

## **CHAPTER 3**

### **Lynch Syndrome: Germline Mutations in MMR Genes**

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## ***Introduction***

Approximately 2-5% of newly diagnosed cases of CRC can be attributed to Lynch syndrome (LS). This syndrome, previously referred to as hereditary nonpolyposis colorectal cancer (HNPCC), is an autosomal dominant disorder with incomplete but high penetrance manifested by early-onset colorectal and uterine cancer and an increased risk of certain extra-colonic cancers, including tumors of the gastrointestinal tract (stomach, small bowel, hepatobiliary tract), urinary collecting system (renal pelvis, ureter), and of the female reproductive system (ovaries) (Table 1).

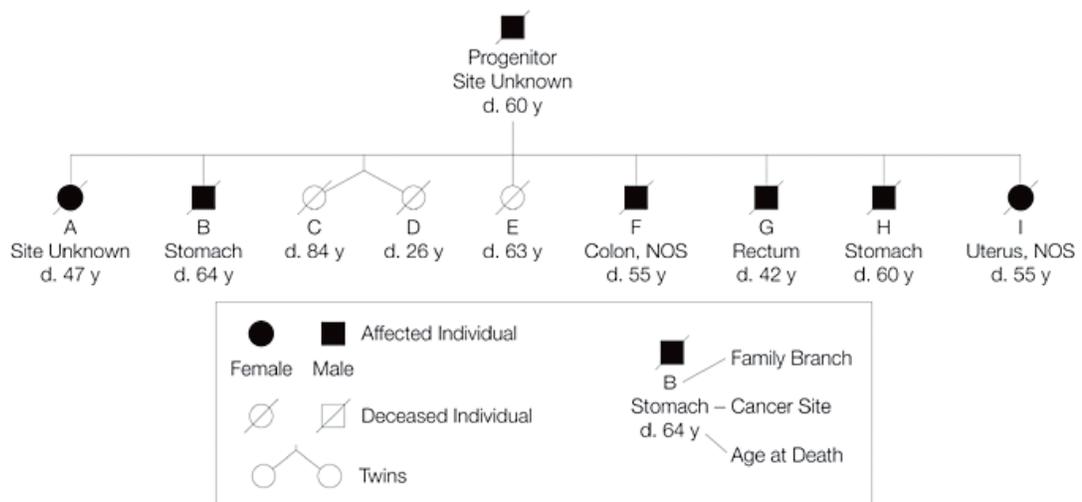
***Table 1. Lifetime cancer risk in LS.***

<b>Cancer</b>	<b>Risk in LS (%)</b>	<b>Risk in the General Population (%)</b>
<b>Colorectal (Men/Women)</b>	<b>68.7/52.2</b>	<b>5-6</b>
<b>Endometrial</b>	<b>27-71</b>	<b>2-3</b>
<b>Ovarian</b>	<b>3-14</b>	<b>1-4</b>
<b>Gastric</b>	<b>2-30</b>	<b>1</b>
<b>Urinary tract</b>	<b>1-12</b>	<b>1</b>
<b>Small bowel</b>	<b>4-7</b>	<b>0.01</b>
<b>Brain</b>	<b>1-4</b>	<b>0.6</b>
<b>Biliary tract</b>	<b>2</b>	<b>0.5</b>

While initial studies of selected Lynch families estimated a 70-80% lifetime risk of colon cancer with a mean age at diagnosis in the mid-40s, more recent studies have suggested a somewhat lower lifetime colorectal cancer risk (52.2% in women and 68.7% in men) and a higher median age at diagnosis of 61.2 years [1-3]. The second most common cancer in LS is endometrial cancer, and women with LS have a 27-71% and 3-14% cumulative lifetime risk of developing endometrial and ovarian cancer, respectively, with a mean age at diagnosis approximately ten years earlier than sporadic cases [4]. The lifetime risk for gastric cancer in LS patients varies between populations, with a particularly high

incidence in areas such as China and Korea that have a high endemic risk of gastric cancer in the general population, with a lifetime risk of approximately 30% [5,6]. The risk for urinary tract, small bowel, brain, and hepatobiliary tumors is approximately 1-12%, 4-7%, 1-4%, and 2%, respectively [4]. Unique variants of the syndrome characterized by the presence of skin tumors (keratoacanthomas, sebaceous neoplasms) or brain tumors (glioblastomas, astrocytomas, and oligodendrogliomas) are termed Muir-Torre and Turcot syndromes, respectively [7].

The first study of a family that represented what is now known as LS began in 1895, when Aldred Scott Warthin, a pathologist at the University of Michigan, initiated one of the most longest cancer family histories ever recorded. He was stimulated to make this study because his seamstress was depressed at the thought of dying prematurely from bowel or womb cancer, as had many of her relatives. Just as she predicted, she died at an early age of metastatic endometrial carcinoma. Warthin published a description of this family, which he called Family “G” in his original article in 1913 (Figure 1) [8].



