and two-thirds of these cancers arise in the proximal colon (Kohlmann and Gruber 2004). HNPCC-related colon cancers carry common histopathological features that include tumor infiltrating lymphocytes, mucin, poor differentiation, signet ring or cribform histology, and the average age of diagnosis is 44 years of age (Kohlmann and Gruber 2004). The second most common cancer in HNPCC individuals is endometrial cancer. Women with HNPCC carry a 20-60% lifetime risk for the development of this cancer

(Kohlmann and Gruber 2004). The average age of diagnosis in individuals with HNPCC-related endometrial cancer is 46 (Kohlmann and Gruber 2004).

The most frequently reported pathology of HNPCC-related gastric cancers is intestinal-type adenocarinoma. The mean age of diagnosis is 56 years of age. The pathology of HNPCC-related ovarian cancer is analogous to that presented in sporadic ovarian cancers. The average age of diagnosis is 42, but 30% of these diagnoses are reported before the age of 40. Other HNPCC-related cancers that present with characteristic features include urinary tract cancers that are transitional carcinomas of the ureter and renal pelvis, also small bowel cancers. A majority of these cancers are adenocarcinomas of the duodenum and jejunum. Finally, the most common type of central nervous system tumor is glioblastoma is also associated in HNPCC patients. Breast cancer, laryngeal cancer, and hematological cancers have also been reported in HNPCC families, but a direct association of these cancers to HNPCC has not yet been demonstrated (Kohlmann and Gruber 2004).

Genetics

HNPCC is caused by mutations in a group of genes that function in the mismatch repair (MMR) pathway; *MLH1*, *MSH2*, *MSH3*, *MSH6*, *PMS1* and *PMS2* (Table 1). The role of the proteins encoded by these genes is well characterized in bacteria. The human genes show homology with their bacterial counterparts and additional orthologs exist in humans. The MMR proteins interact to form complexes that identify and repair mismatched bases and small insertion/deletion mutations within DNA.

Table 1

Human Mismatch Repair Genes

Bacterial MMR Homolog	Human Gene	Chromosomal Localization*	Germline Mutations
	hMSH2	2p16	Yes
	hMSH6	2p16	Yes
	hMSH3	5q14	Not Reported
MutL	*hMLH1*	3p21	Yes
	hPMS1	2q31-33	Yes
	hPMS2	7p22	Yes

*Chromosomal localization in humans

Adapted from Markwitz S. 2000. DNA repair defects inactivate tumor suppressor genes and induce hereditary and sporadic colon cancers. Journal of Clinical Oncology 18:75s-80s.

Discovery of MMR Genes and Their Role in HNPCC

Several HNPCC families meeting the Amsterdam criteria participated in linkage studies to define the genomic mapping of associated genes. In May 1993, positive LOD scores with DNA markers mapped to chromosome 2p was reported in these families (Peltomaki et al. 1993). In November 1993, it was shown that there was a linkage to chromosome 3p in 3 Swedish HNPCC families (Lindblom et al. 1993). It was later shown that a majority of these tumors displayed high microsatellite instability (MSI) that was previously studied in bacteria and yeast (Strand et al. 1993) to lead to positional cloning strategies identifying the human homologue for the MutS gene (*hMSH2*) on chromosome 2p22-21 (Fishel et al. 1993, Leach et al. 1993) followed by the identification of the human homologue of the MutL gene (*hMLH1*) on chromosome 3p (Bronner et al. 1994, Papadopoulos et al. 1994).

In the study by Leach et al., chromosome microdissection was used to obtain highly polymorphic markers from chromosome 2p16. These and other markers were ordered in a panel of somatic cell hybrids and used to define a 0.8 Mb interval containing the HNPCC locus. Candidate genes were then mapped, and one was found to lie within the 0.8 Mb interval that was homologous to *MutS* mismatch repair genes. cDNA clones were obtained and the sequence was used to detect germline mutations in *MSH2*, including those producing termination codons, in HNPCC kindreds. Somatic as well as germline mutations of *MSH2* were identified in tumor cells with high MSI (Leach et al. 1993).

In the study by Fishel et al., the expression of *hMSH2* in E. coli causes a dominant mutation phenotype, suggesting that *hMSH2* interferes with the normal mismatch repair

pathway. A T to C transition mutation was detected in the −6 position of a splice acceptor site in sporadic colon tumors and in two small HNPCC kindreds. These data show that S. cerevisiae *MSH2* mutations cause instability of dinucleotide repeats like those associated with HNPCC to show that *MSH2* is one of the genes responsible for HNPCC (Fishel et al. 1993).

hMLH1, *hPMS1*, and *hPMS2* were identified by having significant similarity to the yeast *MutL* gene and the yeast *MutL* homolog *PMS1* (Prolla et al. 1994, Kramer et al. 1989). Somatic cell hybridization studies localized these genes on chromosomes 3, 2, and 7 (Papadopoulos et al. 1994). In the study by Papadopoulos et al., the precise chromosomal location of *hMLH1* was determined by FISH to locate the gene within band 3p21.3. The relationship between *hMLH1* and the HNPCC locus was determined by physically mapping the region to show the responsible locus is centered at markers D3S1611 and D3S1277. A yeast artificial chromosome (YAC) clone was identified that contained both *hMLH1* and marker D3S1611 to show the gene is within 1cM of the HNPCC locus. The derived marker D3S1611 was found to be located in an intron of *hMLH1*. Mutations of *hMLH1* that would disrupt the gene product were found in affected individuals of HNPCC kindreds to reveal that the gene is responsible for the disease (Papadopoulos et al. 1994).

Function of the MMR System

In *E. coli*, MutS and MutL proteins are involved in two main repair pathways, the methyl-directed long-patch and the very short-patch pathway (VSP). The methyl-directed pathway corrects base-base mismatches, small insertions and deletions that occur during DNA replication (Peltomaki 1997). The VSP pathway functions by correcting G-

T mismatches in nonreplicating DNA resulting from deamination of 5'methlycytosine residues (Peltomaki 1997).

The initial step of this repair process is the binding of MutS to the mismatch base followed by the additional binding of MutL. The formation of this complex activates the binding of MutH, an endonuclease, which makes an incision at a GATC site with an unmethylated adenine located 1 to 2-kb from either side of the mismatch (Peltomaki 1997). The segment containing the mismatch is cut by a 3'-5' or 5'-3' exonuclease and restored with a new segment produced by DNA polymerase (Peltomaki 1997). In yeast, *MLH1* and *PMS1* bind together to form a heterodimer after the detection of the mismatch by *MSH2*, which is followed by the formation of a ternary complex, *MLH1*, *PMS1*, and *MSH2*, that recruit further proteins to repair mismatches as seen in *E. coli* (Peltomaki 1997).

In humans, it is thought that the MMR system operates in a similar way. Unlike *E. coli*, human cells have at least 16 genes that specify MutL-like proteins (Peltomaki 1997). *MSH2* and *MSH6*/*MSH3* make up the mismatch-binding factor in humans. *MSH2* proteins bind to DNA containing base-base mismatches and to substrates containing from one to 14 extra bases. Several studies of yeast strains containing *MSH2*, *MSH3*, and *MSH6* mutations (Strand et al. 1995, Johnson et al.1996, Marsischky et al. 1996, Greene and Jinks-Robertson 1997, Sia et al. 1997) suggest that *MSH2* and *MSH6* are mainly responsible for repairing base-base mismatches. *MSH2* can participate with either *MSH6* or *MSH3* to repair one-and two-base insertion/deletion mismatches. The *MSH2*/*MSH3* heterodimer (MutSβ) is thought to repair insertion/deletions involving repeating units of four to 16 bases (Figure 1). Following recognition of a mismatch by *MutS* related