**Figure 1.** Pedigree of family G: generation I and II.

In 1936, two of his colleagues provided further follow up of this family [9]. Members of Family G had migrated to Michigan from southern Germany during the early and mid-1880s. Henry Lynch described two additional families, families N and M (as they came from Nebraska and Michigan) in 1966 and revisited family G in 1966 and 1971 [10,11]. In the mid-eighties, Finnish, Dutch and Italian investigators started to search for LS families in their respective countries [12-14]. In 1989, the International Collaborative Group (ICG) was set up to promote international research on the Lynch syndrome [15].

The molecular genetic era for LS began almost ten years ago, when Peltomaki *et al.* through linkage analysis, identified a locus on chromosome 2p as a site for a gene predisposing to Lynch [16]. Shortly thereafter, a second locus believed to be etiologic for this syndrome was identified on chromosome 3p by Lindblom in Sweden [17]. Therefore, investigators expected to find a tumor-suppressor gene and searched for loss of heterozygosity among dinucleotide repeats in the critical genetic region. Instead, what was found in all the LS cancers studied were microsatellite alleles that had changed in length as a result of nucleotide insertions or deletions. These modifications were found not only in microsatellites in the critical genetic region but also in microsatellites virtually everywhere in the genome of the tumor. This remarkable phenomenon was termed “replication error” and later renamed “microsatellite instability” [18,19]. The subsequent recognition that MSI is the consequence of defective DNA replication error repair, or “DNA proofreading,” was contributory to the discovery at the 2p and 3p loci of genes for LS, *hMSH2* and *hMLH1*, which encode proteins involved in the identification and repair of DNA mismatch errors [20,21]. The identification of germline mutations in *hMLH1* and *hMSH2* was quickly followed by the discovery that other genes that encode for members of the MMR complex

are mutated in some Lynch families, establishing significant locus heterogeneity for the syndrome. Mutations in at least seven genes have been associated with LS: *hMLH1*, *hMSH2*, *hMSH6*, *hPMS1*, *hPMS2*, *hMLH3*, and *hEXO1*, with the majority of mutations observed clinically in 4 genes. Germline mutations of *hMLH1*, *hMSH2*, *hMSH6*, and *hPMS2* account for 32%, 38%, 14%, and 15% of all known MMR mutations in LS, respectively [22]. Several missense mutations of unclear biological relevance have been identified in *hMLH3* and *hEXO1* genes [23,24]. Genotype-phenotype correlations have begun to emerge in LS (Table 2). *hMLH1* mutation carriers have a higher colorectal cancer prevalence (79% versus 69%) and younger age at cancer diagnosis when compared to *hMSH2* mutation carriers. The prevalence of other LS-associated cancers is greater among *hMSH2* mutation carriers compared with *hMLH1* mutation carriers (24% versus 9%) [25]. Compared with the incidence in the general population, carriers of a germline mutations in *hMSH6* have a 26-fold increased incidence of endometrial cancer, and an 8-fold increased incidence of colorectal cancer, independently of sex and age [26]. Individuals with *hPMS2* mutations have an overall lower risk of Lynch-associated cancers as well as an older age at cancer diagnosis [27].

**Table 2.** Gene-specific cancer risk assessment.

MMR Gene	Cumulative Risk of CRC by age 70	Cumulative Risk of EC by age 70
<b><i>hMLH1</i> Males/Females</b>	<b>~70%/~50%</b>	<b>~30%</b>
<b><i>hMSH2</i> Males/Females</b>	<b>~50%/~40%</b>	<b>~45%</b>
<b><i>hMSH6</i> Males/Females</b>	<b>22%/10%</b>	<b>26%</b>
<b><i>hPMS2</i> Males/Females</b>	<b>20%/15%</b>	<b>15%</b>

The timely recognition of LS is essential to identify patients at high-risk who will require intensive cancer surveillance. Reductions in colorectal cancer mortality can be achieved by colonoscopic screening of individuals with this syndrome [28]. The simplest and most cost-effective way to identify hereditary forms of CRC is the family history. However, although family history can provide important clues to the presence of LS, this is not always reliable because of small family size, a physician's unfamiliarity with the nuances of the syndrome, lack of documentation, or reduced penetrance of the MMR gene mutation in the family. However, advances in the understanding of the molecular basis of the disease have resulted in novel clinical approaches to establish the diagnosis. Approximately 90% of CRCs occurring in LS patients have a characteristic and readily detectable molecular change in the number of DNA microsatellites, making microsatellite instability (MSI) analysis of tumor samples a useful diagnostic tool to screen for LS [29]. In addition, loss of functional MMR protein that results from inactivating mutations may be assessed directly in tumor tissue through immunohistochemical (IHC) analysis for the MMR proteins, and this strategy can also pinpoint which one of the four genes is most likely to be mutated [30]. MSI and IHC analysis can therefore serve as useful "screening" tests for LS, and the results can guide more definitive germline mutational analysis of the appropriate DNA MMR gene. Identification of a germline mutation not only establishes the diagnosis of LS but also provides an invaluable tool for family screening.