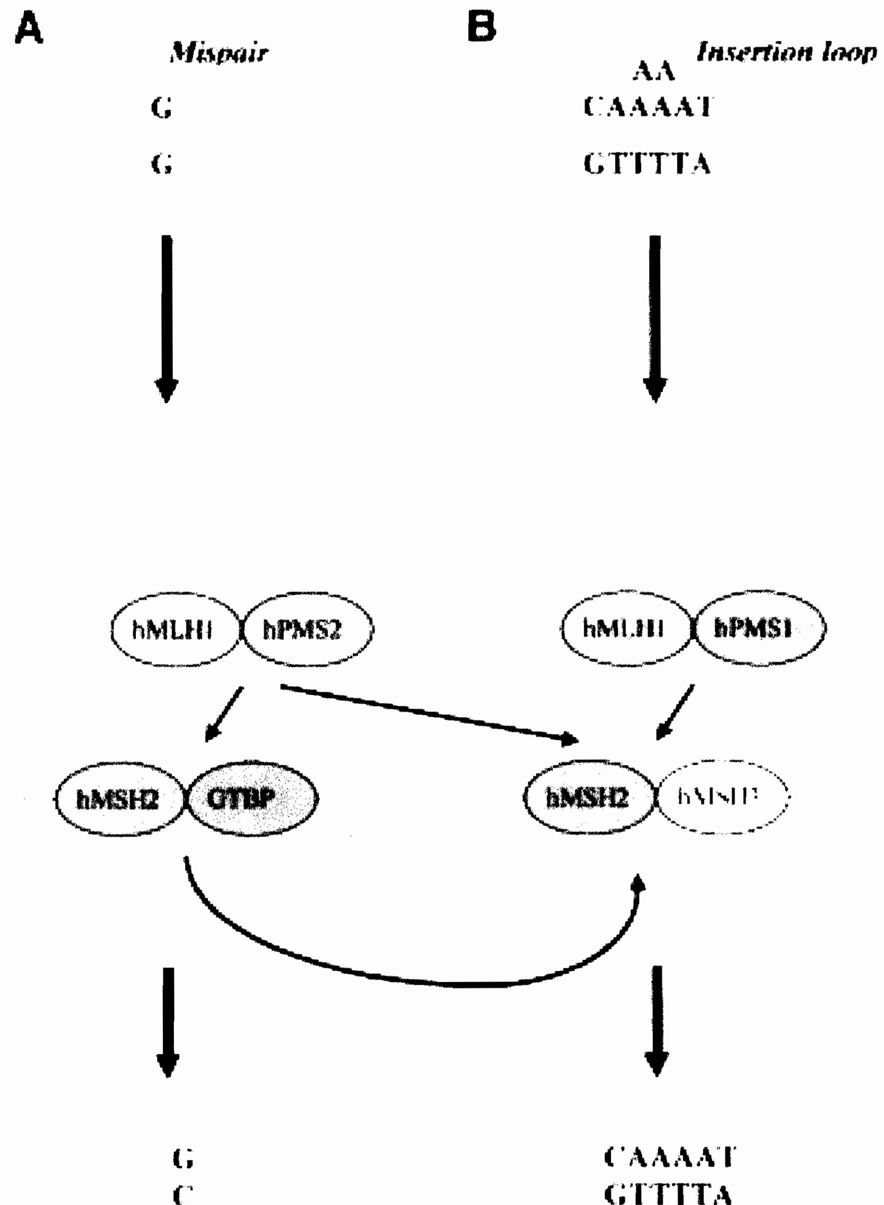

Figure 1. Mismatch Repair Mechanism. A mispaired base is recognized by the hMSH2/GTBP(*MSH6*) complex while an insertion/deletion loop is recognized by the hMSH2/hMSH3 complex. MutL related proteins (hMLH1/hPMS2 and hMLH1/hPMS1 complexes) then interact with the MutS related proteins that are already bound to the mispaired bases.

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proteins, a heterodimeric complex of the *MutL* related proteins (*MLH1*/*PMS1*, *PMS2* in humans) interacts with the *MutS* related proteins bound to the mismatches (Prolla et al. 1994, Li et al. 1995). *MLH1*/*PMS1* binds to the *MSH2*/*MSH3* mismatched complex to increase the efficiency of *MutS* related proteins to recognize a mismatch (Habraken et al. 1997). *MLH1* also forms a complex with *PMS2* to play a role in the repair of insertion/deletion mispairs in the *MSH2*/*MSH3* pathway (Flores-Rozas et al. 1998). MLH1 protein also dimerizes with PMS2 to direct the binding of other proteins including EXO1, helicases, proliferating cell nuclear antigen (PCNA), single-stranded DNA-binding protein (RPA), and DNA polymerase (Kohlmann and Gruber 2004).

There are additional proteins involved in mismatch repair that include DNA polymerase δ, replication protein A, proliferating cell nuclear antigen (PCNA), replication factor C, exonuclease 1, FEN1, and DNA polymerase δ and ε associated exonucleases (Syngal et al. 1999). The "clamp" protein, PCNA, is required to stabilize the *MutS* and *MutL* heterodimers at mismatch sites and during the DNA synthesis step of mismatch repair. After the mismatch is recognized and the complexes have formed, the degradation of the mismatch bases is removed by exonuclease (with assistance from helicase II and SSB proteins) that is initiated from a nick located 1-2 kilobases from the mismatch bases. The resulting excision tract is restored by DNA polymerase.

It may seem that *MSH2* and *MLH1* play equal roles in the repair of single base mismatches, but *MSH2* plays a larger role in the repair of loops of five or more unpaired bases (Peltomaki 1997). Human cells have the ability to repair large loops, which is important because human DNA contains a lot of microsatellites that may produce these loops (Peltomaki 1997).

There might be some redundancy in the functions of the MSH2-MSH3 and MSH2-MSH6 heterodimers. This redundancy could explain the reduced severity of *MSH6* mutations that are believed to be associated with lower penetrance and later age of onset when compared with mutations in *MLH1* or *MSH2*. It is possible that loss of *MSH6* function is mitigated by *MSH3* in the repair of one- and two-base insertion/deletion mismatches.

The Prevalence of Mutations in MMR Genes in HNPCC

While all MMR genes could play a potential role in hereditary risk for colon cancer, most germline mutations identified to date occur in *MLH1* on chromosome 3p22.3 and *MSH2* on chromosome 2p21 (80-90%) (Kohlmann and Gruber 2006). Mutations in additional genes involved in MMR, *MSH6* on chromosome 2p16.3, *MSH3* on chromosome 5q14.1 and *PMS1* and *PMS2* on chromosome 2q32.2 and 7q22.1, are associated with HNPCC, but probably play lesser roles that are defined through ongoing research studies (Hedge et al. 2005).

PMS2 mutations are rare and have been reported in a few families with cancer (Nicolaides et al. 1994, Hamilton et al. 1995, Miyaki et al. 1997, De Rosa et al. 2000, Trimbath et al. 2001), including the identification of four rearrangements in *PMS2* (van der Klift et al. 2005). It has been shown that *PMS2*-mutated human cells display a mutation rate equivalent to or more than that of *MLH1*-mutated human cells (De Vos et al. 2004). The existence of several pseudogenes corresponding to the first five exons, exon 9 and exons 11-15 of *PMS2* could interfere with mutation detection resulting in under diagnosis (De Vos et al. 2004, Nakagawa et al. 2004).

The mechanism through which *PMS2* mutations confer susceptibility to HNPCC-related cancers is still unknown. It has been suggested from recent studies that *PMS2* plays a more important role in HNPCC than previously thought in which the MutLα heterodimer formed by *MLH1* and *PMS2* proteins is a major component of the MMR complex (Nakagawa et al. 2004).

Pseudogenes and *PMS2*

Pseudogenes are a faulty segment of DNA that resembles a known functional gene that have lost their protein-coding ability or are no longer expressed in the cell (pseudogene.org 2007). The first pseudogene was reported in 1977 (Jacq et al. 1977). Since that time most of these pseudogenes have been discovered in humans and other species (Pitman 2004). Three main types of pseudogenes exist, each with distinct mechanisms of origin and distinct characteristic features.

Nonprocessed or duplication pseudogenes are the first classification of pseuodgenes. These genes occur by modification to the DNA sequence of a gene during duplication by mutations, insertions, deletions or frameshifts to result in loss of gene function at the transcription or translation level (Figure 2) (Pseudogene.org 2007). Duplicated pseudogenes usually have all the same characteristics of functional genes, including an intact exon-intron structure and promoter sequences (Pseudogene 2007). The loss of the duplicated gene's function usually has little effect on the organism, since an intact functional copy still exists. Examples of nonprocessed pseudogenes are present in the alpha-globin and beta-globin gene families (Hardison and Miller 1993).

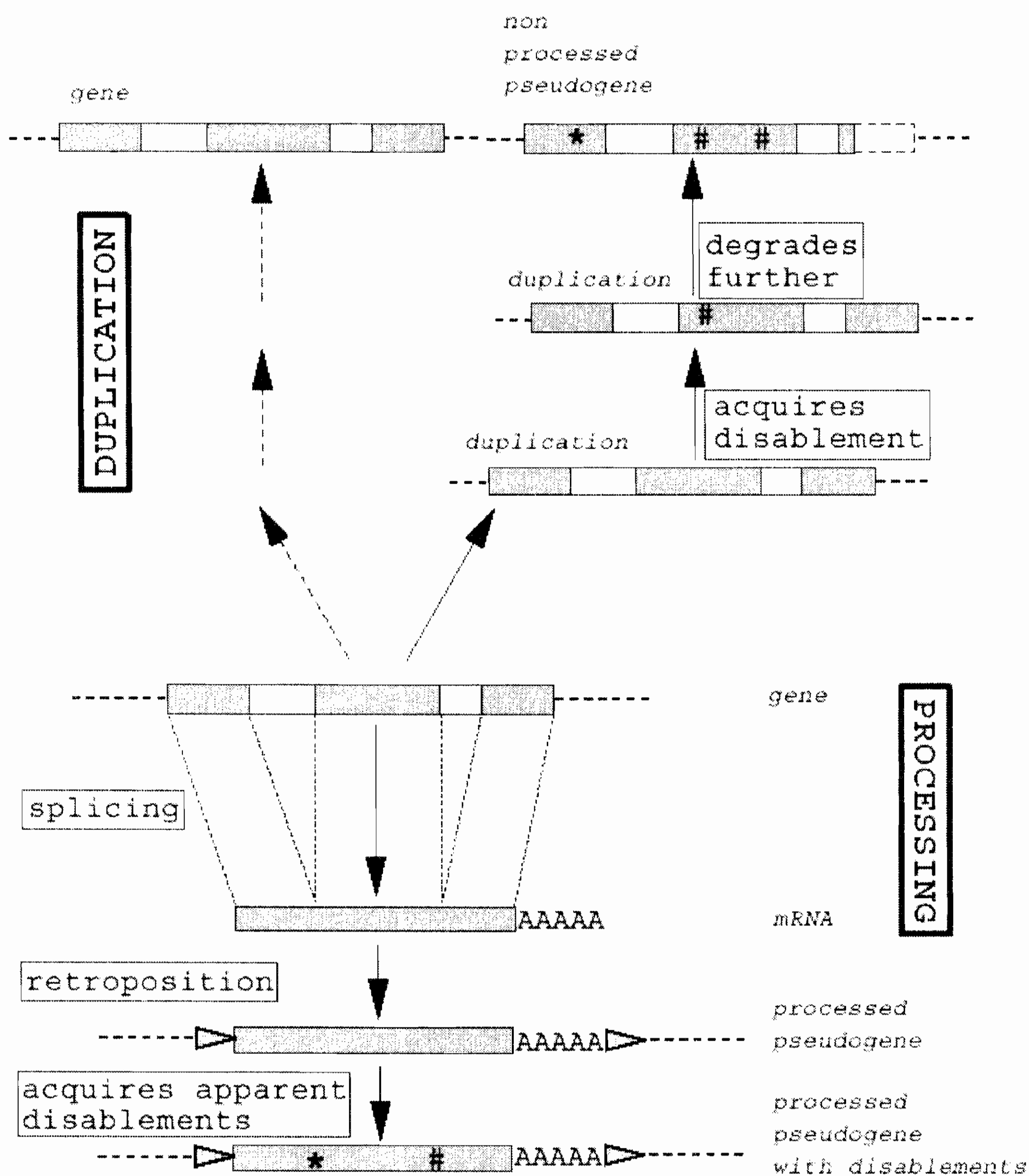

Figure 2. Mechanism of Processed and Duplication Pseudogene Occurrence

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Processed pseudogenes are the second classification of pseudogenes. These genes arise by reverse transcription of an mRNA transcript followed by reintegration of the cDNA into the genome (Pseudogene.org 2007). They are called "processed" because these genes have been spliced and lack introns (Gibson 1994). These genes often have poly (A) tails at their 3'-end, lack promoter sequences and are flanked by short direct repeats. The L1 family of repetitive DNA sequences appears to be the result of this process (Jurka 1989).

Disabled genes or unitary pseudogenes are the third classification of pseudogenes. These genes arise by the same mechanism by which nonprocessed genes become deactivated, but the only difference is that these genes were not duplicated before becoming disabled (Wikipedia 2007).

A novel *PMS2* related gene was identified in chromosome 7p22-23. The gene contains an identical sequence (97%) to exon 9 and exons 11-15 of *PMS2* (Nakagawa et al. 2004). It was shown by Western Blot that the transcript is not translated into a protein, which could be due to a 2-bp deletion and 1-bp insertion in the exon corresponding to exon 11 of *PMS2* (Nakagawa et al. 2004). Other paralogous sequences (at least 13) resemble the 5'end of *PMS2* that extend over exons 1-5 (Nakagawa et al. 2004).

Microsatellite Instability in HNPCC

Mutations in *MLH1* and *MSH2* are associated with a tumor phenotype exhibiting increased genomic instability, characterized by changes in repeat numbers of simple repetitive sequences also referred to as microsatellite instability (MSI). Microsatellites

are regions of DNA (1-5 base pairs, usually repeated 15-30 times) with a repetitive sequence of nucleotides (Jover et al. 2004), which are susceptible to errors during DNA replication that remain nonrepaired in MMR defective cells. Approximately 100,000 microsatellite repeats spread throughout the human genome (Peltomaki 1997) are susceptible to insertions or deletions. A group of two mononucleotides markers, BAT25 and BAT26 and three dinucleotide repeat markers, D2S123, D5S346, and D17S250, has been recommended by the National Cancer Institute to determine MSI in tumor and normal tissues (Umar et al. 2004). A tumor is classified as MSI-high if two or more of the microsatellite markers show instability and is classified as MSI-low if one of the microsatellite markers shows instability (Jagadeesh et al. 2003).

About 90% of familial colon cancers that meet the Amsterdam Criteria are MSI-high (Kohlmann and Gruber 2006). Mutation rates in these repetitive sequences are about 100 times greater in mutated *MLH1* and *MSH2* cells compared with MMR proficient cells (Chao et al. 2006). Most of the *MLH1* and *MSH2* mutations cause MSI-high tumors, but *MSH6* mutations can display an MSI-low phenotype. Functional redundancy in the DNA mismatch repair pathway could explain the MSI-low phenotype of *MSH6* mutations when compared with *MLH1* and *MSH2* (Buttin et al. 2004).

Genomic Rearrangements

The majority of the genetic defects in MMR genes result from point mutations, but genomic rearrangements also account for a significant portion of mutations in HNPCC (van der Klift et al. 2005). Rearrangement mutations are especially prevalent in *MSH2*. It has been shown that rearrangement mutations account for about 30-50% of *MSH2* mutations in HNPCC (Ball et al. 2003). These rearrangements fail detection in

conventional PCR-based methods, such as Denaturing High Performance Liquid Chromatography (DHPLC), Single Strand Conformation Polymorphism (SSCP), and direct DNA sequencing, that are effective at finding single-base substitutions, small deletions and insertions, but are insensitive to large gene rearrangement detection.

Mechanisms of Rearrangements

Four general mechanisms of rearrangements are found in DNA; replication slippage, intrachromosomal crossover event, single strand annealing, and unequal crossing over between sister chromatids. In the first mechanism, replication slippage, DNA polymerase detaches from the DNA strand and reanneals to nearby homologous sequences to result in small deletions or insertions usually seen in microsatellite regions (Figure 3).

There are two mechanisms that results in intrachromosomal deletions. In the first mechanism, intrachromosomal crossover event, homologous sequences are aligned then followed by a break in a single strand to allow strand exchange and recombination between the two homologous sequences. In the second mechanism, single strand annealing, a double strand break forms in a nonhomologous segment between repeats or within a single repeat element (Figure 4). In the double strand break, DNA degradation of the single strands from the 5' exposed ends occurs to result in single stranded segments that anneal to each other followed by processing of the 3' tails and ligation of the nicks to produce the deletions.

The final mechanism results in interchromosomal rearrangements. This incident is mediated by unequal crossing over between misaligned homologous segments on homologous chromosomes or sister chromatids resulting in two reciprocal chromosomal

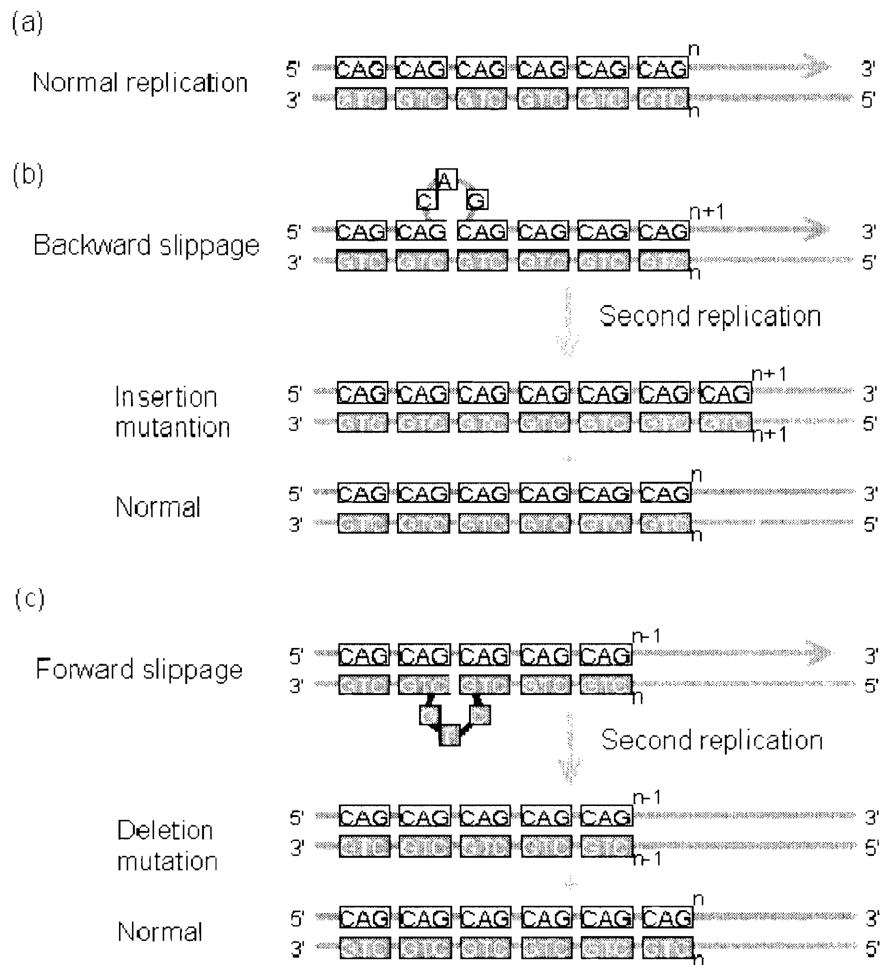

Figure 3. Mutations Caused by Replication Slippage (a) Normal replication. (b) Backward slippage, resulting in the insertion mutation. (c) Forward slippage, resulting in the deletion mutation

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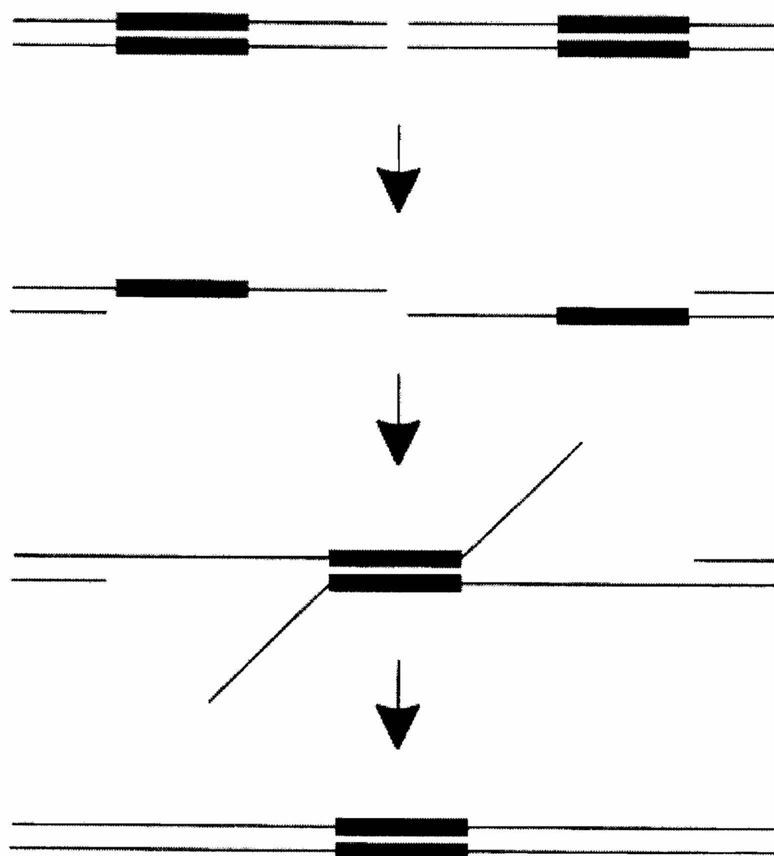

Figure 4. Single-Strand Annealing Model. When a double-strand break is created in vivo, one strand on each side of the DSB is resected in the 5'-to-3' direction, leaving a 3' tail. When complementary sequences on opposite sides are exposed, they can anneal, forming a branched intermediate. The single-stranded tails are removed by a nuclease, the gaps are filled in, and any remaining nicks are ligated, resulting in a deletion product.