## *Diagnosis of MMR Deficiency*

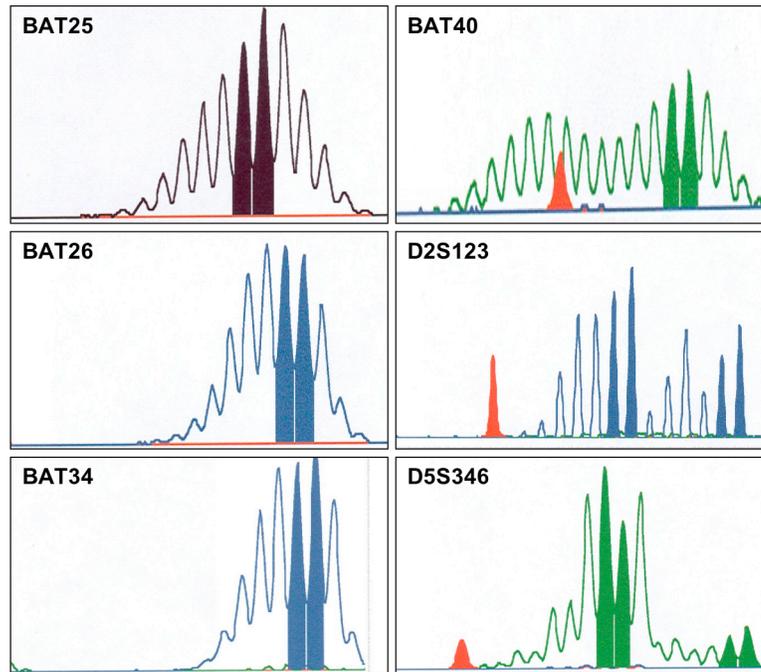
### *Testing for Microsatellite Instability in CRC*

In tumors that develop due to defective DNA mismatch repair, short repetitive DNA sequences known as microsatellites tend to undergo a high level of slippage that results in microsatellite instability (MSI). MSI is reported in as many as 90-95% of colorectal carcinomas and at least 75% of endometrial carcinomas associated with LS, making MSI a sensitive marker for LS-associated tumors. The integrity of mismatch repair is routinely analyzed by extracting DNA from an individual's paraffin-embedded tumor sample as well as control normal tissue, and subjecting that DNA to polymerase chain reaction (PCR) amplification of specific genetic loci (Table 3).

*Table 3. List of commonly used MSI markers.*

Microsatellite markers	Gene near marker /GenBank number	Location of the repeat	Repeat motif
BAT25	<i>c-kit</i>	4q12	TTTT.T.TTTT.(T) <sub>7</sub> .A(T) <sub>25</sub>
BAT26	<i>hMSH2</i>	2p16.3-p21	(T5).....(A) <sub>26</sub>
BAT40	<i>HSD3B2</i>	1p13.1	TTTT.TT..(T) <sub>7</sub> .....TTTT.(T) <sub>40</sub>
NR21	<i>SLC7A8</i>	14q11.2	(T) <sub>21</sub>
NR22	<i>HUMB5A</i>	11p24-25	(T) <sub>22</sub>
NR24	<i>ZNF2</i>	2q11.2	(T) <sub>24</sub>
MONO-27	<i>MAP4K3</i>	2q21	(A) <sub>27</sub>
D2S123	<i>hMSH2</i>	2p16	(CA) <sub>13</sub> TA(CA) <sub>15</sub> (T/GA) <sub>7</sub>
D5S346	<i>APC</i>	5q21/22	(CA) <sub>26</sub>
D17S250	<i>BRCA1</i>	17q11.2-q12	(TA) <sub>7</sub> .....(CA) <sub>24</sub>
D10S197	<i>GAD2</i>	10p	CACCAGA(CA) <sub>7</sub> .A.A.(CA) <sub>12</sub> (AGAAA) <sub>2</sub>
PENTA-C	<i>AL138752</i>	21q22.3	(AAAAG) <sub>3-15</sub>
PENTA-D	<i>AC003656</i>	9p12-13.3	(AAAAG) <sub>2-17</sub>

These PCR products can be separated electrophoretically and differences in fragment size of tumor-derived DNA versus DNA from normal tissue are scored, leading to an assessment of instability (Figure 2).



**Figure 2.** The MSI-curves show instability with 1-2 additional peaks for the markers BAT25, BAT26 and BAT34 and larger size variation for the markers BAT40, D2S123 and D5S346.