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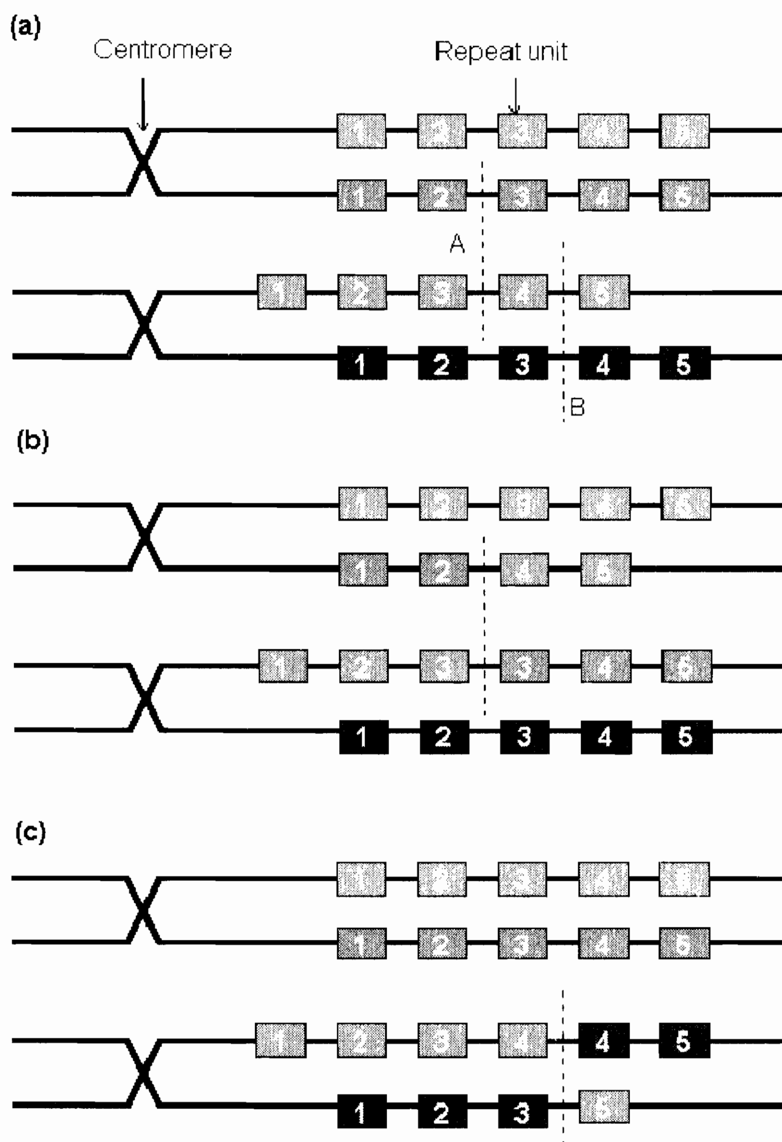

Figure 5. Unequal Crossover and Sister Chromatid Exchange. (a) Two pairs of sister chromatids line up during meiosis. A repetitive region of one chromatid does not line up exactly with its corresponding region in other chromatids.
(b) Strand breaks on nonsister chromatids (along line A) will result in unequal crossover, producing different number of repeat units in these chromatids.
(c) Strand breaks on sister chromatids (along line B) also produce different repeats. In this case, it is called sister chromatid exchange.

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products: one contains a duplication of the region located between the two sites and the other contains a duplication of the region located between the two sites and the other contains a deletion that covers the same exact region (Figure 5). The size of the duplicated region can vary from a few base pairs to tens or even hundreds of kilobases.

Rearrangement Detection Methods

Gene rearrangements have been detected by many methods including Fluorescent In Situ Hybridization (FISH), Southern blot analysis, and PCR amplification assays. The principle of FISH assay is the hybridization of fluorescent probes to denatured complementary target DNA. The hybridized sequences are observed by fluorescent microscopy. It is one of the methods used in the detection of large rearrangements (Honda et al. 2000, Elcioglu et al. 2000). Three cryptic and one complex BCR/ABL 1 rearrangements were detected by FISH assay, which were not visible by conventional cytogenetics (Pelz et al. 2002).

Southern blot assay is another method used to detect large rearrangements (Nordling et al. 1998, Puget et al. 1997, Swensen et al. 1997). The DNA is digested with a restrictive enzyme and separated by gel electrophoresis. The DNA is denatured by NaOH and transferred to nitrocellulose or nylon membrane. The blot is incubated with many copies of a labeled probe to hybridize to the complementary DNA sequence. The pattern of hybridization is visualized by autoradiography or by non-radioactive visualization by colorimetric, fluorescent, or chemiluminescent detection of the probes. The probe reveals the fragments that contain the target DNA sequence and can be mapped back to the genomic sequence and region where the deletion or duplication occurred.

Forty-eight genomic rearrangements in the MMR genes were identified in a cohort of 439 HNPCC families by Southern blot analysis (van der Klift et al. 2005). Twenty-nine of these mutations were found in *MSH2*, 13 in *MLH2*, 2 in *MSH6* and 4 in *PMS2*. In another study, a 2.1-kb deletion of exon 1 in *MSH2*, along with a 5.4-kb deletion of exon 2, a 2.2-kb deletion of exon 3, and a 13-kb deletion of exon 6 was identified by Southern blot analysis (Wijnen et al. 1998).

PCR-based amplification assays have also been used for the identification of rearrangements. These assays are rapid and versatile in vitro methods for amplifying defined target DNA sequences within a source of DNA. One successful assay for the detection of rearrangements is long range PCR. This method involves the use of two polymerases, a nonproofreading polymerase and a proofreading polymerase with 3' to 5' exonuclease activity to remove mismatched bases to achieve the amplification of large DNA fragments. PCR amplification will produce a smaller fragment than the wildtype DNA if a deletion is present or a larger fragment than the wild type if a duplication is present. The deletion of the promoter region of exon 1 and exon 2 in *MSH6* and the duplication of the 3' end of exon 4 and exon 5 in *MSH6* was detected and characterized by long range PCR and DNA sequencing (Plaschke et al. 2003). In another study, thirteen different genomic rearrangements of *MSH2* were confirmed and characterized by long range PCR (Carbonnier et al. 2002).

Southern blotting, the most commonly used procedure to detect genomic deletions and duplications, is offered by a majority of laboratories performing clinical testing for HNPCC patients. However, this technique suffers from major limitations that include the requirement of large amounts of DNA, the need for expert analysis and is time

consuming and laborious. Solutions to these problems have been proposed in the form of assays that detect rearrangement mutations by quantitative PCR.

One assay in particular, multiplex ligation probe amplification (MLPA), is gaining rapid adoption since it has all of the advantages of the PCR-based assays using a single PCR primer set to increase PCR inefficiencies, and is available as a commercial kit. MLPA is a new technique that allows the quantification of multiple nucleic acid sequences in one reaction tube (www.mlpa.com). In MPLA, a mixture of different sized probes are added to the samples and allowed to hybridize overnight to adjacent target sequences then amplified by PCR and quantified. Amplified products are separated by electrophoresis in which the relative amounts of probe amplification products are proportional to the relative copy number of the target sequence.

Thirty-eight germline mutations were detected in 37 of the 126 colorectal cancer families by MLPA. Thirteen genomic deletions were identified in *MSH2* and 4 in *MLH1*, including the deletion of the entire *MLH1* gene detected in two families (Gille et al. 2002). In another study, 12 cases with deletions of one or more exons, six deletions in *MLH1* and six deletions in *MSH2* were identified by MLPA (Taylor et al. 2003).

Genetic Testing

Patient Selection by Family History

Genetic testing for mutations in the genes that cause these cancer syndromes improves patient management. Currently, genetic testing for both HNPCC and FAP is used primarily to confirm diagnoses. Confirmed mutation carriers affected with cancer receive recommendations for more aggressive surgery (American Gastroenterology Association 2003). Unaffected mutation carriers are recommended to undergo increased

cancer surveillance by colonoscopy every 1-2 years (American Gastroenterology Association 2003). Patients that do not carry a known familial mutation can receive surveillance appropriate for the general population. These patients can forgo yearly colonoscopy procedures that are uncomfortable and expensive (American Gastroenterology Association 2003).

Amsterdam Criteria

Genetic testing for HNPCC involves complex and expensive whole-gene mutation scanning and thus is inappropriate for screening the general population. Presently, the most important criteria for selecting patients for clinical genetic testing are based upon family cancer history. In 1990, the International Collaborative Group on HNPCC established the Amsterdam Criteria to identify HNPCC families for research studies. These criteria include three or more family members (one member who is a first degree relative of the other two) with a confirmed diagnosis of colorectal cancer or HNPCC-related cancers, two successive affected generations, one or more colorectal cancers or HNPCC-related cancers diagnosed under 50 years of age, and the exclusion of FAP (Kohlmann and Gruber 2004). These criteria were later modified in the Amsterdam II Criteria to include other HNPCC-related cancers and to recommend that the tumors are verified by a pathological examination (Figure 6).