Initially, the number and location of microsatellite loci used to test for MSI were highly variable. In 1997, in an attempt to facilitate comparison between studies, a National Cancer Institute sponsored workshop recommended a panel of five microsatellite markers, known as Bethesda markers, for the uniform analysis of MSI, consisting of two mononucleotide (*BAT-25* and *BAT-26*) and three dinucleotide (*D5S346*, *D2S123*, and *D17S250*) repeats [31]. Samples with instability in two or more of these markers were defined as MSI-H (for high-frequency MSI), whereas those with one unstable marker were designated as MSI-L (for low-frequency MSI). Samples with no instability in any of the markers were considered to be microsatellite stable (MSS). In the event that more than five

markers are to be tested, instability at  $\geq 30\%$  of analyzed markers would define MSI-H. However, this NCI five-marker microsatellite panel may underestimate the number of MSI-H tumors and overestimate the number of MSI-L tumors, since dinucleotide repeats are less sensitive and less specific for the detection of MMR deficiencies than mononucleotide repeats. In two reports that analyzed MSI using the Bethesda markers, the sensitivities of the dinucleotide repeats were reported to be 72-89% for *D2S123*, 50-81% for *D17S250* and 50-59% for *D5S346* [32,33]. Because these markers are highly polymorphic (i.e., frequently have different sizes between individuals and between both alleles of the same person) the analysis of corresponding normal DNA is required, and this can make the MSI process relatively time consuming and expensive.

Suraweera *et al.* developed a panel of five quasi-monomorphic mononucleotide repeats (*BAT-25*, *BAT-26*, *NR-21*, *NR-22* and *NR-24*), which can be analyzed together in a pentaplex PCR without the need for matching normal samples. This panel performed with a sensitivity greater than 95% and a specificity greater than 98% for the determination of MSI status in a series of 64 MSI-H and 40 MSS colon primary tumors, respectively, whose MSI status was previously established by the analysis of a panel of dinucleotide microsatellite markers [34]. This option was included in the 2004 Revised Bethesda Guidelines for HNPCC and MSI, although the original panel of mono and dinucleotide markers remained unchanged as the primary recommendation [35]. A similar panel of markers that includes five nearly monomorphic mononucleotides (*BAT-25*, *BAT-26*, *NR-21*, *NR-24* and *MONO-27*) for MSI determination and two polymorphic pentanucleotide markers (*Penta C* and *Penta D*) that help confirm that tumor and matching normal samples are from the same individual, has also been introduced. When this assay was applied to a

set of 72 MSI-H and 81 control colorectal tumors that had been previously characterized using a panel of 10 microsatellite markers and IHC analysis for MLH1, MSH2, MSH6 and PMS2, Bacher *et al.* observed that over 97% of MSI-H samples were correctly identified and overall there was 99% concordance in MSI classification between the two methods. Of note, all of the 43 samples classified as MSI-L by the 10-marker panel were scored as MSS with this multiplex system, confirming the high specificity of the MSI multiplex markers for instability in MSI-H tumors only [36].

The performance of the NCI-recommended panel of markers compared to the pentaplex panel of mononucleotide repeats for the detection of MMR-deficient CRCs was addressed by Xicola RM *et al.* [37]. The sensitivity, specificity, and positive and negative predictive values to detect absence of expression of at least one MMR protein were determined using the NCI panel and the pentaplex panel in 531 and 527 colorectal tumors, respectively. Whereas specificity and negative predictive values were high for both panels, the sensitivity and positive predictive values were 76.5% and 65.0% for the NCI panel and 95.8% and 88.5% for the mononucleotide pentaplex panel. Although the two panels were not compared in the same patient populations, the analysis with both sets of markers of all MSI-L tumors and all tumors that showed a discrepancy between MSI and IHC expression of MMR protein confirmed a superior performance of the pentaplex panel of mononucleotide repeats. Furthermore, whereas a substantial number of tumors with an MSI-L phenotype was detected using the NCI panel, this phenotype was absent when the pentaplex panel of mononucleotide repeats was used.

Others have suggested that testing *BAT25* and/or *BAT26* alone would be sufficient to establish the MSI status of a tumor without reference to the germline DNA, being these

markers quasimonomorphic in most Caucasian populations [37-39]. However, it needs to be taken into account that using *BAT26* alone might lead to some underestimation of the true MSI, due to the non-recognition of the relatively infrequent cases with biallelic *hMSH2* deletions. In fact, *BAT26* lies intragenically in *hMSH2* gene and would not be longer amplifiable from MSI CRCs that have a biallelic deletion of *hMSH2*, a relevant reason to use at least one other mononucleotide marker (such as, *BAT25* or *NR24*) as suggested by Laghi *et al.* [40].

### ***Advantages and Limitations of MSI Testing***

There are several advantages and limitations of MSI testing that should be highlighted. One of the main advantages of MSI testing is that it provides a functional analysis of deficient MMR activity. This information is useful in scenarios where there are conflicting findings, such as a strong suspicion of LS based on family history but no DNA MMR mutations are identifiable. A positive MSI test would still be indicative of a diagnosis of LS and suggest that current methods of mutation detection may be inadequate (e.g. mutations in promoter regions or new genes involved in MMR that have yet to be identified). Furthermore, some non-deleterious mutations such as missense or in-frame insertion/deletion mutations are reported, particularly in the *hMLH1* and *hMSH6* genes, which do not lead to a truncated protein and will not be predicted to affect protein translation, stability, and antigenicity. In these cases, MSI could help determine whether there are true functional consequences of these variant mutations.