Bethesda Criteria

In 1997, the Bethesda guidelines were formulated to expand the identification of MMR gene mutation carriers (Berends 2003). These criteria include individuals with colorectal cancer family history that meet the Amsterdam criteria, individuals 45 years

Amsterdam I Criteria

- Three or more relatives diagnosed with colorectal cancer, 1 of whom is a first degree relative of the other 2
- Colorectal cancer involving at least 2 generations
- One or more colorectal cancer diagnosed at 50 years or older

Amsterdam II Criteria

- Three or more relatives diagnosed with HNPCC related cancers, 1 of whom is a first degree relative of the other 2
- Colorectal cancer involving at least 2 generations
- One or more colorectal cancer diagnosed at 50 years or older

Modified Bethesda Criteria

- Individuals in families that meet Amsterdam criteria
- Individuals with 2 HNPCC-related cancers including synchronous and metachronous colorectal cancers or associated extracolonic cancers
- Individuals with colorectal cancer and a first degree relative with colorectal cancer and/or HNPCC-related extracolonic cancer and/or colorectal adenoma; 1 of the cancers diagnosed at age< 50 years and the adenoma diagnosed at age <40 years
- Individuals with colorectal cancer and endometrial cancer diagnosed at age < 50 years
- Individuals with right sided colorectal cancer with an undifferentiated pattern on histology diagnosed at age <50 years
- Individuals with signet ring cell type colorectal cancer diagnosed at age <50 years
- Individuals with adenomas diagnosed at age <40 years

Figure 6. Summary of Diagnostic Criteria for Hereditary Nonpolyposis Colorectal Cancer

Adapted from Jagadeesh D., Syngal S. 2003. Genetic testing for hereditary nonpolyposis colorectal cancer. Current Opinion in Gastroenterology 19(1):57-63.

old or younger with colorectal cancer, or 45 years old or younger with endometrial cancer, or 40 years old or younger with a colorectal adenoma, individuals with colorectal cancer and a first-degree relative with colorectal cancer or an HNPCC-related cancer, 45 years or younger with one of the cancer diagnosed or HNPCC-related cancers and individuals with two HNPCC-related cancers, including synchronous and metachronous colorectal cancer.

These guidelines were later revised stating that patients need to meet one of these criteria: diagnosed with colorectal cancer before the age of 50 years, colorectal cancer with a high MSI morphology that was diagnosed before the age of 60 years, colorectal cancer with one or more first-degree relative with colorectal cancer or other HNPCC-related tumors that was diagnosed before the age of 40 years and colorectal cancer with two or more relatives with colorectal cancer or other HNPCC-related tumors regardless of age (Recognizing Hereditary Cancer 2006).

Patient Selection by Molecular Tumor Analysis

A second approach for identifying candidates for mutation screening involves the molecular analysis of tumors. Colon tumor tissues can be screened by a combination of PCR-based assays to assess MSI and immunohistochemical (IHC) analysis to determine the loss of particular MMR proteins. The gene corresponding to the lost protein is screened by molecular assays using DNA from a blood sample to determine the underlying germline mutation.

The approach of using the prescreened tumors to identify MMR genes for subsequent mutation scanning is incorporated into guidelines prepared by the American Gastroenterology Association (http://www.gastro.org/). The recommended initial test is a

combination of MSI and IHC analysis for *MSH1* and *MSH2* mutations in individuals fulfilling the Amsterdam criteria, the revised Bethesda criteria and in first degree adult relatives of mutation carriers (Umar et al. 2004). If a MSI-high tumor is detected then germline testing is performed by DNA sequencing, conformational sensitive gel electrophoresis (CSGE) or single-strand conformational polymorphism (SSCP) for mutations in *MSH1* and *MSH2* (Umar et al. 2004). IHC is a fast and reliable screening tool for mutation detection in HNPCC individuals. IHC was successful in detecting the loss of *MSH6* antibody expression in 20 of 23 cases that carried underlying *MSH6* mutations (Plaschke et al. 2004)

Molecular Techniques and Mutation Spectrum

Clinical molecular genetic testing for HNPCC is an evolving field that is being driven by requirements for increased sensitivity for mutation detection. Initially, clinical offerings were limited to point mutation and small insertion/deletion screening in *MLH1* and *MSH2*. A variety of techniques were employed for mutation detection including: DDGE, DHPLC, Single Strand Conformation Polymorphism (SSCP), and direct DNA sequencing (Gille 2002). In the United States, direct DNA sequencing has become the predominate method for clinical testing due to high analytical sensitivity.

These PCR-based methods are effective at finding single-base substitutions, small deletions and insertions, but are insensitive to large gene rearrangement mutations, such as genomic deletions or duplications that impact primer annealing sites. When a rearrangement mutation disrupts an amplicon, the mutation remains undetected since only the signal from the normal allele is analyzed. Discoveries of rearrangement mutations in *MLH1* and *MSH2*, where up to one-third of mutations can be

rearrangements, have prompted the incorporation of assays to detect these mutations in clinical tests.

Multiplex Ligation Probe Amplification Analysis

MLPA is rapidly being adopted by clinical laboratories for rearrangement mutation detection. In MPLA, multiple loci can be screened for rearrangement mutations by highly multiplex PCR-based target dosage analysis. The technique is rapid, cost-effective and consumes minimal sample. The application of this novel method is being facilitated by the commercial availability of assay kits for an increasing set of genes and applications including *BRCA1*, *BRCA2*, *MLH1*, *MSH2*, *MSH6*, and *PMS2*.

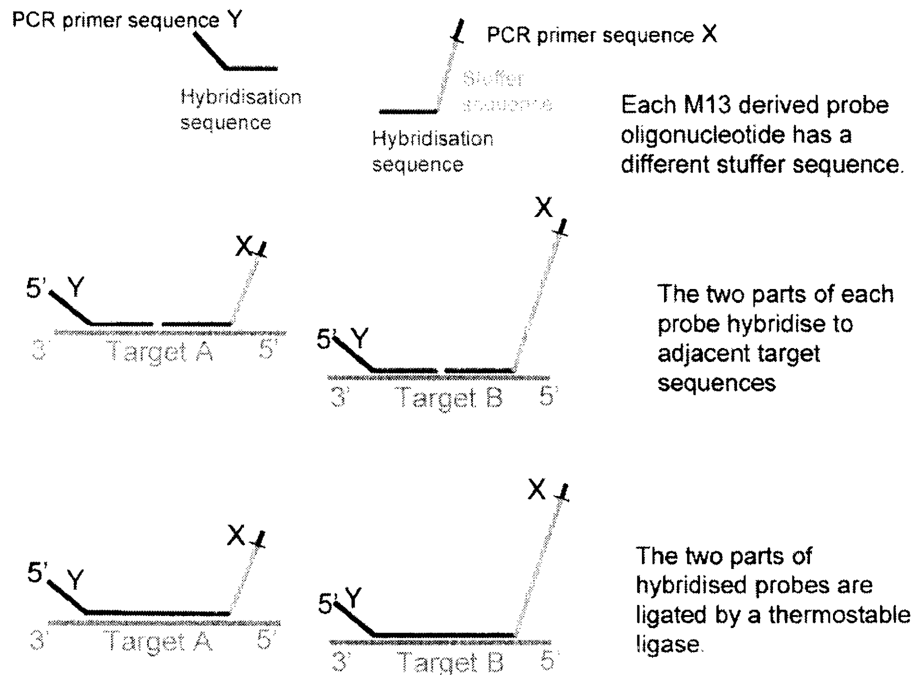
In MPLA, a mixture of different sized probes are added to the samples and allowed to hybridize overnight to adjacent target sequences then amplified by PCR and quantified (Figure 7). Amplified products are separated by electrophoresis in which the relative amounts of probe amplification products are proportional to the relative copy number of the target sequence.

Each probe is made up of two oligonucleotides, one synthetic and one M13 derived, that hybridizes to sites adjacent to the target sequence. The short synthetic oligonucleotide of each probe contains a target-specific sequence of 21-30 nucleotides (nt) at the 3' end and a common 19 nt sequence that is identical to the labeled PCR primer at the 5' end. The long MLPA probe contains a target-specific sequence of 25-43 nucleotides that is cloned into one of the M13-derived SALSA vectors. These tailed probes are ligated so that they may serve as targets in PCR using a single primer pair.

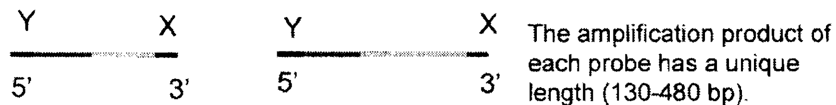
In the study by Taylor et al. (2003), 12 rearrangements were detected in *MSH2* and *MLH1*, in addition to the detection of 13 previously unreported point mutations and

MLPA, Multiplex Ligation-dependent Probe Amplification

- Denatured genomic DNA is hybridized with a mixture of 40 probes.
- Each MLPA probe consists of two oligonucleotides, one synthetic and one M13-derived.



All probe ligation products are amplified by PCR using only one primer pair.



Amplification products are separated by electrophoresis. Relative amounts of probe amplification products reflect the relative copy number of target sequences.

Figure 7. Outline of the MLPA Reaction

Reprint by permission of Schouten J, McElgunn C, Waaijer R., Zwijnenburg D, Diepvens F, Pals G. 2002. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Research 30: e58.

16 point mutations already in the literature. Overall detection sensitivity was increased by 50% and test failures were less than 5% mainly due to low DNA volumes. Gille et al. (2002) detected 16 genomic deletions in 126 Dutch families to also demonstrate that MLPA is a cost effective and robust gene dosage method that can readily be integrated by diagnostic services.

The method is rapid and ready-made commercial kits are available for many genes. The HNPCC MLPA assay checks the dosage of 35 exons against seven control amplicons in a single reaction tube. Washing to remove the nonhybridizing hemi-probes is not necessary since the probes will not be ligated and amplified. Data review is rapid and robust by the use of formatted spreadsheets or by software analysis, such as GeneMarker (SoftGenetics, State College, PA) or Genotyper (Applied Biosystems).

There are also some disadvantages of using MLPA in the detection of genomic rearrangements. First, the use of M13 (single stranded) probes are technically challenging to construct if not included in the commercial kits. Second, polymorphisms or single base mutations in the probe binding regions may affect MLPA results. Nucleotide mismatches at and near the probe binding site may prevent hybridization and ligation to appear as exon deletions. It is recommended that any small fragment deletions, namely single exon deletions, detected by MLPA should be confirmed by another method. MLPA is also very sensitive to DNA quality and should only be used with DNA from the same extraction method as per the manufacturer's instructions.

SALSA MLPA Kit P008 *MSH6/PMS2*

The SALSA MLPA kit P008 *MSH6/PMS2* provides a simple and fast method to detect deletions of one or more exons of *MSH6* and *PMS2*. The kit contains MLPA

probes for each of the 10 *MSH6* exons and probes for 13 of the 15 *PMS2* exons as well as a few probes (3-5) for *MLH1*, *MLH3*, *MSH2*, *MSH3*, and *MYH*. Details of probe sequences can be found on http://www.mrc-holland.com. Some *PMS2* probes contain identical sequences to one or more *PMS2* pseudogenes so some *PMS2* probes have been placed in introns. DNA quality (DQ) control fragments are included in each SALSA probe mix. The DQ fragments provide amplification products that are shorter than the probe amplification products. This provides a warning when not enough DNA was used to obtain reliable results or if the ligation reaction failed.

MSH6 Mutations in HNPCC

According to the database of the International Collaborative Group on HNPCC, *MSH6* mutations account for approximately 10% of all the detectable disease causing mutations in HNPCC families (Peterlongo et al. 2003). Only a few of these mutations are reported from families that fit the Amsterdam criteria or in tumors with an MSI-high phenotype (Peterlongo et al. 2003). Up to 39% of families with mutations in an HNPCC gene do not meet the Amsterdam Criteria (Syngal et al. 2000), which is especially seen in *MSH6*.

The Prevalence of *MSH6* Mutations in HNPCC

From other studies, it has been shown that it is difficult to determine the importance of *MSH6* mutations in HNPCC, since the reported prevalence varies greatly among studies in accordance with selection criteria of patients for research studies (Hendrickson et al. 2005). It has been suggested that germline mutations in *MSH6* are more evident in people with a later age of onset of colorectal cancer whose tumors

display a MSI-low phenotype (Peterlongo et al. 2003). The redundancy of function in the DNA MMR system, specifically *MutS*, could explain the MS-stability in tumors of *MSH6* germline mutation carriers when compared with *MSH2* and *MLH1* (Buttin 2004). *MSH6* kindreds are often characterized by endometrial cancers, lower penetrance of colorectal cancer, and later age of onset when compared with kindred that carry *MLH1* and *MSH2* mutations. It has been reported by many authors that 52-73% of cancers in *MSH6* germline mutation carriers are endometrial cancers (Buttin et al. 2004).

Kolodner et al. identified *MSH6* mutations in families who did not fulfill the Amsterdam and Bethesda criteria, with the average age of diagnosis of 61 years of age. Plaschke et al. reinforce the limitations of the classical criteria for *MSH6* detection in HNPCC individuals, reporting that two-thirds of families carrying *MSH6* mutations would have not been detected by using the both the classical or revised Amsterdam criteria. Since the MSI phenotype is variable in *MSH6* mutation carriers or family history may fail to identify *MSH6* mutations, it is suggested that screening for *MSH6* mutations should be performed when testing is negative for *MSH2* and *MLH1* germline mutations. It may be unreliable to only use MSI for the initial screening of *MSH6* mutations due to the MSI-low phenotype in these tumors.

<u>Genomic Rearrangements in *MSH6*</u>

Genomic rearrangements in *MSH6* play a lesser role in the spectrum of mutations causing HNPCC when compared to mutations in *MLH1* and *MSH2*. It has been shown that 10-20% of patients with *MSH6* negative tumors carry germline rearrangements in this gene (Plaschke et al. 2003). Three patients of 15 who tested negative for MMR mutations and displayed tumors with loss of *MSH6* protein expression were analyzed

using PCR-based relative quantification of *MSH6* fragments (Plaschke et al. 2003). A duplication of the 3' end of exon 4 and exon 5 and a deletion of the functional promoter region and the first two coding exons from one allele of the *MSH6* gene were identified in 2 of the 3 patients (Plaschke et al. 2003). These patients fulfilled the Bethesda guidelines and the tumors were classified as MSI-high (Plaschke et al. 2003).

CHAPTER II

OBJECTIVES OF THIS STUDY

The purpose of this study is to determine the role of *MSH6* mutations in North American patients receiving clinical genetic testing for HNPCC. The relative prevalence of mutations in *MLH1*, *MSH2*, and *MSH6* in these patients was also determined in this study. By augmenting direct DNA sequencing with rearrangement detection by Southern blot analysis and by MLPA, the spectrum of point mutations versus rearrangements across these genes was also determined. Any clinical benefits (increased sensitivity) from pooling these tests together will also be demonstrated along with any clinical characteristics, such as tissue preferences, of genetic mutations among HNPCC individuals.

CHAPTER III

MATERIALS AND METHODS

Patient Sample Selection

The set of 406 specimens were made completely anonymous, while information regarding mutation status and some aspects of family history were retained, were previously subjected to clinical genetic testing for HNPCC by direct sequencing of the coding and flanking splice acceptor/donor regions for *MLH1* and *MSH2* genes. Southern blot analysis of *MLH1* and *MSH2* for rearrangement mutations was also performed. Sixty-four deleterious mutations and 38 genetic variants of uncertain clinical significance in *MLH1* and *MSH2* were identified by DNA sequencing in this sample set. Two hundred seventy-nine samples contained adequate material for *MSH6* mutation prevalence testing by DNA sequencing and MLPA analysis. The samples were grouped into six categories based on cancer frequency reported from patients and their family history (Table 2).

MSH6 Testing

Nucleotide Sequencing

The prevalence of *MSH6* mutations was determined by direct sequencing for the entire coding regions and splice acceptor (20 bps upstream) and donor sites (10 bps downstream) next to the exons. Several design elements were integrated into the assay to

Table 2

Grouping of Patient Samples

Group	Patient EC	Relative EC	Patient CRC	Relative CRC
1	+			
2	-	+		
3	-	-	+	+
4	-	-	+	-
5	-	-	-	+
6	-	-	-	-

Patient samples were grouped into six groups based on cancer frequency of endometrial cancer (EC) and colorectal cancer (CRC) reported from patients and their families (Hendrickson et al 2005)

achieve optimal sensitivity. PCR buffers designed for G-C rich regions, such as in the promoter, were utilized instead of using a standard PCR buffer during PCR (PCRx Enhancer System, Invitrogen, Carlsbad, CA.) Primers were designed to avoid commonpolymorphisms surrounding the exons by sequencing a genetically diverse set of 96 DNAs that were defined by SNP haplotypes to identify these polymorphisms. Dye-primer chemistry (Big Dye, Applied Biosystems, Foster City, CA) was used for nucleotide sequencing and the sequence was screened for both the forward and reverse strands.

A commercially available program (Mutation Surveyor, SoftGenetics, State College, PA) was used for data analysis of direct sequencing results, which was validated in the laboratory by correctly identifying 50 known variants in 240 sequencing chromatograms. Visual confirmation was performed for all *MSH6* mutations identified by the software.

MLPA Analysis

The 279 samples negative for *MLH1* and *MSH2* mutations were subjected to MLPA analysis for the detection of *MSH6* rearrangement mutations. MLPA was conducted according to the manufacturer's protocol using the MLPA probe kit P008 *MSH6/PMS2* of MRC-Holland (http://www.mrc-holland.com). DNA samples (20-500ng) were diluted with water to 5ul and then heated at 98° C for 5 minutes in a 96 well thermocycler with a heated lid. After cooling to 25° C, the DNA was mixed with the probe set and high salt buffer (MLPA buffer). The mixture was re-heated to 95° C for 1 minute and then incubated at 60° C for 16 hours. DNA ligase and ligation buffer were added following probe hybridization, which was incubated at 54° C for 10-15 minutes.

The ligation products were next amplified by PCR using the following PCR conditions: 30 seconds 95° C; 30 seconds 60°C; 60 seconds 72° C for 33 cycles and followed by end incubation at 72° C for 20 minutes. Fragment analysis was employed on automated capillary electrophoresis instruments (MegaBACE 1000, GE Healthcare, Chalfont St. Giles, United Kingdom) using ROX-550 (Bioventures, Inc) as size standard.

Data analysis. Data analysis was performed by examination of the capillary electrophoresis peak profiles using GeneMarker software (Softgenetics, Inc). Data normalization is first performed to increase the low intensities of larger fragments due to low efficiency of PCR reactions and low injection rates during electrophoresis. Data normalization is performed by taking the square root of the intensity ratios then plots the ratios to model a linear regression, using the control probes as reference points. The exponential function, a*e-bz, is used to fit the square root of peak intensities, where z is size, and a and b are fitting constants.

After intensity normalization, the data are plotted into two formats, ratio and regression analysis. Both MLPA analysis methods identify data points as outliers by the taking deviation of each allele peak relative to the average deviation of all peaks. If an individual peak whose residual deviation is higher than three+ times the average deviation then it is defined as an outlier.