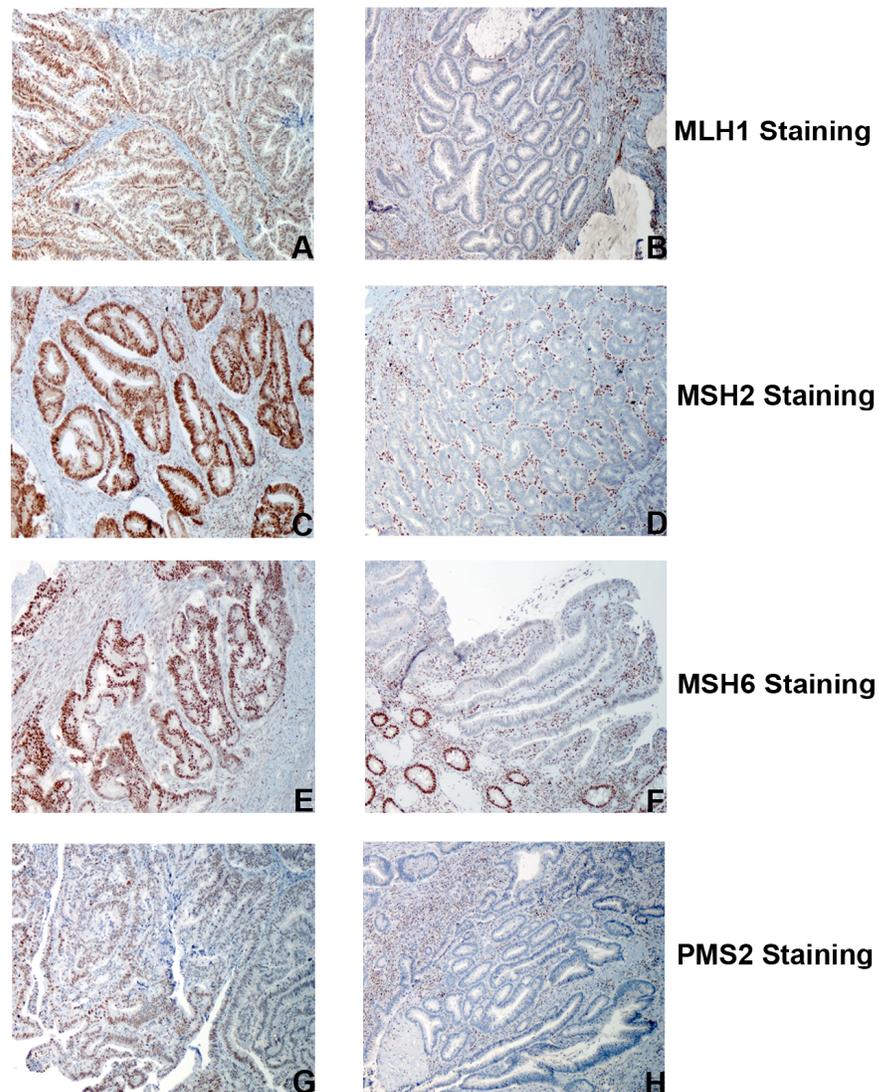
However, MSI testing is labor intensive and more costly than IHC, requires expert pathologic services, and while a hallmark for LS, is not specific for it. Approximately 10-15% of sporadic CRCs also exhibit MSI due to somatic CpG island methylation of the

promoter of the *hMLH1* gene, resulting in transcriptional silencing [41]. LS-associated and sporadic MSI-positive CRCs have many histopathologic features in common, such as mucinous histology, poor differentiation, and the presence of lymphocytic infiltration, but differ in that sporadic MSI CRCs are not associated with a positive family history, and are more common in women at older ages. Recently, a V600E hotspot mutation in exon 15 of the *BRAF* gene, a member of the RAF family of kinases, has shown utility in distinguishing tumors with somatic hypermethylation of *hMLH1* and those arising through a germline mutation. Deng *et al.* found that this specific *BRAF* mutation occurred in 87% of sporadic tumors with hypermethylated *hMLH1*, whereas it was not present in any MSI tumor with a germline *hMLH1* mutation [42]. In addition, *BRAF* mutations were not detected in any tumors from patients with germline *hMSH6* mutations and in tumors from 23 MMR-negative families, 13 of whom fulfilled the Amsterdam criteria and 10 the Bethesda criteria [43]. Collectively, the detection of a *BRAF* mutation in a MSI colorectal cancer suggests a sporadic origin of the disease and not LS. These findings have a potential impact in the diagnostic algorithm of LS, since *BRAF* sequencing can define which cases with abnormal MSI and IHC results do not require further germline testing.

## *Diagnosis of MMR Deficiency*

### *Immunohistochemical Analysis of MMR Protein Expression*

Mutations in MMR genes usually lead to loss of expression of a detectable protein product in the nuclei of tumor cells, providing a rationale for the use of IHC techniques to detect MMR gene mutations. Adjacent normal colonic mucosa can serve as a positive control (Figure 3).



**Figure 3.** Immunohistochemical Staining for MMR Proteins in Colorectal Carcinoma. Positive (A) and absent (B) staining for MLH1; positive (C) and absent (D) staining for MSH2; positive (E) and absent (F) staining for MSH6; positive (G) and absent (H) staining for PMS2.