In MLPA ratio analysis, the intensity ratios of the same probes from the patient samples and the control samples are compared and standardized such that the median point within the data set is 1. The data points that appear outside the threshold lines of the data set represent duplications and deletions. The intensity ratio of a data point greater than 1.33 is identified as a duplication and less than 0.75 is a deletion.

The second analysis occurs as a regression plot method that shows the square root of peak intensity deviations of the patient samples compared with the control samples. The software forms a best fit line and removes a specific number of outliers from the data and forms a new regression line. This is repeated until the regression line has reached a confidence of greater than 99.0% to show that the outlier alleles are truly duplication and deletions. The removed points are placed back into the plot as either outliers or plotted on the regression line. Rearrangement mutations are detected as changes in the ratio of peak intensities from signal amplified across the exons of the gene.

CHAPTER IV

RESULTS

Sixty-four deleterious mutations and 38 genetic variants of uncertain significance were identified by direct sequencing and Southern blot analysis of *MLH1* and *MSH2* in the original 406 specimens, showing an overall total mutation prevalence of 89%. Of the 89%, 25% of these mutations were rearrangements in *MLH1* and *MSH2* identified by Southern Blot analysis. Seventeen *MLH1* mutations and 31 *MSH2* mutations were detected along with 27 genetic variants of uncertain significance in *MSH1* and 11 in *MSH2*. Three rearrangements were detected in *MLH1* and 13 rearrangements were detected in *MSH2* (Table 3 and Table 4).

MSH6 Results

Sequencing results identified six protein truncating mutations showing an overall *MSH6* mutation prevalence of 11% (limited by mutation detection by direct DNA sequencing). The six deleterious mutations were distributed along the coding sequence of the gene. Three of the mutations, 2150delTCAG, R10689X, 3859delCAAG, were previously characterized (Kolodner et al. 1999, Plaschke et al. 2002, Goodfellow et al. 2003) and the remaining three appeared to be novel mutations, Q4X, 1816insA, 2230insG (Table 5). Four of the six mutations, Q4X, 2230insG, R10689X, 3859delCAAG were identified in patients that were diagnosed with endometrial cancer.

Table 3

Summary of All Mutations Detected in *MLH1*, *MSH2*, and *MSH6*

Mismatch Repair Gene	*MLH1*	*MSH2*	*MSH6*
Point Mutations	17	31	6
Rearrangements Mutations	3	13	0
% of Mutations in HNPCC	41%	48%	11%
Genetic VUS	27	11	8

Table 4

Summary and Prevalence of All Mutations

Group	Patient EC	Relative EC	Patient CRC	Relative CRC	Group totals n=406	*MLH1*/ *MSH2* mutation count n=64	%	Tested for *MSH6* n=279	*MSH6* mutation count n=6	%
1	+				54	17	26.6	30	4	66.7
2	-	+			44	10	15.6	32		
3	-	-	+	+	169	23	35.9	105	1	16.7
4	-	-	+	-	47	6	9.4	40	1	16.7
5	-	-	-	+	61	3	4.7	49		
6	-	-	-	-	31	5	7.8	23		

The summary of all mutations detected in *MLH1*, *MSH2*, and *MSH6* and the prevalence of these mutations in groups 1-6. Groups 1-6 are based on cancer frequency reported from patients and their family history (Hendrickson et al 2005). EC-endometrial cancer, CRC-colorectal cancer.

Table 5

Deleterious Mutations and Variants of Unknown Significance in *MSH6* Identified by Sequencing

Mutation	Severity	Exon
10C>T (Q4X)	Deleterious	1
1816insA	Deleterious	4
2150delTCAG [1]	Deleterious	4
2230insG	Deleterious	4
3202 C>T (R1068X) [2]	Deleterious	5
3959delCAAG [3]	Deleterious	9
IVS3-7C>A	Unknown	4
663A>C (E221D)	Unknown	4
866GC>AA (G289E)*	Unknown	4
1106C>T (T369I)	Unknown	4
1856A>C (E619D) [4]	Unknown	4
2025G>C (E675D)	Unknown	4
2057G>A (G686D)	Unknown	4
3911G>A (R1304K)	Unknown	9

The six protein truncating mutations along with 8 missense variants of uncertain clinical importance were identified in *MSH6* by direct sequencing. Six of the mutations are deleterious and 4 of the 12 mutations were previously identified in other studies as noted (1). Kolodner et al. 1999 (2). Plaschke et al. 2002 (3). Goodfellow et al. 2003 (4). Plaschke et al. 2004. Numeric designation corresponds to transcript location based on GenBank BC004246, initiated from first adenine of the start codon. Amino acid change shown in parentheses. All mutations and variants observed once each.* Assumes nucleotide changes are in cis (Hendrickson et al 2005).

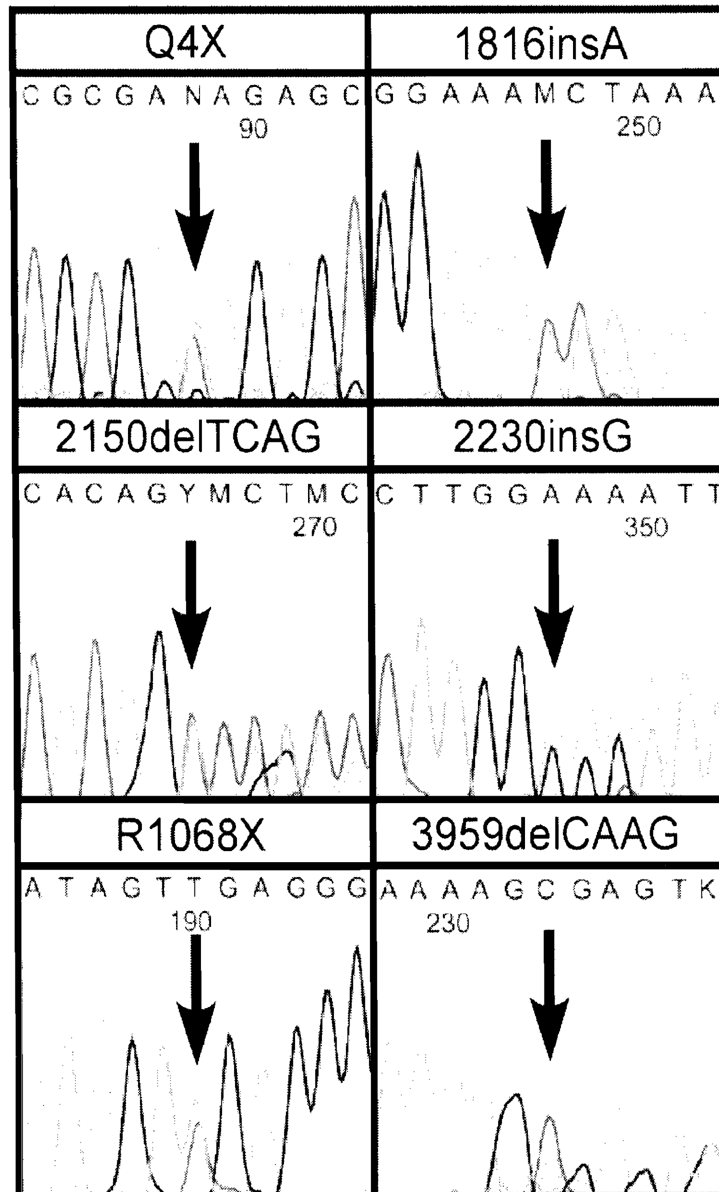
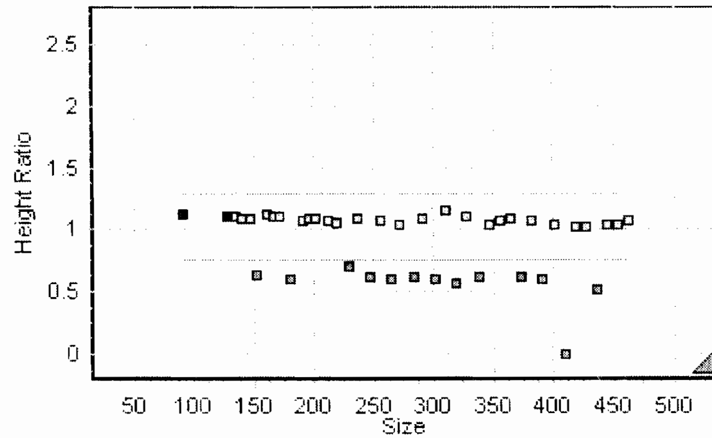


Figure 8. Summary of Sequencing Electropherograms of the Six Protein Truncating *MSH6* Mutations Identified. The arrow indicates the location of each mutation; Q4X resides in exon 1; 1816insA, 2150delTCAG and 2230insG reside in exon 4; R1068X resides in exon 5; 3959delCAAG resides in exon 9 (Hendrickson et al 2005).

The increased frequency of *MSH6* mutations in patients diagnosed with endometrial cancer is demonstrated by other studies suggesting a connection of *MSH6* mutations with this cancer type. A statistical analysis to verify an increased association of endometrial cancer with *MSH6* compared with *MLH1/MSH2* mutation carriers showed that the results are suggestive, but not significant in this study (Fisher's exact test, p=0.062). Additionally, eight variants of unknown clinical significance were found, one of which was previously identified (Plaschke et al. 2004). There were no rearrangement mutations detected in *MSH6* by MLPA analysis. Only one sample remained unresolved for *MSH6* rearrangement mutations due to uninterpretable results. Due to the requirements for making the samples anonymous, additional information such as age of onset, cancer type and exact number of affected family members were made unavailable.