Knowing how mismatch repair proteins interact during DNA repair can help interpret IHC results and guide germline testing. MMR proteins form heterodimers. MSH2 dimerizes with either MSH6 or MSH3, depending on the length of the basepair mismatch, and then recruits heterodimers of MLH1 and PMS2 or MLH1 and PMS1 to excise the mismatched nucleotides. MSH2 and MLH1 proteins are the obligatory partners of their respective heterodimeric complexes, and mutations result in the loss of both the obligatory and secondary partner proteins by immunohistochemistry. However, the converse is not true, since loss of staining of MSH6 or PMS2 alone is typically observed with germline mutations in each of these respective genes. Other proteins, such as MSH3, MLH3, and PMS1, may compensate in the binding to the obligatory proteins (Table 4).

**Table 4.** Immunohistochemistry Testing Results Based on Germline Mutation

Germline mutations in:	Protein staining			
	MLH1	MSH2	MSH6	PMS2
<i>hMLH1</i>	Absent	Present	Present	Absent
<i>hMSH2</i>	Present	Absent	Absent	Present
<i>hMSH6</i>	Present	Present	Absent	Present
<i>hPMS2</i>	Present	Present	Present	Absent

Since *hMSH2* and *hMLH1* mutations account for more than 70% of all known MMR mutations in LS, efforts to evaluate the utility of IHC in detecting the syndrome have focused primarily on MSH2 and MLH1. As recently reviewed by Shia, the sensitivity of MLH1 and MSH2 IHC in predicting germline mutations in the corresponding genes is approximately 85% [44]. More than one third of the *hMLH1* alterations are missense mutations that may result in catalytically inactive but antigenically intact proteins and false positive staining by IHC. The addition of PMS2 IHC led to the identification of 23% more patients with an *hMLH1* mutation in a study by de Jong *et al.* [45]. When 35 tumors

associated with a known *hMLH1* germline mutation were tested by IHC, 21 tumors showed absence of both MLH1 and PMS2 staining, while 8 tumors exhibited negative staining for PMS2 only. Furthermore, MSH6 immunostaining not only permits the detection of *hMSH6* mutations but also supports the detection of *hMSH2* mutations. The initial 2-antibody panel (MLH1 and MSH2) has evolved into a 4-antibody panel (MLH1, MSH2, MSH6, and PMS2) with an increased sensitivity of 94% in predicting a gene mutation.

### ***Advantages and Limitations of IHC Testing***

The major advantage of this assay is that IHC is widely available as part of the routine services in general pathology and therefore does not depend upon the involvement of a molecular genetics laboratory. The recent automation of immunostaining assures consistent and reproducible immunostaining procedures, allowing comparison of immunostaining patterns between different tissues and cases. Another specific advantage of IHC is that tumors with *hMSH6* mutations frequently lack or have low levels of MSI due to a functional redundancy in the DNA MMR system. Indeed, MSH2 may form heterodimers with either MSH6 to repair single-nucleotide mismatches or with MSH3 to repair small insertion and deletion mismatches larger than one nucleotide. Therefore, when *hMSH6* is mutated, the MSH2/MSH3 dimer is still functional, and MSI may not be apparent. However, IHC will reveal loss of MSH6 staining.

One inherent potential shortcoming is that the technique is somewhat subjective and depends upon the quality of tissue preparation, staining, and interpretation of the results. Interestingly, abnormal staining patterns may be due to tissue preservation and the tumor microenvironment. For example, tissue hypoxia or oxidative stress may diminish the function of MMR proteins, even in genetically MMR-proficient tissues, leading to a focal

loss or weak staining [48,49]. Secondary abnormalities in MMR genes may also lead to rare staining patterns. For example, the existence of mononucleotide repeats in the coding sequences of several MMR genes, such as *hMSH2*, *hMSH6* and *hPMS2*, may result in abnormal immunohistochemical staining when mutations in these microsatellites occur as a consequence of a germline mutation of a different MMR gene [50].

## ***Diagnosis of MMR Deficiency***

### ***Detection of MMR Gene Mutations***

Most germline mutations have been identified in the *hMSH2* (38%) and *hMLH1* (32%) genes, with *hMSH6* and *hPMS2* mutations each accounting for approximately 15% of all known LS mutations [22]. There are no consensus hot spots for mutations, and a full spectrum of nonsense, frameshift, splice, and missense point mutations have been described. Prescreening techniques, such as single-strand conformation polymorphism (SSCP), conformation sensitive gel electrophoresis (CSGE), denaturing gradient gel electrophoresis (DGGE), or denaturing high-performance liquid chromatography (DHPLC) can be used, and such a strategy permits DNA sequencing to be targeted to specific abnormal regions. Each of these strategies takes advantage of the different physical properties of DNA fragments containing a polymorphism or mutation that distinguishes them from normal sequences. Currently, the cost and ease of large-scale DNA sequencing have obviated the need for these indirect approaches. However, certain classes of gene mutations are not detected by DNA sequencing, and these include large genomic deletions, genomic rearrangements, and genomic duplications. In these cases, the gold standard is Southern blotting. However, this approach is time consuming and expensive, typically uses radionuclides, and requires a large amount of DNA. Alternative methods such as multiplex ligation-dependent probe amplification (MLPA) have been developed. This is a relatively inexpensive, simple, and reproducible PCR-based method that uses the same equipment used for DNA sequencing [51,52]. In a Finnish study, 45 mutation-negative individuals with clinical and immunohistochemical findings suggestive of LS were evaluated by MLPA, or in some cases, long-range genomic PCR. Twenty seven percent were found to

have large genomic rearrangements caused by deletions of one or several exons of the *hMLH1* or more frequently, *hMSH2* gene [53]. Large genomic alterations account for 5-30% and 10-60% of all *hMLH1* and *hMSH2* mutations, respectively, with this wide range of frequencies due, in part, to the fact that many studies examined small sample populations [51,54-58]. Baudhuin *et al.* utilized both Southern blotting and MLPA techniques in a consecutive series of 365 unrelated cases and found that although the majority of the mutations identified in *hMLH1* and *hMSH2* genes were point mutations and small insertions/deletions, large genomic alterations were present in 17.9% and 45.3% of the *hMLH1* and *hMSH2* mutation-positive carriers [59].

A diploid to haploid conversion analysis, in which maternal and paternal alleles are separated prior to mutation screening, is a more sensitive method for detecting mutations undetectable by routine sequencing. Somatic cell hybrids between human and rodent cells are first created. These hybrid cells lose individual human chromosomes, thereby allowing the analysis of a haploid human genome, one chromosome at a time. Casey *et al.* performed a blinded comparison of conventional DNA sequencing and conversion analysis to identify mutations in *hMLH1*, *hMSH2*, and *hMSH6* genes in 89 CRC patients suspected of carrying a mutation in MMR genes due to their family history, age at diagnosis, MSI status and/or loss of MLH1, MSH2, or MSH6 protein expression of their tumors [60]. Conversion analysis increased the diagnostic yield of genetic testing by 56% compared with genomic DNA sequencing alone. Despite this potential, this technique has not come into routine clinical use due to the high expense and technical demands of creating somatic cell hybrids.

### ***Recurrent and Founder Mutations***

Most germline mutations reported in the MMR genes are unique. A mutation that arises *de novo* with high frequency is defined as recurrent. Mutations that occur once and are then passed on to succeeding generations are designated founder mutations and are typically limited to a certain geographic area or a certain ethnic group. One of the best-known recurrent mutations that accounts for approximately 11% of all *hMSH2* germline mutations is an A → T transversion in the donor splice site of intron 5 that leads to transcriptional skipping of exon 5 [61]. The recurrent nature of this mutation is explained by the fact that the adenine is the first in a stretch of 26 adenines, creating a “hot spot” for the slippage of DNA polymerase during replication. In Newfoundland, this mutation behaves as a founder mutation, having been introduced by an early settler some time after 1610, and it accounts for 20-25% of all LS mutations in this region [62]. A genomic deletion of exon 16 of the *hMLH1* gene, dating back over 1000 years, accounts for more than 50% of all LS cases in Finland [63]. The 1906 G → C mutation in the *hMSH2* gene has been documented as a founder mutation in Ashkenazi Jews, accounting for almost 20% of LS in Ashkenazi Jewish families [64]. The American Founder Mutation (AMF), a genomic deletion of exons 1-6 of *hMSH2*, has been traced back to 1727 to a German immigrant and his wife. This mutation accounts for up to 10% of the estimated total population of LS carriers in the U.S. [65]. The most important aspect of recurrent and founder mutations is that testing for these particular alterations as a first step in appropriate populations may lower the cost of a molecular diagnosis. Indeed, in populations where a high proportion of all LS are caused by a founder mutation, such as Finnish (>50%), Jewish (~20%), and Newfoundlander populations (20-25%), this testing algorithm is already in use.

### ***Homozygous Biallelic MMR Mutations***

Rarely, carriers of two independent MMR gene mutations have been described. These individuals exhibit a unique clinical phenotype including hematological malignancies and/or brain tumors, CRC in childhood, and features reminiscent of neurofibromatosis type 1 (NF1), mainly café au lait spots. In 1999, there were two simultaneously published reports of offspring in LS families who developed hematological malignancies and signs of NF1 at a very early age. DNA sequence analysis and allele-specific amplification revealed homozygous *hMLH1* germline mutations [66,67]. Gallinger *et al.* first reported on children with homozygous *hMLH1* gene deficiency and NF1 features who developed early-onset gastrointestinal cancers in the first two decades of life [68]. In all these reports, the children were conceived from consanguineous matings between first cousins. This syndrome has been referred as childhood cancer syndrome (CCS), Lynch III syndrome, “CoLoN” syndrome (Colon tumors or/and Leukaemia/Lymphoma or/and Neurofibromatosis features), and very recently, Constitutional Mismatch Repair Deficiency (CMMR-D) syndrome [69-72]. As reviewed by Wimmer and Etzler, patients carrying homozygous *hMLH1* or *hMSH2* mutations have an earlier age of malignancy (mean age 3.5 years versus 9 years) and more frequently develop hematological tumors than patients with biallelic *hMSH6* or *hPMS2* mutations, who have a higher incidence of brain and typical LS-associated tumors [72].