PMS2 Deletion

The deletion of the entire *PMS2* gene was identified by MLPA in a patient diagnosed with colorectal cancer, has a family history of colorectal cancer, but no history of endometrial cancer (Figure 9). With the exception of exons 13- 15, the data from this sample appeared consistent with a whole gene deletion mutation. This finding was repeated by MLPA to confirm the deletion of exons 1-15 in this sample. From the MLPA analysis, exons 13-15 of the *PMS2* gene appeared deleted or duplicated in numerous patient samples. It was apparent that these probes were hybridizing to pseudogenes homologous to exons 13-15 of the *PMS2* gene. These results were excluded due to the high variability of these probes. Exons 1-12 had an average coefficient of variability (CV) of 6.5% (maximum CV=9.3%), not including the deletion sample, while exons 13-15 had CV values of 28.4%, 22.9% and 36.6%.



Probe Name	Size	Allele ratio
PMS2_ex01	152	0.613
PMS2_ex02	180	0.585
PMS2_ex05	229	0.684
PMS2_ex06	247	0.619
PMS2_ex07	265	0.596
PMS2_ex08	283	0.603
PMS2_ex09	301	0.609
PMS2_ex10	319	0.569
PMS2_ex11	337	0.593
PMS2_ex13	373	0.592
PMS2_ex14	391	0.595
PMS2_ex15	409	0.000
PMS2_ex12	436	0.515

Figure 9. MLPA Allele-Ratio Graph Showing the Deletion of the *PMS2* Gene. Data points below the threshold line, having an intensity allele ratio of less than 0.75, represent a deletion. Analysis of results was completed using commercial software (GeneMarker, SoftGenetics) and a synthetic population derived control.

The deletion of the *PMS2* gene in this sample set was an additional finding in this study. Germline mutations in *PMS2* have been reported as a rare cause of HNPCC. Only six germline mutations (Nakagawa et al. 2004) and four rearrangements (van der klift 2005) are identified in *PMS2*. This finding shows that the rate of *PMS2* mutations is still unknown at this point due to testing difficulties imposed by the pseudogenes. More research is needed since little is known about the role of *PMS2* in HNPCC.

CHAPTER V

CONCLUSION

An accurate prevalence of *MSH6* mutations in HNPCC can't be reported since results vary significantly between studies due to patient selection by researchers and stringent cancer type ascertainment combined with founder mutations. Plaschke et al. reinforces the limitations of the classical criteria for *MSH6* detection in HNPCC individuals, which reported that two-thirds of families carrying *MSH6* mutations would have not been detected by using the Amsterdam criteria. Several studies reported that *MSH6* mutations are more prevalent in kindreds that don't fulfill the Amsterdam or Bethesda criteria (Wijnen et al. 2003, Plaschke et al. 2004). These data also suggest that families selected by the Amsterdam or Bethesda criteria for HNPCC testing appear to select against patients that would benefit from *MSH6* testing. Because the 279 specimens were made fully anonymous, the remaining patient information did not permit the samples to be evaluated by clinically accepted standards, such as the Amsterdam or Bethesda criteria. This study cannot confirm that these criteria select against *MSH6* mutations in these patients.

In these experiments, the purpose of this study was to determine the role of *MSH6* mutations in North American patients receiving clinical genetic testing for HNPCC. This study proposed to answer several questions arising from the role of *MSH6* mutations in

this sample set: What are the relative prevalence of mutations in *MLH1*, *MSH2*, and *MSH6* in these patients and the spectrum of these mutations (point mutations verses rearrangements) across the three genes? Are there clinical benefits from augmenting tests with rearrangement detection? Do the clinical characteristics of genetic mutations vary in MMR genes among HNPCC individuals?

In the clinical testing of the 406 samples, *MLH1* and *MSH2* mutations accounted for 89% of all detectable HNPCC associated mutations in North American patients receiving clinical HNPCC testing. Of the 89%, 25% of these mutations were identified as rearrangement mutations in *MLH1* (5%) and *MSH2* (20%) by Southern blot analysis. *MSH6* mutations accounted for 11% of all HNPCC associated mutations in this sample set. MLPA analysis did not identify any rearrangement mutations in the *MSH6* gene. This demonstrates rearrangement mutations occur less frequently in *MSH6* when compared to point mutations but could be due to the small sample size of this study. This study can confirm that rearrangement mutations in *MLH1* and *MSH2* account for about 25% of all mutations in HNPCC when compared with other studies. These findings also confirm that the majority of MMR mutations occur in *MLH1* and *MSH2* and less frequently in *MSH6* in HNPCC families.

It has been shown that *MSH6* kindreds are often distinguished by endometrial cancers, lower penetrance of colorectal cancer, and later age of onset when compared with kindreds that carry *MLH1* and *MSH2* mutations. It has been shown that 52-73% of cancers in *MSH6* germline mutation carriers are endometrial cancers (Buttin et al. 2004) with the average age of onset of 61 years of age (Kolodner et al). In the study by Buttin et al., 58% of *MSH6* mutations were detected in patients with endometrial cancer with a

lower penetrance in colorectal cancer. Four out of six mutations in *MSH6* were found in patients diagnosed with endometrial cancer. The data appear suggestive, but not significant as shown by a statistical analysis (Fisher's exact test, $p=0.062$). There was insufficient patient information to show if the families associated with *MSH6* mutations displayed a later age of onset.

By augmenting tests with rearrangement detection by MLPA, a deletion of the *PMS2* gene was identified in a patient diagnosed with colorectal cancer, had a family history of colorectal cancer, but no history of endometrial cancer. This mutation would not have been detected with only the incorporation of conventional methods such as DDGE, DHPLC, or direct sequencing for mutation detection.

The MutLα heterodimer formed by the *MLH1* and *PMS2* protein is a major component of the MMR complex (Nakagawa et al. 2004). Germline mutations in *PMS2* are rare and have been reported in only a few families with cancer (De Vos et al. 2003, van der Klift 2005) despite its important role in the MMR pathway. It has been shown that *PMS2* mutated human cells display a mutation rate equivalent to or higher than *MLH1* mutated human cells, but reported *PMS2* mutations are low (De Vos et al. 2004). More data needs to be obtained to understand the mechanism of which *PMS2* mutations predispose individuals to HNPCC. One possible reason for the under diagnosis of *PMS2* mutations could be the existence of pseudogenes corresponding to the first five exons, exon 9, and exons 11-15 of PMS2 to interfere with analysis.

CHAPTER VI

DISCUSSION

According to the HNPCC database, *MSH6* is the third most common gene associated with HNPCC. *MSH6* mutations account for 10% of all mutations in HNPCC, which is confirmed by this study (Kariola et al. 2002). *MSH6* mutations are linked to families that display less common clinical features associated with HNPCC (Wijnen et al. 1999), such as a higher incidence of endometrial cancers, lower penetrance of colorectal cancer and a later age of onset when compared with kindreds carrying *MLH1* and *MSH2* mutations. By using the classical criteria for the selection of individuals for HNPCC testing, many individuals harboring *MSH6* mutations could be overlooked. *MSH6* mutations require the same or more stringent level of surveillance as for any other MMR mutation in HNPCC kindreds (Offit 2004), but require a broader selection criterion.

Overall this study shows that disease causing *MSH6* mutations are rarer among HNPCC families when compared with *MSH2* and *MLH1* mutations. In this study, the prevalence of *MSH6* mutations was determined in a clinical population of North Americans receiving HNPCC clinical testing. This study shows testing in a more practical clinical population of families that would benefit from *MSH6* testing, since several studies reports *MSH6* mutations are more prevalent in kindreds that don't fulfill Amsterdam or Bethesda criteria (Wijen et al. 2003, Plaschke et al. 2004).

At this time, it is not clear how *MSH6* analysis should be incorporated into HNPCC clinical testing; therefore it is suggested that *MSH6* sequence analysis should be done in HNPCC families when no other mutations are identified in *MLH1* and *MSH2* and in families with a history of endometrial cancer as well as colorectal cancer regardless of age. *MSH6* analysis should also include the screening of genomic rearrangements when no other mutations are detected in other MMR genes. It has been shown by this study that mutations in *MSH6* play a lesser but important role in the cause of HNPCC. Since MLPA has been proven to be rapid and simple, this assay could be incorporated into a clinical setting as an initial screening tool for mutation detection.

It has been shown that there are clinical benefits from combining conventional detection with rearrangement detection by MLPA. It has increased sensitivity of mutation detection and can be used as a fast, simple, reliable screening tool for rearrangement detection. Most PCR-based methods are effective at detecting point mutations, but are insensitive to detecting large gene rearrangements. Southern blot analysis is most commonly employed for the detection of genomic deletions and duplications, but it is time consuming and laborious, requires large amounts of DNA, and requires expert analysis. Screening for genomic deletions in MMR genes, especially in *MSH2* and *MLH1*, is essential for the diagnosis of HNPCC. Since MLPA is rapid, efficient, and a simple technique for the detection of genomic deletions in MMR genes, it could be incorporated into the initial screening process for MMR gene mutation analysis. Without the screening for genomic rearrangements in *MSH2* and *MLH1*, up to 30% of these mutations could go undetected due to the incorporation of methods that are insensitive to genomic rearrangements.

Published reports regarding *MSH6* mutations describe research specimens that were selected by various criteria that make correlations to the clinical population undergoing testing difficult. This is not unusual in the field of human genetics where initial discoveries occur in highly selected specimen sets. It is now known that the Amsterdam or Bethesda criteria select against *MSH6* mutations and more informed decisions to develop the correct patient criteria for *MSH6* testing will require additional clinical data for the systematic analysis of point and rearrangement mutations in *MSH6*. This study could not properly display the prevalence of *MSH6* rearrangements versus point mutations due to the small sample size of this study. More research is needed to determine the importance of *MSH6* rearrangements in HNPCC. Results from this study and other recently published studies support the conclusion that *MSH6* testing is an important part of genetic testing for HNPCC individuals. The need to correctly identify all mutations in HNPCC individuals and HNPCC-like families is important since the cost of clinical testing is high and also to prevent unnecessary testing in unaffected individuals.

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