### ***Germline Epimutations***

There are recent reports of germline methylation of the *hMLH1* and *hMSH2* gene promoter, resulting in transcriptional silencing of the affected allele in tissues derived from all three embryonic germ cell lineages. Epimutations of the *hMLH1* gene were first

described in 2001, and affected individuals developed tumors that exhibited MSI, loss of expression of the MLH1 protein, and in some cases somatic loss of the wildtype allele [73-76]. Some kindreds had a positive family history of colorectal or other Lynch-related cancers, though they generally did not satisfy the Amsterdam criteria [74,75]. One patient had a very low (<1%) proportion of spermatozoa with *hMLH1* promoter methylation, implying the potential for transmission to the offspring, but no intergenerational transmission has been demonstrated in his family members. Hitchins *et al.* recently evaluated 24 patients with early onset MSI positive colorectal or endometrial cancers without germline mutations in MMR genes. They found two unrelated women who had a germline *hMLH1* epimutation. A son of one of these patients exhibited partial methylation of *hMLH1*, consistent with transmission of the epimutation, but methylation of *hMLH1* was not present in his sperm, indicating reversion of the epimutation during spermatogenesis. Although the maternal allele was inherited by several other children, there was no evidence of methylation, indicating a reversion of the epimutations to normal status, and in fact biallelic expression of *hMLH1* was found [77]. Although it is a very rare cause of LS, a germline epimutation of *hMLH1* should be suspected in individuals who have a family history of LS-associated cancers that exhibit MSI and loss of MLH1 staining in the absence of a germline mutation of *hMLH1*. In many of these cases, the first clue may be the demonstration of MSI within the normal control tissue.

Fewer cases of germline *hMSH2* epimutations have been reported. A unique set of Dutch and Chinese families developed early-onset colorectal or endometrial cancers, all with MSI and MSH2 protein loss, but without germline mutations in the *hMSH2* gene [78,79]. Deletions that disrupt the 3' end of the adjacent *TACSTD1* gene were identified,

and this led to inactivation of the downstream *MSH2* gene through the induction of methylation of the *MSH2* promoter [79].

### ***Polymorphisms with variable penetrance***

Recent genome wide association studies (GWAS) have identified several polymorphisms in DNA MMR genes that are associated with increased colorectal cancer risk. Because these polymorphisms may result in minor reductions in DNA repair capacity, it has been hypothesized that these polymorphisms may increase the risk of developing colorectal cancer [80]. Using GWAS, Lipkin *et al.* identified a new *hMLH1* variant, 415 G → C, which results in the amino acid substitution D132H that attenuates MLH1 function. This variant confers a clinically significant susceptibility to CRC and accounts for 1.3% of all CRC cases in Israel [81]. The *hMLH1* -93 G → A polymorphism is located in the gene promoter, potentially reducing MLH1 transcription and thereby reducing overall DNA repair function. This variant allele, either in the homozygous or heterozygous state, is associated with a higher risk of developing MSI tumors among patients from Ontario and Newfoundland [82]. Finally, the *hMSH6* variant 116 G → A is associated with an increased risk of colon cancer among men but not women [80].

### ***Genetic Test Results***

When LS is suspected, genetic testing is ideally initiated in a proband with an early onset Lynch-associated cancer. A positive test confirms the diagnosis, but the absence of a mutation is a “true negative” result only when a mutation has been previously identified in another family member. A particularly difficult result to explain is the so-called “variant of uncertain significance (VUS)”, which is usually a single-nucleotide substitution that results

in a missense mutation. In contrast to mutations that result in a prematurely truncated protein, a VUS results in a single amino acid substitution and the resulting change in protein function is not known. Most such variants are benign polymorphisms, but determining the functional and clinical significance of a specific VUS with certainty is difficult. Since DNA variants are found in many CRC families, it is important to register the variants found worldwide in an accessible database.

A positive test result may result in anxiety and depression, possibly due to concerns about discrimination in insurance coverage or the workplace. Furthermore, some carriers may feel guilty and isolated from their families, and this can occur with either a positive or negative test result. If a mutation is identified, it is then feasible for family members at-risk to undergo “mutation-specific testing”, which has lower costs than the initial full gene testing. Despite major advances in diagnostic capabilities, there will be families in whom no testing is feasible (i.e. no tumor tissue or affected relative available for testing), or in which testing is negative but in which the clinical suspicion for LS is still strong. No single test or combination of tests is completely sensitive or specific for diagnosing or excluding the syndrome. Clinical judgment should not be overridden by laboratory results if the clinical presentation for Lynch syndrome is compelling.

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